Planta (1995) 197: 333–342

Planta
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ent-Kaurene biosynthesis in a cell-free system from wheat (*Triticum aestivum* L.) seedlings and the localisation of *ent*-kaurene synthetase in plastids of three species

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Received: 22 December 1994/Accepted: 1 February 1995

Abstract. A cell-free system capable of converting [^{14}C]geranylgeranyl diphosphate to *ent*-[^{14}C]kaurene and to an unidentified acid-hydrolysable compound was obtained from the basal portions of 5-d-old shoots of wheat seedlings (*Triticum aestivum* L.). By means of marker enzyme activities, the synthesis of *ent*-kaurene and the unknown compound could be quantitatively assigned to a plastid fraction obtained by Percoll-gradient centrifugation of the homogenate. The enzyme activities were located within the plastids, probably in the stroma, because they withstood trypsin treatment of the intact plastids, and the plastids had to be broken to release the activity, which was then obtained in soluble form. Plastid membranes had no activity. Plastid stroma preparations obtained from pea (*Pisum sativum* L.) shoot tips and pumpkin (*Cucurbita maxima* L.) endosperm also yielded *ent*-kaurene synthetase activity, but did not form the unknown compound. The exact nature of the active plastids was not ascertained, but the use of methods for proplastid isolation was essential for full activity, and the active tissues are all known to contain high proportions of proplastids, developing chloroplasts or leucoplasts. We therefore believe that *ent*-kaurene synthesis may be limited to these categories. Mature chloroplasts from the wheat leaves did not contain *ent*-kaurene synthetase activity and did not yield the unknown component. Incorporation of [^{14}C]geranylgeranyl diphosphate into *ent*-[^{14}C]kaurene and the unknown component was assayed by high-performance liquid chromatography with on-line radiocounting. *ent*-[^{14}C]Kaurene was identified by Kovats retention index and full mass spectra obtained by combined gas chromatography-mass spectrometry. The unknown component was first believed to be copalyl diphosphate, because it yielded a compound on acid hydrolysis, which migrated like copalol on high-performance liquid chromatography and gave a mass spectrum very similar to that of authentic copalol. However, differences in the mass spectrum and in retention time on capillary gas chromatography excluded identity with copalol. Furthermore, the unhydrolysed compound was not converted to *ent*-kaurene by a cell-free system from *C. maxima* endosperm as copalyl diphosphate would have been.

Key words: Chloroplast (developing) – *Cucurbita* (endosperm) – Gibberellin (biosynthesis) – *ent*-Kaurene (biosynthesis) – *Pisum* (seedlings) – Proplastid – *Triticum* (seedlings)

Introduction

Gibberellin (GA) biosynthesis follows the terpenoid pathway up to the formation of geranylgeranyl diphosphate (GGPP). This intermediate is cyclised via copalyl diphosphate to *ent*-kaurene in two steps catalysed by *ent*-kaurene synthetase (KS) A and B, respectively. Although these are distinct enzymes, we will refer to KS as catalysing the over-all reaction from GGPP to *ent*-kaurene. Since *ent*-kaurene is a precursor of GAs, the GA pathway separates from the terpenoid pathway at this point making it a likely site of regulation and an interesting process to study.

ent-Kaurene biosynthesis in higher plants has mainly been investigated in preparations from developing (immature) seeds, because these organs produce the largest amounts of GAs and contain the highest concentrations of GA biosynthetic enzymes. Thus, KS was partially purified and characterised from the endosperm of developing seeds of *Marah macrocarpus* (Frost and West 1977; Knotz et al. 1977), which finally led to resolution of the A- and B-activities (Duncan and West 1981). Endosperm of

Abbreviations: ADH = alcohol dehydrogenase; AMO 1618 = 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenyl piperidine-1-carboxylate; BSA = bovine serum albumin; DTT = dithiothreitol; GA_n = gibberellin A_n; GAPDH = $NADP^+$-glyceraldehyde 3-phosphate dehydrogenase; GC-MS = combined gas chromatography-mass spectrometry; GGPP = all trans-isomer of geranylgeranyl diphosphate; KS = *ent*-kaurene synthetase; MDH = malate dehydrogenase; MAA = mevalonate activating activity; SOR = shikimate oxidoreductase

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developing *Cucurbita maxima* seeds is also very high in KS activity (Graebe 1969) and has served as starting material for the purification of KS B (Saito et al. 1993). Cell-free systems from developing seeds of *Pisum sativum* (Anderson and Moore 1967) are less active, but have proved useful for investigating physiological problems. These and other aspects of *ent*-kaurene biosynthesis are discussed in several reviews (Graebe and Ropers 1978; Hedden et al. 1978; Coolbaugh 1983; Graebe 1987).

One of the most important functions of *ent*-kaurene biosynthesis is to supply precursors for the production of endogenous GAs that regulate plant growth. To understand this process, enzyme extracts capable of *ent*-kaurene biosynthesis are needed from vegetative organs. This has been difficult to realise, because the activity is very low and often subject to inhibition. Preparations that convert mevalonate and, in some cases, GGPP and copalyl diphosphate to *ent*-kaurene have been obtained from castor-bean seedlings (Robinson and West 1970a,b), pea seedlings (Coolbaugh et al. 1973) and etiolated maize seedlings (Hedden and Phinney 1979). Other preparations from vegetative plant parts or tissue cultures converted copalyl diphosphate, but not GGPP, to *ent*-kaurene, i.e. only the B-activity of KS was found (Yafin and Shechter 1975; Shen-Miller and West 1982; West et al. 1982). This was either attributed to the presence of inhibitors in the homogenates or to the absence of KS A activity, perhaps as a form of GA biosynthesis regulation. Indeed, Shen-Miller and West (1982, 1985) found that homogenates of sunflower seedlings inhibited KS activity from *M. macrocarpus* endosperm. The inhibitory action was weakened by dialysis (Shen-Miller and West 1982), and KS activity was obtained if the sunflower seedlings were stored in liquid N_2 before homogenisation (Shen-Miller and West 1985).

Although *ent*-kaurene synthesis is always found in the supernatant after high-speed centrifugation of homogenates prepared without osmoticum, it was early suggested that the enzymes might be located within plastids in the intact cell. This would be logical, since GGPP is exclusively formed in plastids (Gray 1987). Nevertheless, attempts to demonstrate *ent*-kaurene synthesis in plastids met with the same problem as in homogenates from seedlings; plastid preparations from several species failed to convert GGPP to *ent*-kaurene, although they converted copalyl diphosphate to *ent*-kaurene more or less efficiently (Simcox et al. 1975; West et al. 1982; Railton et al. 1984). Only one plastid preparation, obtained by sucrose density centrifugation of a homogenate from endosperm of *M. macrocarpus*, contained both KS A and KS B activities (Simcox et al. 1975). These workers estimated that the plastid fraction of the endosperm contained a significant portion of the total *ent*-kaurene synthetic activity found in the crude homogenate. The activity was presumably stromal, because it was solubilised by osmotic breakage of the plastids, and the particulate fraction had no activity. Railton et al. (1984) obtained low KS B activity only from pea shoot chloroplasts. Although this activity was present in the stroma, it seemed to require thylakoid membranes for full activity. In another case, chloroplast preparations obtained by differential centrifugation of a homogenate from pea seedling shoots may have had some KS activity (Moore and Coolbaugh 1976). However, conversion was extremely low and did not depend on enzyme concentration. The low yield did not permit conclusive identification of the *ent*-kaurene produced. Thus, the compartmentation of *ent*-kaurene synthesis remained unclear. However, the most recent results have brought new evidence for a location of KS in plastids. Sun and Kamiya (1994) cloned a cDNA coding for KS A from *Arabidopsis thaliana* and expressed this gene in *Escherichia coli*. The corresponding protein (86 kDa) had features typical for chloroplast proteins. Furthermore, the translation product obtained in vitro was incorporated into and processed by isolated pea chloroplasts to a protein with a molecular mass (76 kDa) identical to that of the corresponding cDNA product in transgenic plants.

A system for the location of native KS is still needed. We report a method for the preparation of cell-free systems with stable, active and reproducible KS activity from wheat seedlings. The activity was localised in the meristematic region at the shoot base, which primarily contains proplastids. Using isolation conditions suited to recover small and sensitive proplastids, we could demonstrate that KS activity is quantitatively located within plastids from wheat seedling bases as well as from pea seedling shoots and pumpkin endosperm.

Materials and methods

Plant material. Caryopses of wheat (*Triticum aestivum* L. cv. Jubilar) were a generous gift from Dr. W. Rademacher (BASF Research Station, Limburger Hof, Germany). The grains were soaked in distilled water for 30 min, placed in a biosnacky germination dish (Biokosma, Konstanz, Germany) and grown for 5 d in a growth chamber under 14 h photoperiod at 22°C day and 18°C night. Light was supplied by Osram L65 W/25 white universal tubes, located 80 cm above the plants and giving a photon fluence rate of 162 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Entire shoots, base portions (lowest 5 mm consisting of scutellum, mesocotyl and meristem) or apical parts (beginning just above the meristem) were used for preparations as indicated.

Seeds of *Pisum sativum* L. cv. Torsdag (WB 2157) were originally (1983) obtained from Weibullsholm Plant Breeding Institute (Landskrona, Sweden) and had thereafter been propagated at our institute. Seeds were soaked for 5 h in distilled water and grown in vermiculite for 10 d under the conditions described above for wheat seedlings. The apical portion from the youngest fully expanded leaf to the tip was harvested.

Cucurbita maxima L. cv. Riesenmelone, gelb genetzt (seeds from van-Waveren, Göttingen, Germany) were grown in the Botanical Garden, University of Göttingen, during the summers of 1992 and 1994. The stage of seed development is given as the length of the cotyledons in per cent of the seed lumen (Graebe 1972).

Labelled compounds. R-[2-^{14}C]mevalonic acid lactone ($1.85 \cdot 10^{12}$ $Bq \cdot mol^{-1}$; Amersham-Buchler, Braunschweig, Germany) was hydrolysed in 5 mM KOH and used as a substrate for synthesis of [$^{14}C_4$]geranylgeranyl diphosphate (GGPP) by the method of Oster and West (1968) with modifications. Endosperm of immature *C. maxima* seeds (harvest 1992; cotyledons 40% of the seed lumen) was homogenised lightly, filtered through three layers of gauze and centrifuged at 5000 $\cdot g$ for 5 min. The supernatant (20 ml) was dialysed one time for 60 min and twice for 45 min against 1 l 0.05 M potassium phosphate buffer (pH 8.0) containing 5 mM $MgCl_2$. The reaction mixture contained 9 ml dialysed preparation, 5 mM $MgCl_2$, 10 mM ATP, 7.5 mM phosphoenolpyruvate, 5 mM dithiothreitol (DTT), 0.02 mM [2-^{14}C]mevalonate and 1 mM

AMO 1618 (Dr. W. Rademacher, BASF) in total 10 ml. After 90 min incubation at 30°C, the reaction was terminated by the addition of 0.4 ml 5 M KOH, and the products were extracted four times with 5 ml water-saturated collidine (2,4,6-trimethyl pyridine; Fluka, Neu-Ulm, Germany). Water and ionic materials were displaced from the combined collidine phases by the addition of 60 ml diethyl ether, which had been freed of peroxides immediately before use. The ethereal upper phase was washed three times with 3 ml 0.1 M NH_4CO_3 each, the aqueous phases were combined and applied to a DEAE-Sephadex A-25 column (4 cm long, 1 cm inner diameter; Pharmacia, Freiburg, Germany) equilibrated with 0.1 M NH_4HCO_3. After the column had been washed successively with 50 ml 0.1 M and 50 ml 0.25 M NH_4HCO_3, [^{14}C]GGPP was eluted in ten 5-ml fractions with 0.35 M NH_4HCO_3. The fractions with significant radioactivity were combined and lyophilised 14 h to remove both water and NH_4HCO_3. The dry powder was dissolved in 5 mM KOH, 2 mM EDTA and 0.05% (v/v) Tween 20 (polyoxyethylenesorbitanemonolaurate; Schuchhardt, München, Germany) and diluted to a final concentration of 7.5 μM [$^{14}C_4$]GGPP. This procedure routinely yielded approx. 25 nmol of [$^{14}C_4$]GGPP with a specific radioactivity of $6.73 \cdot 10^{12}$ Bq·mol^{-1} (as determined by gas chromatography-mass spectrometry (GC-MS)) and containing more than 40% of the radioactivity that had been added as [2-^{14}C]mevalonate. The preparation was more than 95% radiochemically pure, the impurity being primarily [$^{14}C_4$]geranylgeraniol. We will refer to these compounds as [^{14}C]GGPP and [^{14}C]geranylgeraniol, except where the presence of four ^{14}C-atoms per molecule is important for the interpretation (Table 7).

Cell-free extracts. All steps were conducted at 4°C. Cell-free extracts were prepared from wheat shoots or shoot segments (usually 12 g fresh weight) by homogenising the frozen parts with 0.2 g insoluble polyvinylpyrrolidone (PVP-40T, MW 40000; Sigma, München, Germany) per g fresh weight for 1 min in a prechilled Waring blender set at the high speed. The frozen powder was suspended in 2 ml 0.05 M potassium phosphate (pH 6.9), 5 mM $MgCl_2$, 5 mM DTT (reaction buffer) per g fresh weight and stirred for 5 min. The homogenate was filtered through eight layers of gauze and centrifuged at 200 000·*g* for 90 min. The resulting supernatant was brought to 50% $(NH_4)_2SO_4$-saturation by adding saturated solution with gentle stirring on ice over 5 min. The suspension was stirred for another 5 min, centrifuged at 20 000·*g* for 20 min and the pellet was dissolved in 2.5 ml of reaction buffer. This solution was desalted through a PD-10 column (Pharmacia) containing Sephadex G-25 medium gel, which was equilibrated and eluted with reaction buffer, resulting in the final preparation (3 ml). On the average, 11 mg protein were obtained from 1 g plant material (fresh weight).

Plastid and stroma preparations. Excised parts from wheat or pea seedlings were homogenised twice in isolation medium [2 ml·(g fresh weight)$^{-1}$] consisting of 0.6M sorbitol, 0.02M 2-(N-morpholino) ethanesulfonic acid (Mes)-KOH (pH 6.3; Serva, Heidelberg, Germany), 5 mM EDTA, 5 mM DTT, 0.1% (w/v) Insoluble polyvinylpyrrolidone and 0.1% (w/v) bovine serum albumin (BSA; fraction V; Sigma). The first time, three 1-s bursts were given with the Waring blender set at the low speed. The homogenate was filtered through eight layers of gauze and three layers of nylon mesh (25 μm; Schütt, Göttingen, Germany). The residue was resuspended in the same volume of medium, blended at high speed for 3 s, and filtered as the first time. The filtrates were combined and sampled (1 ml) for later determination of enzyme activities. The rest was centrifuged at 200·*g* for 3 min to remove intact cells. The supernatant was transferred to a 96-ml centrifuge tube, where it was underlayered with 10 ml 20% (w/v) Percoll (Pharmacia) and 5 ml 80% (w/v) Percoll, both in isolation medium. The gradient was centrifuged at 6000·*g* for 10 min in a swing-out rotor (HSS 13.94 in Suprafuge 22; Heraeus, Osterode, Germany). The diffuse, turbid, pale green plastid zone above the 80% Percoll cushion was collected carefully, resuspended by stirring very gently in 30 ml isolation medium, without PVP and BSA, and once more centrifuged at 6000·*g* for 10 min through 10 ml 20% Percoll (w/v) in the modified medium to produce a plastid pellet. Both the plastid pellet and the previously diverted sample of the homogenate were treated by five up-and-down strokes in a Thomas-homogenizer (Schütt) containing 6 ml 0.02M Mes-KOH (pH 6.3), 5 mM EDTA and 5 mM DTT to lyse intact plastids. The resulting homogenates were centrifuged at 200 000·*g* for 90 min to sediment all membranes. Supernatant protein, in the case of the plastid pellet mainly stroma protein, was purified and concentrated by precipitation with 50% $(NH_4)_2SO_4$, centrifuging and desalting through PD-10, all as described for the cell-free extract. Three milliliters of solution containing 300–480 μg stroma protein were obtained from 12 g (fresh weight) wheat shoot bases, and the same volume containing 3.2 mg of stroma protein were obtained from 15 g (fresh weight) pea shoot tips. Bovine serum albumin, which was contained in the sample of the original homogenate, remained in the supernatant after the ammonium-sulfate precipitation.

Endosperm (10 ml) from immature seeds of *C. maxima* (harvest 1994; cotyledons 20–25% of the seed lumen) was homogenised in 10 ml isolation medium, without PVP and BSA, by squeezing the sample through a nylon net tied around the open end of a 20-ml plastic syringe. This procedure was done totally four times with successively smaller mesh: 200 μm, 100 μm, 40 μm, 25 μm. After sampling the homogenate, the plastids were enriched by gradient centrifugation and lysed as described above. Ammonium-sulfate precipitation and gel filtration were omitted, because enzyme activity was high in the pumpkin system and all assays could be done with the 200 000·*g* supernatant preparation. Three milliliters of solution containing 1.4 mg stroma protein were obtained.

Protease treatments of intact wheat plastids and of unprotected stroma preparations. For the data shown in Table 3, part of the plastid fraction was diverted after the first gradient centrifugation at 6000·*g*. This sample was resuspended in 0.02M Hepes-KOH (pH 7.6), 0.6M sorbitol, 3 mM $CaCl_2$ and 2.7 mg·ml^{-1} trypsin (bovine pancreas, type III, 10200 units·mg^{-1}; Sigma) and was gently shaken at 12°C for 1 h. Second centrifugation, lysis and stroma protein purification followed as in the normal preparations. To show the inhibitory action of the protease (Table 4), stroma protein was prepared and concentrated by the standard method, except that the PD-10 column was equilibrated and eluted with 0.05M phosphate buffer of pH 7.3 (rather than pH 6.9), containing 5 mM $MgCl_2$ and 5 mM DTT. The final preparation was incubated for 1 h at 30°C with 0.01 mg·ml^{-1} or 0.1 mg·ml^{-1} thermolysin (protease Type X; Sigma) or trypsin before starting the *ent*-kaurene synthetase (KS) assay with [^{14}C]GGPP (see below). Where indicated, 0.13 mg·ml^{-1} trypsin inhibitor (from egg white, 23 IU·mg^{-1}; Serva), was mixed with trypsin before adding both to the incubation mixture.

Assay of ent-Kaurene synthetase. The enzyme preparations were diluted with 0.05M potassium phosphate (pH 6.9) containing 5 mM $MgCl_2$ and 5 mM DTT (reaction buffer) to give a final maximal protein concentration of 0.1 mg·ml^{-1} (wheat and pea) or 5 μg·ml^{-1} (pumpkin). The reaction was started by adding [^{14}C]GGPP (final concentration 0.25 μM) to give a total volume of 0.5 ml. The mixture was incubated at 30°C for maximally 3 h (wheat and pea) or for 30 min (pumpkin). The reaction was terminated by adding 50 μl 5M KOH. *ent*-Kaurene and alcohols that had arisen through dephosphorylation were extracted twice with 0.5 ml actone and 1.5 ml petroleum spirit (boiling range 40–60°C) and a third time with 2 ml petroleum spirit only. After this extraction, 100 μl 5M H_2SO_4 were added to the remaining incubation mixture, which was incubated at 40°C for 1 h to hydrolyse any remaining allylic diphosphates. Liberated alcohols were then extracted as described for the first extraction. Radioactivity was determined in aliquots of each extract by liquid scintillation (PW 4700; Philips, Eindhoven, The Netherlands), purely organic samples being counted in Quicksafe N and aqueous solutions in Aquasafe 300 (both from Zinsser Analytic, Frankfurt, Germany). The extracts were dried under a stream of N_2 on ice (*ent*-kaurene is volatile at room temperature), and the product composition was determined by HPLC. The residues were resuspended in 150 μl methanol-acetone (1:1) and applied to a C_{18} reverse-phase

column (15 cm long, 8 mm i.d., 4 μm; Novapak Liquid Chromatography Cartridge in a RCM 100 radial compression system; Waters, Eschborn, Germany), which was eluted with a gradient from 75% methanol in water to 100% methanol delivered by a two-pump HPLC system (models 501 and 510; Waters) at a flow rate of 1 ml·min^{-1}. The effluent was monitored on-line by an HPLC radioactivity monitor (model LB 506 C-1; Berthold, Wildbad, Germany).

To obtain samples for identification by GC-MS, a whole stroma protein preparation (3 ml) from wheat seedling shoot bases was incubated with 1.5 nmol [^{14}C]GGPP as above for 14 h at 30°C. The reaction products were extracted by the standard procedure, except for omitting the acid hydrolysis, because the petroleum-spirit extract already contained 90% of the applied radioactivity. The dried extract was dissolved in 2 ml petroleum spirit (boiling range 40–60°C) and applied to a silica cartridge (Waters), from which *ent*-[^{14}C]kaurene was eluted with 20 ml petroleum spirit, followed by the ^{14}C-labelled hydrolysis product of compound X and [^{14}C]geranylgeraniol, which were eluted by 20% (v/v) acetone in petroleum spirit. The fractions were dried and purified by HPLC as described above. The three compounds were collected in separate fractions, extracted with petroleum spirit, dried, derivatised, where appropriate, and subjected to GC-MS analysis (data for *ent*-kaurene and compound X in Table 7). A second sample of *ent*-[^{14}C]kaurene was prepared from 20 standard incubations of wheat base homogenate and subjected to GC-MS with the same result (data not shown). The GC-MS data for [^{14}C]geranylgeraniol shown in Table 7 was obtained by enzymatic hydrolysis of precursor [^{14}C]GGPP. This compound (0.275 nmol) was dissolved in 1 ml 0.1M glycine (pH 8.6), containing 1 mg alkaline phosphatase (Serva), and incubated for 3 h at 30°C. The reaction product was extracted with petroleum spirit, dried, derivatised and analysed by GC-MS.

Marker enzyme assays. Enzyme activites were assayed in the final protein preparations, except the activity of NADP$^+$-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was measured in the 200 000·*g* supernatant of the lysing medium, omitting $(NH_4)_2SO_4$ precipitation. This was done, because GAPDH activity is inhibited by phosphate buffer and was high enough not to require concentration. Since the stroma preparations and the diverted samples of homogenates were both treated the same way, omission of the precipitation step did not influence the comparison of enrichment factors.

Alcohol dehydrogenase (ADH) activity was assayed in 1 ml containing 50–200 μl protein preparation, 0.1M Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 5 mM DTT and 2 mM NAD$^+$. The reaction was started with 15 mM ethanol and recorded at 340 nm (Smith and ap Rees 1979).

NADP$^+$-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity was determined by measuring the reverse reaction in 1 ml containing 200–400 μl 200 000·*g* supernatant, 0.1M Hepes-KOH (pH 8.0), 20 mM KCl, 30 mM $MgCl_2$, 2 mM EDTA, 5 mM DTT, 5 mM ATP, 0.3 mM NADPH and 600 nkat 3-phosphoglycerate kinase. The reaction was started with 5 mM 3-phosphoglycerate, and the reaction rate was recorded at 340 nm (Stitt et al. 1989).

Malate dehydrogenase (MDH) activity was determined by measuring the reverse reaction in 1 ml containing 10–50 μl protein preparation, 0.07M potassium phosphate buffer (pH 7.5), 5 mM $MgCl_2$, 0.25 mM NADH and 5 mM DTT. The reaction was started with 2.3 mM oxalacetic acid and recorded at 340 nm (Cooper 1981).

Mevalonate activating activity (MAA) was determined by conversion of mevalonate to compounds extractable after acid hydrolysis and was assessed in a mixture (0.5 ml) of 5 mM $MgCl_2$, 10 mM ATP, 7.5 mM phosphoenol pyruvate, 5 mM DTT, 5 μM [2-^{14}C]mevalonate (1.85·10^{12} Bq·mol^{-1}) and 20–50 μg protein. After 3 h of incubation at 30°C, 50 μl 5M HCl was added, and the incubation was continued for another hour to release the organic moieties of allylic diphosphates. The solution was neutralised with 60 μl 5M KOH, products were extracted twice with 0.5 ml acetone and 1.5 ml petroleum spirit and one time with 2 ml petroleum spirit alone. The volume of the combined organic extracts was reduced under N_2, and the radioactivity was measured by scintillation counting. The method actually indicates a combination of mevalonate activation, isopentenyl diphosphate isomerase and prenyl transferase activities.

Shikimate oxidoreductase (SOR) activity was assayed in 1 ml mixtures containing 50–200 μl protein preparation, 0.1 M glycine-KOH (pH 9.5) and 0.24 mM NADP$^+$. The reaction was started with 3 mM shikimate and the rate was recorded at 340 nm (Fiedler and Schultz 1985).

Protein determination. Protein concentrations were determined according to Sedmak and Grossberg (1977) using BSA as a standard.

Combined gas chromatography-mass spectrometry. Dried HPLC-fractions containing putative alcohols were trimethylsilylated by heating with 10 μl N-methyl-N-trimethylsilyltrifluoracetamide (Macherey-Nagel, Düren, Germany) at 90°C for 40 min. The derivatised samples and underivatised samples, expected to contain *ent*-kaurene and dissolved in 10 μl petroleum spirit (boiling range 40–60°C), were analysed using a Incos 500 GC-MS system (Finnigan Bremen, Germany) equipped with a Hewlett-Packard 5890 (Series II) gas chromatograph. Samples (1 or 2 μl) were co-injected with a solution of Parafilm in hexane (0.5 μl) for determination of Kovats retention indices (Gaskin et al. 1971) into a HP-5MS capillary column (30 m long, 0,25 mm inner diameter, 0,25 μm film thickness; Hewlett-Packard, Waldbronn, Germany) at an oven temperature of 80°C. The split valve (40:1) was opened after 0.7 min, after which the temperature was increased by 10°C·min^{-1} to 190°C and then by 4°C·min^{-1} to 260°C. The He inlet pressure was 115 kPa and the injector, transfer-line and source temperatures were 250, 260 and 130°C, respectively. Mass spectra were acquired over a period of approx. 6 min around the expected retention time at an electron energy of 70 eV and a current of 600 μA from mass 60 to 320 at 0.84 s per scan for *ent*-kaurene and from mass 60 to 400 at 0.55 s per scan for trimethylsilylated alcohols. Specific radioactivity was calculated from mass spectra by the method of Bowen et al. (1972).

Results

Establishing a cell-free system from wheat seedlings. Experiments were designed to obtain KS activity in enzyme preparations from 5-d-old wheat seedlings, sufficiently high for quantitative analysis. Since inhibitory substances and high phosphatase activity had been found in other tissues, we tried several different purification steps at once. The success of this approach is evident from Table 1. Two products, *ent*-kaurene and an unknown compound (compound X), yielding a diterpene alcohol on acid hydrolysis, were formed in almost equimolar amounts from [^{14}C]GGPP. The low-speed (5000·*g*) supernatant of the homogenate showed no detectable activity, unless it was gel-filtered through Sephadex G-25. Ammonium-sulfate precipitation at this stage did not improve incorporation. Ultracentrifugation, followed by gel filtration, approximately doubled the specific activity, and ammonium sulfate precipitation prior to gel filtration enhanced it another 60–75%. Ultracentrifugation probably improved the yield by removing particle-bound phosphatase activity. Thus, GGPP was almost entirely hydrolysed in 3 h if the incubation was contaminated with the turbid lower portion of the supernatant, whereas it remained intact to at least 60% if the clear upper supernatant was used (data not shown). Centrifugation at 200 000·*g*, ammonium-sulfate precipitation and desalting through Sephadex G-25 were chosen as standard conditions.

Table 1. Incorporation of [^{14}C]GGPP into *ent*-kaurene and an unidentified diterpenoid component, compound X, in cell-free preparations from 5-d-old wheat seedlings. Supernatants after centrifugations at 5000·*g* (S5) or 200000·*g* (S200) were desalted by filtration through Sephadex G-25 (G-25), or supernatant protein was precipitated with $(NH_4)_2SO_4$ and then desalted. Standard assay for KS with a protein concentration of 0.1 mg·ml^{-1}. n.d., not detected

Preparation	Protein yield [mg·(g FW)$^{-1}$]	Enzyme activity [fkat·(mg protein)$^{-1}$]	
		ent-Kaurene	Compound X
S5	22	n.d.	n.d.
S5, G-25	22	0.8	0.6
S5, $(NH_4)_2SO_4$,G-25	14	0.6	0.8
S200, G-25	16	1.2	1.8
S200, $(NH_4)_2SO_4$, G-25	11	2.1	2.9

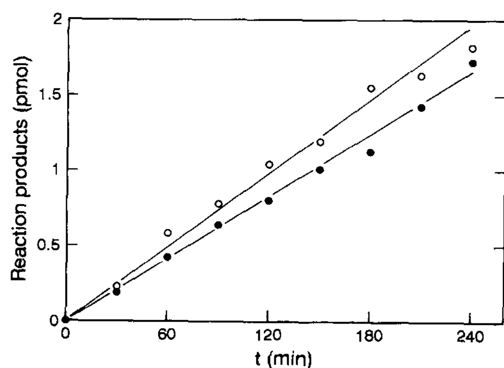

Fig. 1. Synthesis of *ent*-[^{14}C]kaurene (●) and compound X (○) from [^{14}C]GGPP in a cell-free system from shoots of 5-d-old wheat seedlings as a function of incubation time. Standard assay with 0.1 (mg protein)·ml^{-1}. Compound X was assayed as its hydrolysis product

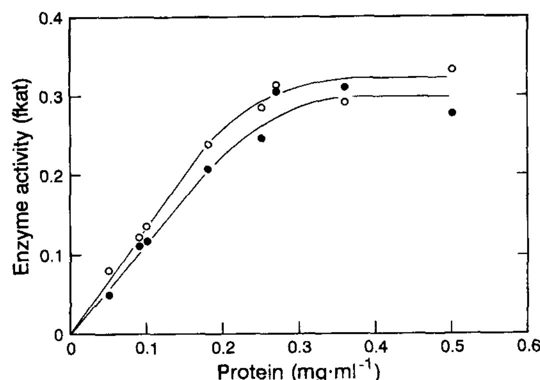

Fig. 2. Synthesis of *ent*-[^{14}C]kaurene (●) and compound X (○) from [^{14}C]GGPP in a cell-free system from shoots of 5-d-old wheat seedlings as a function of protein concentration. Standard assay (3 h) with protein concentrations as indicated

Constant reaction rates were obtained for at least 4 h with standard preparations at a protein concentration of 0.1 mg·ml^{-1} and a temperature of 30°C (Fig. 1). Reaction rates were approximately proportional to protein concentrations up to 0.2 mg·ml^{-1} within 3 h (Fig. 2). The standard conditions chosen, 3 h incubation at 0.1 (mg protein)·ml^{-1}, resulted in maximally 20% conversion of the substrate. The presence of BSA (1 mg·ml^{-1}) in the incubation mixture totally inhibited both KS activity and the formation of the unidentified product (data not shown).

Conversion of [^{14}C]GGPP was exclusively found in extracts from the 5-mm basal segment of the wheat shoot (Table 2). This portion of the plants included scutellum (largely degraded), mesocotyl and meristem. There was no activity in preparations from the apical part consisting of the coleoptile and primary leaves. When the two preparations were combined, activity was diluted, but not inhibited. This confirmed that there was no activity in the apical parts and showed that extracts from this part of the plants were not inhibitory when prepared as described.

Preparation of active plastids. Since no mature chloroplasts were to be expected in the parts of the seedlings that were active, the classical chloroplast isolation methods were critically evaluated. Proplastids and developing chloroplasts are smaller and more sensitive and require careful handling. Indeed, preliminary experiments showed that sedimentation of a pellet by plain differential centrifugation in sorbitol yielded a sticky, coagulating mass, from

Table 2. Synthesis of *ent*-kaurene and compound X in cell-free systems from different parts of 5-d-old wheat seedling shoots. Preparations originating from the lowest 5 mm (base), consisting of scutellum (largely degraded), mesocotyl and meristem, or from the parts above the meristem (top) of the same plants were assayed separately or mixed (1:1). Standard assay with 0.1 (mg protein)·ml^{-1}. n.d. not detectable

Plant	Enzyme activity [fkat·(mg protein)$^{-1}$]	
	ent-Kaurene	Compound X
Base	5.6	6.6
Top	n.d.	n.d.
1/2 Base, 1/2 top	2.8	2.2

which no intact plastids could be recovered. The plastids were therefore centrifuged through 20% Percoll onto a cushion of 80% Percoll, and the optimal osmolarity of the homogenisation medium was sought. At least 0.5 osmol·l^{-1} was required during isolation for a maximum yield of the plastid marker enzyme NADP$^+$-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in subsequent stroma preparations (Fig. 3). Identical results were obtained for the stroma marker enzyme shikimate oxidoreductase (SOR; data not shown). Protein yields and the activities of both KS and synthesis of compound X were maximal at 0.6 osmol·l^{-1}. This concentration was used in subsequent experiments for tissue homogenisation and plastid resuspension.

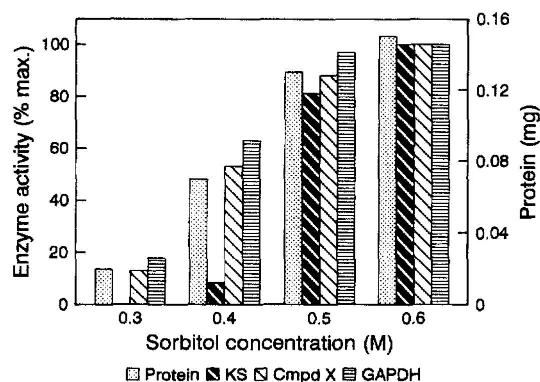

Fig. 3. Comparison of protein yields, KS and GAPDH activities and compound-X synthesis in stroma preparations from plastids isolated from wheat shoot bases at different osmolarities of the homogenisation medium. Because of different orders of magnitude, enzyme activities are normalised to their highest measured values (100%), which were 5.4 fkat for KS, 17.1 fkat for the synthesis of compound X and 1.1 nkat for GAPDH. Stroma marker SOR (maximal activity 0.6 nkat) gave the same picture as GAPDH. Twelve grams (fresh weight) of plant material were used for each preparation, and the centrifugations were at 6000·*g*

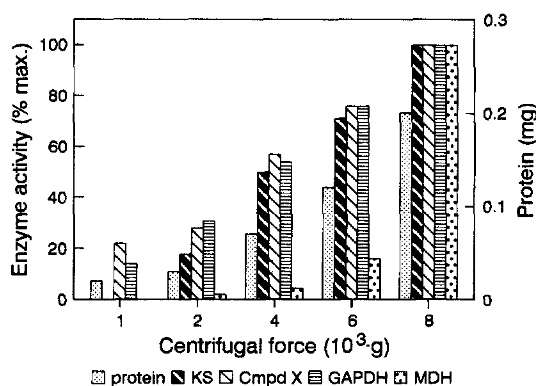

Fig. 4. Comparison of protein yields, KS, GAPDH and MDH activities and compound-X synthesis in stroma preparations from plastids isolated from wheat shoot bases at different centrifugal forces. Enzyme activities are normalised to their highest measured values, which were 6.7 fkat for KS, 15.0 fkat for the synthesis of compound X, 1.7 nkat for GAPDH and 76 nkat for MDH. Stroma marker SOR (maximal activity 0.8 nkat) gave the same picture as GAPDH. The homogenisation medium was 0.6 osmolar. The supernatant of each centrifugation was subjected to the next higher centrifugation step; cumulative yields are shown

Differential centrifugation through 20% Percoll at gravitational forces from 1000·*g* to 8000·*g* yielded further significant amounts of intact plastids in each successive supernatant as assayed by GAPDH activity and protein contents in subsequent stroma preparations (Fig. 4). *ent*-Kaurene synthetase activity and the synthesis of compound X paralleled this behaviour. The 8000·*g* pellet contained the highest activity of KS, but also an intolerably high contamination by mitochondria as represented by malate dehydrogenase (MDH). The specific activity of MDH in the 8000·*g* pellet was as high as in the uncentrifuged homogenate (not visible from the diagram), while the 6000·*g* sediment contained only 20% of this value and 71–75% of the KS activity. Sedimentation at 6000·*g* was employed in further experiments.

After the methods had been optimised, the effects of omitting component steps were examined in an attempt to simplify the procedure. However, all steps were found to be necessary. It particularly deserves to be pointed out that no activity was obtained if the ammonium-sulfate precipitation of the stroma protein in solution was omitted.

Localisation of KS and organelle marker enzyme activities in plastids. Plastids were prepared from wheat shoot bases by the optimised methods and lysed. The membrane fraction was sedimented by ultracentrifugation, stroma protein in the supernatant was concentrated and purified by ammonium-sulfate precipitation. These stroma preparations contained 2–5% of the total GAPDH and 0.5–1.5% of the total SOR activities of the homogenate. Both are considered plastid stroma markers. As seen from Table 3, the specific activities of KS, compound X formation, GAPDH and SOR in the stroma preparation were several times higher than the corresponding activities in the homogenate, whereas the activity of MDH (mitochondria) decreased by 80% and that of ADH (cytosol) by over 90% as compared with the homogenate. If the isolated intact plastids were treated with trypsin, which was removed again before lysing the plastids, the remainder of cytosolic marker ADH was removed, whereas the activity of KS and that of the plastid markers rather increased. The activation of mevalonate to acid-hydrolysable diphosphates (MAA) was measured for comparison. Obviously these activities are entirely cytosolic. The experiment without protease treatment was done three times yielding nearly identical results, including the lower enrichment of SOR activity in stroma preparations in comparison with those of GAPDH, KS and the synthesis of compound X.

A control experiment showed that a concentration of trypsin as low as 0.1 mg·ml^{-1} completely destroyed both KS activity and the synthesis of compound X, if it was added to the unprotected stroma preparations (Table 4). In contrast, the KS activity shown in Table 3 was protected inside the plastids even at 27-fold higher concentration of trypsin. Thermolysin, which is often used to verify the location of enzymes inside plastids, was less active and did not completely inhibit the exposed enzymes at 0.1 mg·ml^{-1}. The use of trypsin inhibitor showed that the inhibition of enzyme activities by trypsin was a specific protease effect (Table 4). The experiments protocolled in Table 4 were done at a pH 7.3 rather than at pH 6.9 in order to optimise the activity of trypsin. The higher pH also favored the formation of compound X over that of *ent*-kaurene.

Mature green chloroplasts from the upper half of leaves (5d old), isolated by the same method as the active plastids from the lower portion, had no KS activity and no synthesis of compound X, although they did have GAPDH and SOR activities (data not shown).

ent-Kaurene synthetase activity in the plastids of other species. The method used to prepare plastids from wheat shoots was successfully used to prepare plastids with KS activity from pea shoots. The results (Table 5) are very

Table 3. Localisation of *ent*-kaurene synthetase (KS) and the synthesis of unidentified compound X in plastids of *Triticum aestivum* shoots. Stroma protein was prepared from plastids isolated from the shoot bases of 5-d-old wheat seedlings by methods ensuring a high proplastid yield. A portion of the plastids was incubated with trypsin (2.7 mg · ml^{-1}) for 1 h, which was removed again before osmotic breakage and stroma preparation. Homogenate and stroma protein concentrations were adjusted to 0.05 to 0.08 mg ml^{-1} for the KS assay to stay within the linear range. Marker enzymes were GAPDH and SOR for stroma, MDH for mitochondria and glyoxysomes and ADH for cytosol. The conversion of [2-^{14}C]mevalonate into acid-labile compounds (MAA) was assayed for comparison. n.d. not detectable

Preparation	Enzyme activity [fkat · (mg protein)$^{-1}$			Enzyme activity [nkat · (mg protein)$^{-1}$]			
	KS	Cmpd X	MAA	GAPDH	SOR	MDH	ADH
Homogenate	8.6	13	42	1.4	2.2	480	2.2
Stroma	39	100	0.72	8.7	5.7	97	0.14
Stroma from protease-treated plastids	82	93	n.d.	16	7.0	100	n.d.
				Enrichment factor			
Stroma/homogenate	4.5	7.7	0.02	6.2	2.6	0.2	0.06
Stroma (protease)/homogenate	9.5	7.2	0.00	11	3.2	0.2	0.00

Table 4. Protease inhibition of KS activity and compound-X synthesis. Stroma protein preparations from shoot bases of 5-d-old wheat seedlings were preincubated for 1 h with thermolysin or trypsin, or simultaneously with trypsin and trypsin inhibitor, before the KS assay was started. Stroma protein concentration was 0.06 mg · ml^{-1} in each case. n.d., not detectable

Treatment [(mg protease) · ml^{-1}]	Enzyme activity [fkat · (mg protein)$^{-1}$]	
	KS	Compound
Control (0)	28	54
Thermolysin (0.01)	27	53
Thermolysin (0.1)	13	26
Trypsin (0.01)	15	24
Trypsin (0.1)	n.d.	n.d.
Trypsin (0.01) + inhibitor	31	57
Trypsin (0.1) + inhibitor	29	57

similar to those obtained with wheat (Table 3), except that compound X was not formed and KS activity was somewhat lower in the pea shoot preparation.

The results of corresponding experiments with endosperm from immature seeds of *Cucurbita maxima* are shown in Table 6. This endosperm is an excellent material to achieve a high yield of plastids, because it is semi-liquid and no strong cell walls have to be broken during homogenisation. Even without $(NH_4)_2SO_4$ precipitation, 10^3 times higher specific activity of KS than in wheat plastids was measureable. Like in the cell-free systems from pea, no compound X was formed. Most preparations had no GAPDH activity. The stroma preparation appeared as pure as the preparations from pea and wheat, as judged by marker enzyme activity, and the enrichment factor for KS was as high as for plastid marker SOR.

Identification of products. Full mass spectra and Kovats retention indices of the products were obtained by capillary GC-MS and compared with those of authentic standards (Table 7). One sample of *ent*-[^{14}C]kaurene was obtained from 20 standard incubations of homogenate protein (200 000·*g* gel-filtered supernatant) originating from wheat seedling shoot bases. This incubation gave a clear mass spectrum for *ent*-kaurene (data not shown), but the spectrum for the hydrolysis product of compound X was uninterpretable because of contamination with fatty acids. Another sample was obtained from 3 ml stroma protein preparation subjected to 14 h incubation, which yielded clear spectra for both *ent*-kaurene and the compound X hydrolysis product (Table 7). Because of the long incubation time, compound X was already hydrolysed, so that the diterpene organic component could be extracted, purified and derivatised without prior hydrolysis. This component had the same retention time as copalol on HPLC and a mass spectrum very similar to that of copalol. However, the mass spectra do show differences, particularly with respect to the prominent ion at *m/z* 210 for the X moiety, and the capillary GC retention times are different (Table 7). Furthermore, unhydrolysed compound X, formed in a wheat stroma preparation, was not converted to *ent*-kaurene by the *C. maxima* preparation, as copalyl diphosphate would have been (Table 8). As a further difference, the KS A inhibitor AMO 1618 only partially inhibited the formation of compound X, whereas it completely inhibited the formation of *ent*-kaurene in both wheat and pumpkin plastid preparations. As expected for an inhibition of KS A, no copalyl diphosphate accumulated in the incubations with AMO 1618.

Discussion

ent-Kaurene synthetase (KS) was obtained in active, stable and reproducible form from vegetative tissues of wheat and pea. Immature pumpkin seeds were already known as a prime source of KS (Graebe 1969; Saito et al. 1993). The KS activity of the investigated tissues is apparently entirely located in plastids, because it was enriched in the plastid fractions to at least the same extent as the activities of SOR and GAPDH, which are frequently used plastid stroma markers (e.g. Fiedler and Schultz 1985; Stitt et al. 1989). Furthermore, KS activity was protected against inactivation by trypsin in the intact plastids, whereas it was entirely lost if the plastids were lysed before

Table 5. *ent*-Kaurene synthetase activity, mevalonate incorporation into acid-labile compounds and marker enzyme activities in protein preparations of homogenate and plastid stroma from the shoot tip of 10-d-old pea seedlings. Compound X was not formed in the pea system. Homogenate and stroma protein concentrations were adjusted to 0.1 mg·ml^{-1} for the KS assays. Marker enzymes as in Table 3

Preparation	Enzyme activity [fkat·(mg protein)$^{-1}$]		Enzyme activity [nkat·(mg protein)$^{-1}$]			
	KS	MAA	GAPDH	SOR	MDH	ADH
Homogenate	3.1	55	8.3	0.65	31	1.6
Stroma	17	0.9	17	1.9	8.8	0.06
			Enrichment factor			
Stroma/homogenate	5.5	0.02	2.0	2.9	0.28	0.04

Table 6. *ent*-Kaurene synthetase and marker enzyme activities in protein preparations of total homogenate and plastid stroma from endosperm of immature seeds of *Cucurbita maxima*. Protein concentration had to be diluted to only 5 μg·ml^{-1} for the KS assay to remain within the linear range. Marker enzymes as in Table 3

Preparation	Enzyme activity [pkat·(mg protein)$^{-1}$]	Enzyme activity [nkat·(mg protein)$^{-1}$]		
	KS	SOR	MDH	ADH
Homogenate	3.4	0.36	33	4.8
Stroma	21	2.3	10	0.24
	Enrichment factor			
Stroma/homogenate	6.2	6.4	0.30	0.05

protease treatment. The plastid preparation was to some extent contaminated with mitochondria as shown by the MDH activities, while cytosolic ADH activity was almost absent in the first plastid preparation and totally lost after protease treatment (Table 3). Mevalonate-activating activity appeared totally cytosolic in all three species investigated.

The plastid fractions from wheat and pea shoots contained proplastids, young developing chloroplasts and mature chloroplasts in different proportions. It was beyond the scope of our work to distinguish which of these plastids contained KS activity and which did not. Nevertheless, the high osmolality and relatively high centrifugal forces needed to obtain active preparations suggest location in proplastids or similar organelles (Guillot-Salomon et al. 1987; Baumgartner et al. 1989). The pumpkin endosperm contained only leucoplasts. The heterogeneous plastid populations from the vegetative tissues, taken together with the unequal intracellular location of enzymes in these tissues, may explain the less than perfect agreement between the enrichment of some marker enzymes and KS activities, and between marker enzyme activities themselves. Such variations are known from other work as well. For example, Fiedler and Schultz (1985) found that all detectable forms of SOR in spinach were localised in chloroplasts, whereas Mousdale and Coggins (1985) found that a portion of the same enzyme in pea shoot cells was extraplastidic. In our experiments, SOR activity in wheat plastid preparations was enriched less than KS and GAPDH activity, suggesting that a considerable part of the SOR activity might be extraplastidic.

It appears that *ent*-kaurene synthesis is restricted to growing tissues like immature endosperm, cotyledons, scutella and meristems, so that the use of these tissues as starting material already constitutes a certain concentration of KS. Thus, apices and the youngest internodes yield the most-active cell-free systems for *ent*-kaurene synthesis in pea shoots (Chung and Coolbaugh 1986), and younger tissues also synthesise *ent*-kaurene most actively in photoperiodically induced spinach plants (Zeevaart and Gage 1993). In *Ricinus* seedlings, *ent*-kaurene production is maximal 2.5–3 d after sowing (Robinson and West 1970b), and also barley seedlings synthesise *ent*-kaurene maximally at 3 d after imbibition (Groβelindemann et al. 1991). The most active *ent*-kaurene synthesis in 4-d-old wheat seedlings was found in scutellum (Lenton et al. 1994), which, although not a dividing tissue, is a very specialised tissue void of mature chloroplasts. The gramineous shoot is an excellent material in which to study tissues of different developmental stages because meristematic activity is limited to the basal part of the shoot, and more than 80% of the mitotic cells are situated within the first 5 mm (Baumgartner et al. 1989). This is also the region where we found KS activity in young wheat shoots. Since the scutella of our wheat seedlings were largely assimilated at the time of harvest, we believe that the activity mainly resided in the meristem.

Our results agree with those of Simcox et al. (1975), showing the association of KS activities with a plastid fraction of *Marah macrocarpus* endosperm, a tissue which only contains leucoplasts and has similar strong KS activity as *C. maxima* endosperm. A location of KS in plastids also agrees with the results of Sun and Kamiya (1994), who have cloned the *GA1* gene from *Arabidopsis thaliana*, showing that it encodes KS A and that the protein product obtained by translation in vitro is imported into isolated pea chloroplasts.

Failures to demonstrate substantial KS activity in cell-free systems and plastids from vegetative tissues or the observation of KS B activity alone may now be evaluated. One important factor is the membrane-bound phosphatase activity, which, in insufficiently centrifuged homogenates, hydrolyses GGPP before it is converted to copalyl diphosphate. This latter intermediate appears less sensitive to hydrolysis, which may be the reason why KS B activity was sometimes obtained where KS A activity was not. In the case of plastids, methods more suited to isolate mature chloroplasts than to sediment and maintain proplastids and leucoplasts in active form may have been used. Furthermore, our stroma preparations were only active after they had been concentrated and purified by ammonium-sulfate precipitation. This latter procedure was only used by Duncan and West (1981) showing that purified KS A from *M. macrocarpus* could be coupled with

Table 7. Identification by GC-MS of *ent*-[$^{14}C_4$]kaurene and analytical data for the ^{14}C-labelled component of compound X, both biosynthesised from [$^{14}C_4$]geranylgeranyl diphosphate by preparations of wheat plastid stroma protein. Data for [$^{14}C_4$]geranylgeraniol, obtained by enzymic hydrolysis of the substrate, and for authentic standards are included for comparison. RT, retention time; KRI, Kovats retention index

Sample	Isotope	HPLC-RT (min)	KRI	Characteristic ions at *m/z* (% relative intensity of base peak)
Enzymatic *ent*-kaurene	$^{14}C_4$	27.67	2061	280(M$^+$, 18), 265(25), 237(21), 221(20), 207(09), 193(13), 179(18), 165(21),71(100)
Authentic *ent*-kaurene[a]	$^{2}H_2$	26–28	2061	274(M$^+$,21), 259(47), 229(46), 213(35), 203(13), 189(29), 177(21), 163(34), 69(100)
Compound-X hydrolysis product[b]	$^{14}C_4$	13.83	2331	370(M$^+$, < 0.2), 355(< 0.2), 280(1.2), 265(4.6), 237(1.1), 223(1.3), 210(96), 195(15), 193(12), 165(31), 139(44), 125(55), 111(80), 97(57), 73(100)
Authentic copalol[b,c]	^{12}C	13.95	2302	362(M$^+$,0.35), 347(1.2), 272(5.9), 257(25), 229(4.9), 215(3.6), 203(4), 201(3.6), 189(5.4), 187(6.6), 161(12), 137(38), 123(23), 109(29), 95 (40), 73(100)
Precursor geranylgeraniol[b]	$^{14}C_4$	12.67	2258	370(M$^+$, < 0.2), 355(0.2), 280(0.4), 267(0.5), 237(0.7), 209(3.8), 195(1.6), 139(20), 125(15), 111(18), 97(20), 71(100)
Authentic geranylgeraniol[b,c]	^{12}C	11–13	2258	362(M$^+$,0.27), 347(0.23), 272(0.81), 259(0.73), 229(2.4), 203(4.9), 189(3.5), 135(22), 121(17), 107(22), 93(27), 69(100)

[a] *ent*-[17,17-$^{2}H_2$]kaurene (Prof. L.N. Mander)
[b] Trimethylsilylether derivative
[c] Unlabeled compounds used for mass spectra, [^{3}H]copalol for HPLC retention time (Dr. Y. Kamiya)

Table 8. Demonstration that compound X is not converted to *ent*-kaurene by KS from *Cucurbita maxima* endosperm. Stroma protein preparations from wheat shoot bases and from *C. maxima* endosperm were incubated with [^{14}C]GGPP for 3 h and 1 h, respectively. In mixed incubations, the wheat system was first incubated alone for 3 h, thereafter *C. maxima* enzyme was added and the incubation continued for another 1 h. Total recovery of radioactivity was 94%

Incubation	Enzymic reaction products (% of recovered radioactivity)				
	GGPP	Geranyl-gerniol	Cmpd X	Cmpd X hydrolysis product	*ent*-Kaurene
3 h wheat	51	29	10	1.4	8.7
1 h pumpkin	1.2	3.1	0	0	96
3 h wheat, 1 h pumpkin	0.9	37	11	1.9	49
3 h wheat + AMO 1618	69	26	4.0	0.4	0
1 h pumpkin + AMO 1618	96	3.8	0	0	0
3 h wheat, 1 h pumpkin + AMO 1618	34	44	11	1.8	9.1

a B-enzyme preparation obtained by ammonium-sulfate fractionation of a spinach leaf chloroplast stromal preparation to give *ent*-kaurene from GGPP. Simcox et al. (1975), getting KS B but not KS A activity in plastids from etiolated pea shoot tips, did not report centrifuging their lysed plastid fractions before they were assayed. Moore and Coolbaugh (1976), reporting KS activity hardly above background in plastid preparations from pea shoot tips, described their preparations as containing 26% of the chlorophyll present in each original sample. This suggests high phosphatase activity. Railton et al. (1984) found B activity, but not AB activity, in chloroplast lysates from light-grown shoots of *P. sativum*, *Helianthus annuus*, *Phaseolus coccineus*, *Hordeum vulgare* and *Zea mays*. The chloroplasts were isolated in 0.33 M sorbitol and sedimented at 2000·*g* for 40 s, i.e. by methods used to obtain mature chloroplasts. Under these conditions, no AB-activity was obtained in our preparations either (Figs. 3, 4). As for cell-free systems without isolated chloroplasts, an active preparation from young tissue of pea shoots employed centrifugation at 150 000·*g* and dialysis (Chung and Coolbaugh 1986), whereas a less-active system from *Zea mays* seedlings had only been sedimented at 40 000·*g* for 30 min, and no further purification of the preparations was mentioned (Hedden and Phinney 1979). Shen-Miller and West (1984, 1985) only obtained substantial KS activity in cell-free systems from sunflower seedlings after the seedlings had been stored for some time in liquid nitrogen. We cannot explain that.

Compound X, the second product from GGPP in the wheat system, was first believed to be copalyl diphosphate for the reasons mentioned under *Results*, but the spectra of the respective hydrolysis products were different and so were the retention times on capillary GC. Since the mass spectra of the compounds were very similar, we suspected that the unknown compound might be an artifact of the extraction procedure. However, subjecting authentic copalol to the standard extraction method did not yield the unknown product. Conversely, the unknown diterpene, and not copalol, was extracted from incubation mixtures after enzymatic hydrolysis without acidification. Further proof that compound X was not copalyl diphosphate was obtained by incubating wheat and pumpkin stroma preparations together. Under these conditions,

copalyl diphosphate would have been converted to *ent*-kaurene, but it was not. Finally, the formation of compound X was less inhibited by AMO 1618 than the formation of *ent*-kaurene. We have not been able to match the mass spectrum of the organic component of compound X with known diterpene spectra.

We thank Mrs. Gudrun Bodtke and Mrs. Dorothee Dasbach for able technical assistance, Prof. L.N. Mander (Australian National University, Canberra, Australia) for *ent*-[$^2H_2$]kaurene and Dr. Yuji Kamiya (RIKEN, Saitama, Japan) for geranylgeraniol and copalol. The work was supported by the Deutsche Forschungsgemeinschaft.

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