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Developmental and Stress Regulation of Gene Expression for Plastid and Cytosolic Isoprenoid Pathways in Pepper Fruits[1]

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Plant cells synthesize a myriad of isoprenoid compounds in different subcellular compartments, which include the plastid, the mitochondria, and the endoplasmic reticulum cytosol. To start the study of the regulation of these parallel pathways, we used pepper (*Capsicum annuum*) fruit as a model. Using different isoprenoid biosynthetic gene probes from cloned cDNAs, we showed that only genes encoding the plastid enzymes (geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and capasanthin-capsorubin synthase) are specifically triggered during the normal period of development, at the ripening stage. This pattern of expression can be mimicked and precociously induced by a simple wounding stress. Concerning the cytosol-located enzymes, we observed that the expression of the gene encoding farnesyl pyrophosphate synthase is constitutive, whereas that of farnesyl pyrophosphate cyclase (5-epi-aristolochene synthase) is undetectable during the normal development of the fruit. The expression of these later genes are, however, only selectively triggered after elicitor treatment. The results provide evidence for developmental control of isoprenoid biosynthesis occurring in plastids and that cytoplasmic isoprenoid biosynthesis is regulated, in part, by environmental signals.

The biosynthesis of plant isoprenoids occurs concomitantly in several subcellular compartments, which include the plastid, the ER cytosol, and the mitochondria. Under normal conditions, the proximal IPP precursor is engaged through the action of soluble and specific isomerases and prenyltransferases into the synthesis of prenyl PPi's, which are further converted by membrane-bound enzymes into carotenoids prenylquinones, sterols, and ubiquinones. In some plant families, including the Solanaceae, stress and elicitor treatments redirect the basic pathway and trigger a massive synthesis of isoprenoid phytoalexins. For example, steroid glycoalkaloid biosynthesis is suppressed and sesquiterpene phytoalexin biosynthesis is induced in arachidonic acid-elicited potato tuber (Kuc, 1982; Tjamos and Kuc, 1982). However, integrative regulation of these parallel biosynthetic pathways occurring in the same cell is poorly understood (West et al., 1979; Gray, 1987; Chappell, 1995; Weissenborn et al., 1995). Evidence to date suggests that plastid autonomy for IPP synthesis is limited (Heintze et al., 1994; McCaskill and Croteau, 1995). This raises a question regarding to what extent the plastid and the cytosolic pathways compete for IPP when massive synthesis of carotenoids in plastids is developmentally triggered and sesquiterpene phytoalexin biosynthesis in the cytosol is induced by elicitation.

Pepper (*Capsicum annuum*) fruits offer an opportunity to study in parallel the regulation of the plastid and cytosolic isoprenoid pathways. First, during the ripening stage, a massive synthesis of ketocarotenoids, which include capsanthin and capsorubin, takes place (Camara et al., 1995) through the sequential action of several enzymes (Fig. 1). Second, this pathway can be redirected to the intense synthesis of lycopene using CPTA (Coggins et al., 1970). Third, under stress conditions, active synthesis of capsidiol through the intermediacy of 5-epi-aristolochene synthase occurs in the cytosol (Watson and Brooks, 1984). It is not known whether this phenomenon involves turning off squalene synthesis, as shown for tobacco cell cultures (Threlfall and Whitehead, 1988; Vögeli and Chappell, 1988) and potato (Brindle et al., 1988; Zook and Kuc, 1991). Prominent features of these parallel isoprenoid pathways in pepper fruits are shown in Figure 1.

Using pericarp discs excised from pepper fruit as a model, we present a comprehensive analysis of the inducibility of the key genes encoding the plastid and the cytosolic isoprenoid biosynthetic enzymes under normal and stress conditions in relation to CPTA treatment. The results are discussed with regard to the possible channeling of IPP and temporal regulation of the plastid and the cytosolic pathway of isoprenoid biosynthesis in plant cells.

MATERIALS AND METHODS

Plant Material and Chemicals

Bell pepper (*Capsicum annuum* cv Yolo Wonder) plants were grown under controlled greenhouse conditions. The

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Abbreviations: CPTA, 2-(4-chlorophenylthio)triethylamine hydrochloride; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GPP, geranyl pyrophosphate; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; IPP, isopentenyl pyrophosphate.

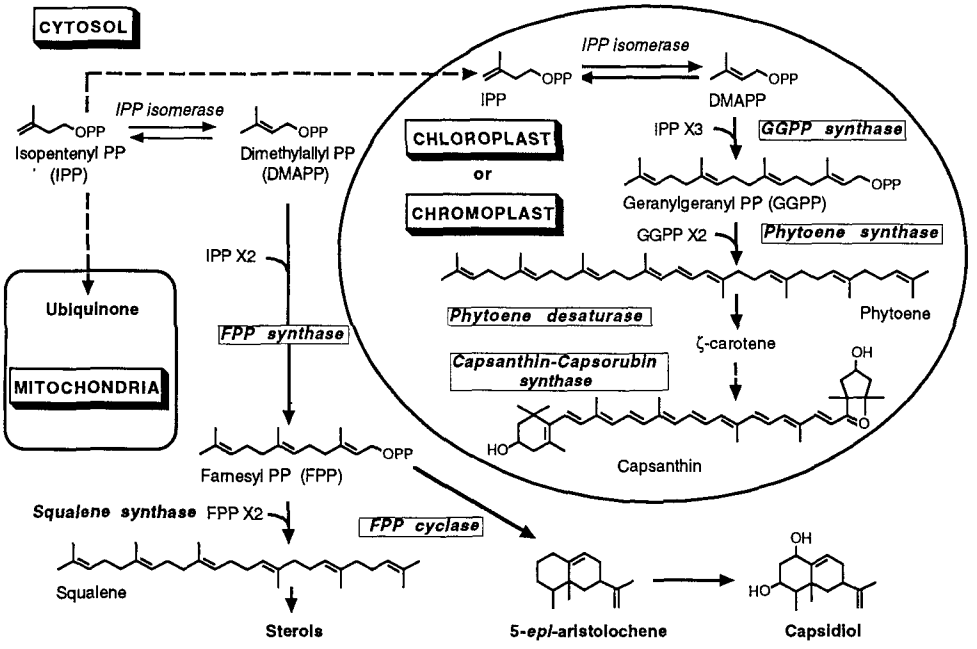


Figure 1. Simplified pathway of cytosolic and plastidial isoprenoid biosynthesis. The isomerization of IPP to DMAPP is followed by prenyl transfer reactions, which yield FPP in the cytosol and GGPP in the plastid. Although the basic mitochondrial prenyl PPi leading to the ubiquinone side chain is unknown, cytosolic FPP is converted through the intermediacy of squalene into sterols or used for sesquiterpene formation such as capsidiol. In contrast, GGPP is used in the plastid for the synthesis of carotenoids (phytoene to capsanthin). The individual enzymes are indicated in italics and in boxes for those considered in this study. Dashed arrows indicate IPP originating from the cytosol.

different stages were defined according to the diameter of the fruits and are referred to as A (0.5–1 cm), B (3 cm), C (7–8 cm, green adult stage), D (brownish), and E (red). All chemicals were purchased from Sigma, unless otherwise stated. [^{14}C]IPP (52.7 mCi mmol^{-1}) and [^{14}C]mevalonate (56 mCi mmol^{-1}) were from Amersham. Unlabeled prenyl PPis were synthesized according to a previously described procedure (Camara, 1985).

Elicitation Procedure and Analysis of Products

After sterilization with 2% sodium hypochlorite, pericarp discs (15 mm in diameter) were excised aseptically using a cork borer, and the test solutions were added as 50 μL of solution in concentric grooves (10 mm in diameter) formed in each disc. Subsequently, six discs were incubated in 12-well titration plates (Corning, Corning, NY) as described previously (Campbell et al., 1990; Deruère et al., 1994).

Elicitation of capsidiol biosynthesis was done by treating the pericarp discs with 50 μL of a sterile solution of *Trichoderma viride* cellulase (Onozuka R-10; Serva, Heidelberg, Germany) in water (1 mg/mL). For in vivo labeling experiments, each disc was fed with 50 μL of water containing [^{14}C]mevalonate (0.25 μCi). After 2 h of incubation, the discs were extracted by methanol:chloroform (1:1, v/v). The chloroform extract was saponified by KOH in ethanol, and the products were analyzed by TLC on Silicagel G F254 plates (Merck, Darmstadt, Germany) developed in chloroform:methanol (19:1, v/v). Autoradiography was done with a Phosphorimager (Fuji, Tokyo, Japan), and capsidiol was revealed by vanillin sulfuric acid spray (10 mg/mL) (Guedes et al., 1982). Capsidiol quantitation was achieved, according to a purified standard, by GC on an HP 5890 apparatus (Hewlett-Packard) equipped with a flame ionization detector and a capillary column operated at 100 to 300°C with an increment of 10°C/min. Nitrogen served as the carrier gas.

Prenyltransferase and Phytoene Synthase Assays

Fruit pericarps were homogenized (1 g/mL) with Tris-HCl buffer (50 mM, pH 7.6) containing 0.33 M Suc and 2 mM DTT in a Waring Blendor (four 5-s bursts at full speed). The brei was squeezed through three layers of nylon Blutex (50-μm apertures) and centrifuged at 4,000*g* for 5 min. The pellet was removed and the supernatant was further subjected to centrifugation at 100,000*g* for 1 h. The resulting supernatant was used as the source of FPP synthase. For GGPP synthase and phytoene synthase, purified plastids prepared as described previously were used (Camara, 1993).

The incubation medium (100 μL final volume) contained, unless otherwise stated, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM DTT, 50 mM Tris buffer (pH 7.6), [^{14}C]IPP (0.05 μCi), 1 mM DMAPP, and a defined amount of proteins (1–10 mg). After 30 min at 25°C, the reactions were stopped by phosphatase hydrolysis (Koyama et al., 1985) followed by chloroform:methanol (2:1, v/v) extraction. The products were analyzed by TLC on Silicagel 60 F254 plates developed with benzene:ethylacetate (90:10, v/v), followed by autoradiography using a Phosphorimager. The identity of the products was

determined by comparison with authentic unlabeled prenols detected by sulfuric acid pulverization and heating at 100°C.

HPLC Analysis of Carotenoids

HPLC analysis of carotenoids was achieved on a C_{18} μBondapak column (Waters) developed with acetonitrile: ethyl acetate:chloroform (70:20:10, v/v) (Camara, 1985).

Gel Electrophoresis and Immunoblotting

SDS-PAGE and immunoblotting were carried out according to standard techniques as described previously (Hugueney et al., 1992) using antibodies directed against the purified enzymes. Blotted FPP and GGPP synthase proteins were revealed using the enhanced chemiluminescence of Amersham. Protein determinations were carried out using a Bio-Rad protein assay kit.

Cloning of Pepper FPP Synthase cDNA

Two oligonucleotides, CTCCAAGCTTATTTCCTTGTGCTTGATGACATTATGG(upstream) and GGGATCAGCAAAACAATCCAGATAATCATCCTGCACTTGGAA-(downstream), designed according to the *Arabidopsis thaliana* FPP synthase cDNA sequence (Delourme et al., 1994), were used for pepper FPP synthase cDNA amplification. Total RNA (10 μg) isolated from green pepper fruit were reverse transcribed in the presence of 10 ng of downstream primer with Superscript II reverse transcriptase (GIBCO-BRL) according to the manufacturer's instructions. A 2-μL aliquot of the reverse-transcription reaction was amplified in the presence of 100 ng of both upstream and downstream primers, using the following protocol: 94°C (2 min), 30 cycles at 94°C (1 min), 50°C (1 min), and 72°C (1 min). The PCR product was radiolabeled and used to screen a *C. annuum* cDNA library as described previously (Hugueney et al., 1992). Positive phages were purified and their inserts were subcloned in the *Not*I site of pBluescript KS plasmid vector and sequenced manually according to a previously described procedure (Zhang et al., 1988) or using an automated sequencer (Applied Biosystems).

For the expression in *Escherichia coli*, a *Pst*I-*Sac*I fragment of the pBluescript KS containing the pepper FPP synthase cDNA was cloned in *Pst*I-*Sac*I sites of the pUC19 plasmid vector. Expression was carried out in *E. coli* strain JM109, grown at 37°C to an A_{600} of 0.6 before the addition of 1 mM isopropyl-β-D-thiogalactopyranoside. The induced cultures were further incubated for 4 h and pelleted by centrifugation at 5,000*g* for 5 min. The pellet was washed with Tris-HCl buffer (pH 7.6) containing 1 mM EDTA and 1 mM DTT and lysed by five cycles of 10 s in the same buffer using a Sonic Power Sonifier 350 equipped with a one-half-inch tip (power setting 2, duty cycle 70%; Branson, Danbury, CT). The resulting homogenate was centrifuged at 10,000*g* for 10 min, and the supernatant was used for enzymatic assay.

Northern Blot Analysis

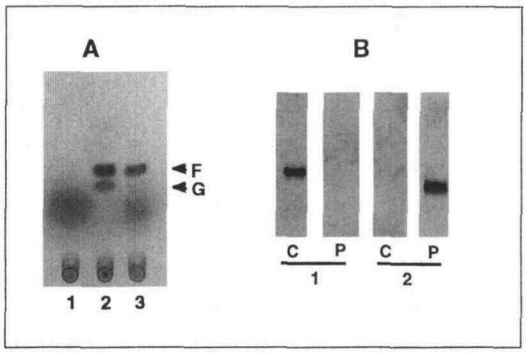
Total RNAs isolated as described previously (Verwoerd et al., 1989) were used for northern blot analysis (Hugueney et al., 1992). Hybridizations were performed using standard procedures at 65°C in 2× SSC (Sambrook et al., 1989). Membranes were then washed at 65°C in 0.5× SSC. Northern blot analysis was performed using a Phosphorimager.

RESULTS AND DISCUSSION

Developmental Regulation of Plastidial and Cytosolic Isoprenoid Pathways

To investigate the regulation of isoprenoid biosynthesis in developing and stressed pepper fruits, we used cDNA clones for plastidial GGPP synthase *Ggps* (Kuntz et al., 1992), phytoene synthase *Psy* (Römer et al., 1993), phytoene desaturase *Pds* (Hugueney et al., 1992), capsanthin-capsorubin synthase *Ccs* (Bouvier et al., 1994), a sesquiterpene cyclase *Fpc* (Facchini and Chappell, 1992), and a pepper FPP synthase (*Fps*) for cytosolic isoprenoid biosynthesis. FPP synthase cDNA was isolated using the PCR strategy described in "Materials and Methods." Expression of FPP synthase in *E. coli*, followed by in vitro analysis of the incubation products, revealed that only GPP and FPP are formed, depending on the concentration of DMAPP (Fig. 2A) as shown for the purified pepper FPP synthase (Hugueney and Camara, 1990). The specificity of FPP synthase as a typical cytosolic prenyl transferase marker is further demonstrated by the absence of characteristic transit peptide motifs in contrast to the plastidial GGPP synthase (Fig. 2B). This trend is also followed by the absence of antigenic cross-reactivity with the plastid stroma proteins (Fig. 2C).

Using the above-mentioned molecular probes we followed the expression of *Ggps*, *Psy*, *Pds*, *Ccs*, *Fps*, and *Fpc* genes during different stages of pepper fruit development. During stage A the pericarp tissue is subject to intensive cellular divisions that yield the final cell population. Following the division period, the fruit cells enter into an active phase of expansion (stage B) until the mature green or adult stage is reached (stage C). Subsequently, the ripening period is triggered and is phenotypically characterized by the apparition of transient brown zones in the pericarp (stage D), which finally yield the full red color characteristic of the final stage of pepper fruit development (stage E). Northern blot analysis of the expression of the different isoprenoid biosynthetic genes (Fig. 3) during the cell division period (stage A) and the growth period (stage B) of pepper fruit development revealed that transcripts corresponding to *Ggps*, *Psy*, *Pds*, and *Ccs* are barely detectable, whereas *Fps* is apparently expressed constitutively, which is in agreement with the potential use of FPP for cytosolic sterol biosynthesis, which generally parallels the growth stages of seedlings (Green and Baisted, 1972) or fruits (Gillaspy et al., 1993) and the requirement for de novo synthesis of new membranes. The expression of *Ggps*, *Psy*, *Pds*, and *Ccs* was detectable only during the ripening period (C–E) (Fig. 3). During this period the chloroplasts differentiate into chromoplasts, accumulating massive



C

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      1                                                  50
Ggps  MRSMNLVDLW AQQACLVFNQ TLSYKSFNGF MKIPLKNSKI NPKLNKKRPF
Fps   .......... .......... .......... .......... ..........

      51                                                 100
Ggps  SPLTVSAIAT TKEDERIEAA QTEEPENFKI YVTEKAISVN KALDEAIIVK
Fps   .......... ........MS DFKSKEMEAY KVLKSELLND PGFE..FTDD

      101                                                150
Ggps  EPHVIHEAMR YSLLAGGKRV RPMLCLAACE LVGGNQE... ....NAMAAA
Fps   SREWVDKMLE YN.VPGGKLN RGLSVIDSYS LVDDGKELTR DEIFKASALG

      151                                                200
Ggps  CAVEMIHTMS LIHDDLPCMD NDDLRRGKPT NHKIYGEDVA VLAGDSLLAF
Fps   WCIEWLQAYF LVLDDI..MD NSHTRRGQEC WYKVEKVGMI AVNDGILLRN

      201                                                250
Ggps  AFEHIVNSTA GVTPSRIVGA VADLAKSIGT EGLVAGQVAD IKCTGNASVS
Fps   HISRLLKNH. .FRPKSYYVD LLDLFNEVEF QT.ASGQMID LITTHVGEKD

      251                                                300
Ggps  LETLEF.... .IHVHKTAAL LESSVVLGAI LGGGTNVEVE .KLRRFARCI
Fps   LSKYSLPIHC RIVQYKTAYY SFYLPVACAL LMAGENLDNH VDVKNILIEM

      301                                                350
Ggps  GLLFQVVDDI LDVTKSSEEL GKTAGKDLVV DKTTYPKLLG LEKAKEFAAE
Fps   GIYFQVQDDY LDCFADPEVL GKIGTD...I QDFKCSWLVV ..KALELCNE

      351                                                400
Ggps  LNREAAQQLE GFDSRKAAPL IALAD..... .....YIAYR DN.......
Fps   EQK..KILYE NYGKDDAACI AKIKTLYNDL KLEEVFLEYE KKTYEKLANS

      401                  425
Ggps  .......... .......... .....
Fps   IAAHPSKAVQ AVQLSFLGKI YKRQK
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Figure 2. Molecular and biochemical characterization of pepper FPP synthase. A, Prenyltransferase activity in *E. coli* expressing the *C. annuum* FPP synthase cDNA. Prenyltransferase activity in control JM109 *E. coli* transformed with pUC19 (lane 1) and in JM109 transformed with pUC19 harboring the pepper FPP synthase cDNA (lanes 2 and 3). The incubation mixtures contained [^{14}C]IPP and 1 mM DMAPP (lane 2) or 100 μM DMAPP (lane 3) as an allylic primer. After phosphatase hydrolysis, the products were purified by TLC and detected by autoradiography. F (farnesol) and G (geraniol) indicate the migration of the authentic compounds. B, Immunoblot analysis of FPP synthase and GGPP synthase in pepper fruit extracts. After electrophoresis, 25 μg of total cytosolic (C) and plastid (P) proteins were blotted and incubated with anti-FPP synthase (1) or anti-GGPP synthase (2) antibodies. Immunoreactive bands were detected using an enhanced chemiluminescence kit (Amersham). C, Comparison of the peptide sequence of *C.annuum* FPP synthase (Fps, accession no. X84695) and GGPP synthase (Ggps). Black boxes indicate identical amino acid residues.

amounts of carotenoids (Camara et al., 1995). Although expressed earlier, *Fps* transcripts accumulate constitutively during the ripening period. The gene *Fpc* was not expressed during the whole period of normal fruit development (results not shown). The distinct expression patterns of *Ggps* and *Fps* genes, which encode the two main plant cell prenyltransferases, suggest that the plastidial and the cytosolic pathway of isoprenoid biosynthesis, which use GGPP and FPP, respectively, as a building block, are differentially regulated during plant development. A finding consistent with this interpretation is the observation that during the germination of pumpkin seeds FPP synthase activity is highest during the initial growth period. GGPP synthase increases later and FPP synthase decreases (Shinka et al., 1974).

With regard to the cytosolic origin of IPP (Kleinig, 1989), one may consider that the distinctly temporal regulation of the plastid and the cytosolic pathways could be due to the fact that the bulk of IPP is alternatively channeled by an unknown mechanism toward different subcellular sites of isoprenoid biosynthesis according to the developmental requirements. The coordination of this phenomenon must be very complex, since transgenic overexpression of cytosolic HMG-CoA reductase in tobacco only increased the level of sterols without affecting the metabolism of plastid isoprenoids (Chappell et al., 1995; Schaller et al., 1995).

Stress Regulation of Plastidial and Cytosolic Isoprenoid Pathways

Treatment of pepper discs isolated from mature green or red fruit with cellulase leads to capsidiol accumulation to the same magnitude (Fig. 4A). This phenomenon is specific, since we noted no accumulation of capsidiol in the absence of cellulase (Fig. 4B). Furthermore, addition of organelle protein synthesis inhibitors chloramphenicol (200 μg/mL) and lincomycin (200 μg/mL) during the elicitation procedure did not abolish the induction of capsidol formation in contrast to cycloheximide (100 μg/mL), thus demonstrating the prevalent nucleocytoplasmic control of the inducibility (Fig. 4B).

It has been previously reported that the elicitation of sesquiterpene phytoalexin synthesis in tobacco cell cultures (Threlfall and Whitehead, 1988; Vögeli and Chappell, 1988) and potato (Brindle et al., 1988; Zook and Kuc, 1991) is accompanied by a suppression of squalene synthase activity. Accordingly, we measured the incorporation of mevalonic acid into the sterol fraction of cellulase-elicited pericarp discs. Compared to the control preparation, cellulase

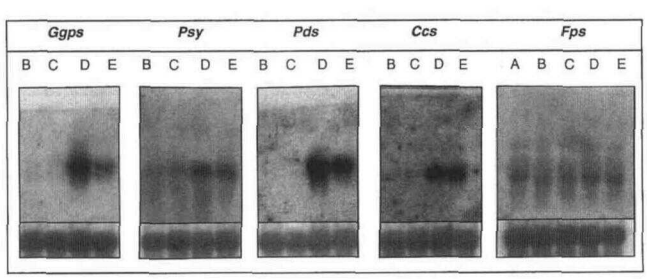

Figure 3. Northern blot analysis of mRNAs encoding GGPP synthase (*Ggps*), phytoene synthase (*Psy*), phytoene desaturase (*Pds*), capsanthin-capsorubin synthase (*Ccs*), and FPP synthase (*Fps*) during various stages of fruit development, including fertilized ovary (stage A), young fruit (stage B), mature green fruit (stage C), semiripened fruit (stage D), and red fruit (E). Each lane contained 20 μg of total RNA. The RNA blots were subsequently hybridized with a *C. annuum* 25S rRNA-specific probe to ensure the presence of equivalent amounts of RNA.

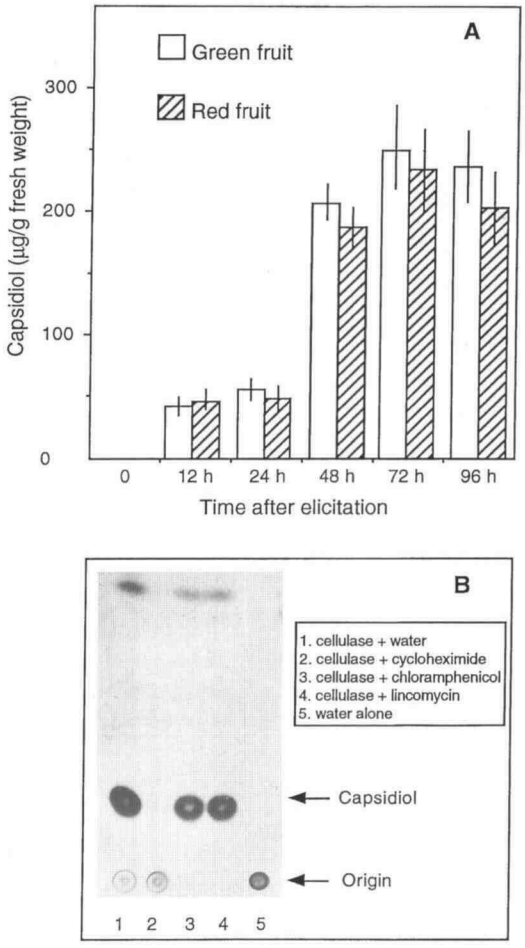

Figure 4. Induction of capsidiol formation in green and red pepper fruits following cellulase elicitation and effects of protein synthesis inhibitors. A, Effect of cellulase elicitation on the accumulation of capsidiol in green and red fruit pericarps. Six pericarp discs, aseptically cut from pepper fruit, were treated either with 50 µL of a sterile solution of *T. viridae* cellulase (1 mg/mL) or with 50 µL of sterile water and incubated for the indicated time before determination of the capsidiol content. B, Effect of protein synthesis inhibitors on the formation of capsidiol. Six pericarp discs, aseptically cut from green pepper fruits, were treated either with 50 µL of a sterile solution of *T. viridae* cellulase (1 mg/mL) or with 50 µL of sterile water and labeled mevalonate (56 mCi $mmol^{-1}$, 0.25 µCi/disc) and incubated for 12 h in the presence of the indicated inhibitors. At completion the discs were homogenized in 5 mL of water, using a Waring Blendor (four 5-s bursts at full speed). After the sample was centrifuged at 2000*g* for 5 min, capsidiol was extracted from the supernatant and purified by TLC before autoradiography.

treatment induced a strong inhibition of mevalonic acid incorporation into the putative sterol fraction migrating with unlabeled stigmasterol (Fig. 5A). These data suggest that in elicited pepper pericarp isoprenoid precursors are probably channeled toward the synthesis of capsidiol (Fig. 5A), and this redirection of carbon may be mediated by a suppression of squalene synthase activity.

To what extent this redirection of carbon could affect the plastid isoprenoid pathway is presently unknown. This issue was addressed using two strategies. In the first case, we evaluated the activity of the plastidial GGPP synthase and phytoene synthase in control and elicited pericarp discs using labeled IPP. In the second case, we artificially boosted the plastid carotenoid pathway with CPTA, which is known to induce massive synthesis of lycopene (Coggins et al., 1970), and analyzed in parallel the cellulase-elicited capsidiol formation. This procedure facilitates the quantitative analysis, since mevalonic acid or any other labeled precursor is poorly incorporated in vivo into the carotenoid fraction during short incubation times (Treharne et al., 1966).

Plastid fractions isolated from cellulase-elicited discs and control discs were incubated in vitro with labeled IPP before extraction and analysis of the reaction products. The data obtained after 12 to 48 h of elicitation indicates that no significant difference exists between the synthesis of GGPP and phytoene in control and elicited discs (Fig. 5B). Therefore, GGPP synthase and phytoene synthase activities are not likely down-regulated after elicitation. Therefore, phytoene synthase, in contrast to its cytosolic homolog squalene synthase, is not subject to direct down-regulation by cellulase elicitation, a mechanism that probably operates only when competing enzymes are localized in the same cellular compartment, as was noted in the case of the down-regulation of steroid glycoalkaloid biosynthesis (Tjamos and Kuc, 1982).

As an additional test we assessed the in vivo effect of cellulase elicitation by analyzing the change in the carotenoid content after elicitation. To this end, we made use of CPTA to artificially induce the accumulation of lycopene (Coggins et al., 1970). This procedure seemed reasonable, since we noted in a preliminary experiment that incubation of the pericarp discs with CPTA alone did not induce the formation of capsidiol, nor did CPTA affect cellulase induction of capsidiol (Fig. 5C). The time course of carotenoid accumulation indicates that the formation of lycopene is rapidly switched on when CPTA is added alone. On the other hand, in a parallel incubation containing CPTA plus cellulase, an immediate down-regulation of lycopene formation was observed concomitantly with the accumulation of capsidiol (Fig. 5C). Since we did not previously observe any direct effect of cellulase on the carotenogenic enzymes GGPP synthase and phytoene synthase (Fig. 5B), the down-regulation of the carotenogenic pathway observed in the in vivo experiments is likely due to a limited availability of carbon precursors dedicated to isoprenoid biosynthesis in the plastid.

Two plausible mechanisms may be envisaged in relation to the putative cytosolic (Kleinig, 1989) or plastidic (Heintze et al., 1994; McCaskill and Croteau, 1995) origin of IPP. One may consider that following elicitation the plastid carbon metabolism is altered, thus leading to a limited plastid IPP synthesis, while the cytosolic isoprenoid pathway is activated. This hypothesis is supported by the reported down-regulation of ribulose bisphosphate carboxylase during plant pathogen interactions (Walters and Ayres, 1984; Roby et al., 1988). Alternatively, one may consider that the plastidic and the cytosolic pathways compete for the same IPP pool. This fact, together with the

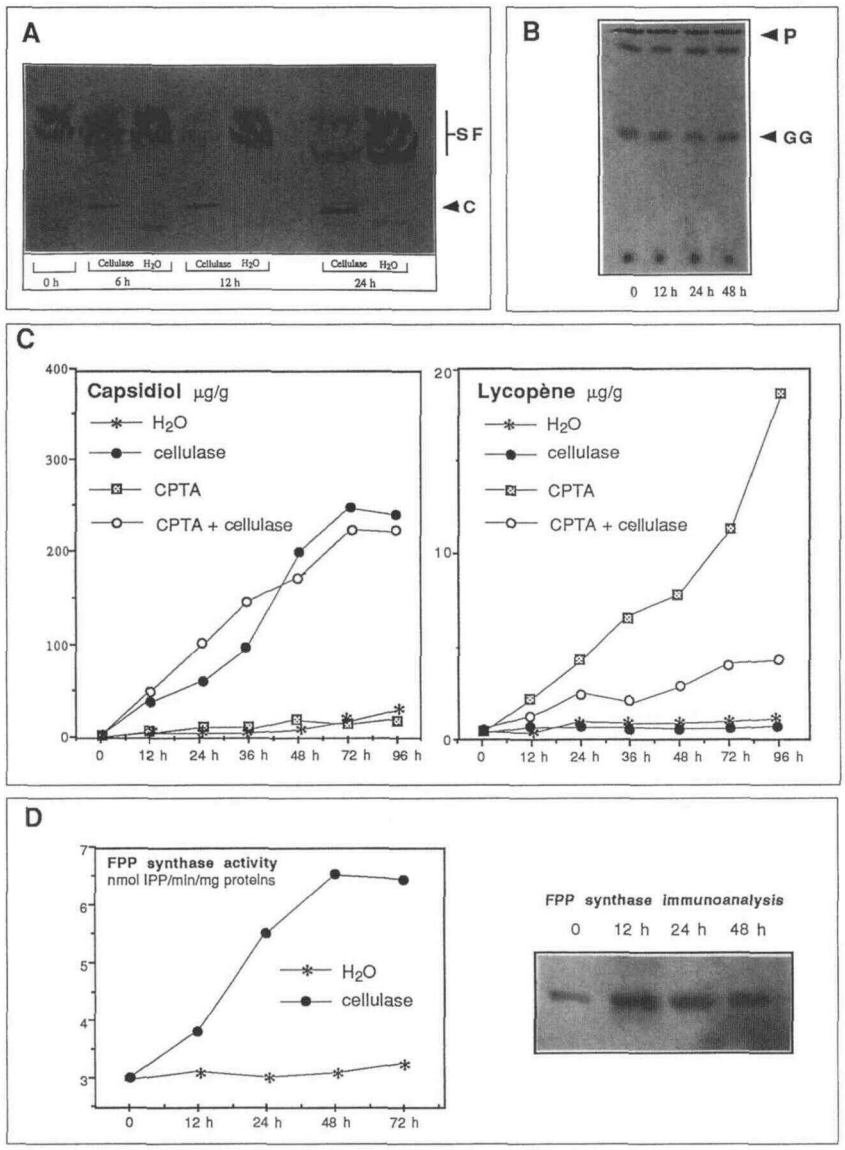

Figure 5. Biochemical modification of the isoprenoid pathway induced by cellulase elicitation and CPTA. A, In vivo incorporation of [^{14}C]mevalonate in pepper fruit pericarp discs following elicitation with cellulase. Six pericarp discs, aseptically cut from pepper fruit, were treated either with 50 μL of a sterile solution of *T. viridae* cellulase (1 mg/mL) or with 50 μL of sterile water. After 0 (C, control), 6, 12, or 24 h of incubation in the continuous presence of mevalonate (56 mCi mmol^{-1}, 0.25 μCi/disc), the discs were then extracted with methanol/chloroform and, after saponification, the organic phase was analyzed by TLC. The positions of the the radioactive sterol fraction (SF) and of capsidiol were determined using unlabeled stigmasterol and capsidiol. B, Biosynthesis of GGPP and phytoene after cellulase elicitation. The enzymic activities were evaluated using purified plastids isolated from pepper fruit pericarp discs taken at 0 (control), 12, 24, and 48 h after elicitation with cellulase, as described in "Materials and Methods." The positions of geranylgeraniol (GG) and phytoene (P) are indicated. C, Capsidiol and lycopene accumulation in pericarp discs treated with water, cellulase, and/or CPTA. Six pericarp discs were treated with water, cellulase, 10 mM of CPTA, or both cellulase and CPTA and were incubated for the times indicated. At the completion, the discs were extracted with methanol/chloroform before HPLC analysis of lycopene and GC determination of the capsidiol content. D, Induction of FPP synthase activity in pepper fruit following elicitation by cellulase. Left, FPP synthase activity was analyzed as described in "Materials and Methods" using radioactive IPP and pericarp discs after 0 (control), 12, 24, 48, and 72 h of elicitation with cellulase. Right, Immunoreactive FPP synthase in total proteins (50 μg) isolated from the same discs were probed with anti-FPP synthase antibodies.

putative cytosolic origin of IPP (Kleinig, 1989), suggests that the pool of cytosolic IPP is channeled toward the synthesis of capsidiol at the expense of the carotenogenic pathways in the elicited pericarp tissue. Whatever the prevailing mechanism, it is interesting to note that the specific activity of FPP synthase and the immunologically detectable FPP synthase present in the fruit pericarp increased in parallel during the elicitor treatment (Fig. 5D). Further analysis at the gene level strengthens this idea concerning activation of the cytosolic pathway, since the expression of *Fps* and that of *Fpc* were drastically induced concomitantly with the elicitation with cellulase (Fig. 6). Therefore, our data suggest that during cellulase elicitation FPP synthase is synthesized de novo and enhances the pool of FPP required for the FPP cyclase. The increase in FPP synthase activity may be partly responsible for the diversion of IPP away from the plastidial isoprenoid pathway.

Concerning the expression of carotenogenic genes, we noted that under our experimental conditions *Ggps*, which

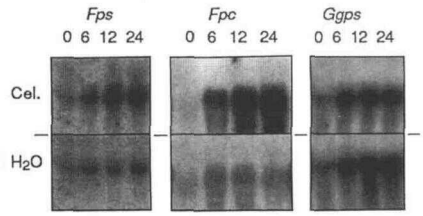

Figure 6. Northern blot analysis of the expression of mRNAs encoding isoprenoid biosynthetic enzymes following elicitation of pepper fruit. Pericarp discs were treated with cellulase (Cel.) or sterile water (H_2O) for 0 (control), 6, 12, and 24 h. Total RNAs extracted from the discs were then hybridized with cDNA probes for FPP synthase (*Fps*), FPP cyclase (*Fpc*), and GGPP synthase (*Ggps*).

encodes the plastidial prenyltransferase, displayed the same pattern of induction in cellulase-treated discs and in control discs (Fig. 6). A similar result was observed with the gene *Ccs*, which encodes the last enzyme of the plastid carotenoid biosynthesis pathway (results not shown). Since the pericarp discs were punched from the intact fruit before the deposit of the cellulase solution, we reasoned that the elicitation procedure used induces a mechanical wounding. Consequently, the potential inducing effect of mechanical injury in the absence of any cellulase treatment was tested. Intact, mature green fruits still attached to the plants were wounded with a scalpel, and the expression of *Ggps* and *Ccs* were compared with that of *Fps* and *Fpc* after 6 and 12 h of incubation (Fig. 7). Only the genes *Ggps* and *Ccs*, which encode the enzymes of the plastid pathway, were induced by the mechanical injury, whereas mRNA levels for *Fps* were constitutive and below the detection limit for *Fpc*. Furthermore, mechanical wounding did not induce any of the symptoms characteristic of cellulase elicitation, i.e. the accumulation of capsidiol, and the down-regulation of sterol synthesis (results not shown).

Two conclusions can be drawn from these results. First, in the cellulase-elicited pericarp discs, two nonequivalent processes are superimposed upon each other, a wounding effect due to mechanical injury and an elicitor effect due to cellulase. Second, elicitor treatment triggers expression of cytosolic isoprenoid biosynthesis genes, whereas mechanical wounding activates plastid isoprenoid biosynthesis genes. Our observation is consistent with the fact that wounding, in contrast to elicitor treatment, does not generally induce the formation of pathogenesis-related proteins (Ward et al., 1991). In this context, it is worth noting that plant genes encoding cytosolic HMG-CoA reductases can be classified as elicitor- and wound-inducible genes (Yang et al., 1991; Choi et al., 1994). A very similar phenomenon has been observed in yeast, which has two HMG-CoA reductases (HMG1 and HMG2) differentially regulated by heme. These two enzymes are involved in sterol biosynthesis but are apparently localized in different compartments (Wright et al., 1988; Casey et al., 1992).

Obviously, the regulation of plant isoprenoid metabolism is hampered by the paucity of information concerning the subcellular compartmentation of the different enzymes.

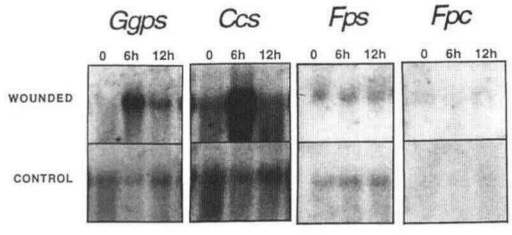

Figure 7. Northern blot analysis of the expression of mRNAs encoding isoprenoid biosynthetic enzymes following mechanical wounding of pepper fruit. Mature green pepper fruit still attached to plants were locally wounded with a sterile scalpel. Total RNAs from the wounded regions or control regions (not wounded) of the fruit pericarp were prepared 0, 6, and 12 h after wounding and then hybridized with cDNA probes for GGPP synthase (*Ggps*), capsanthin-capsorubin synthase (*Ccs*), FPP synthase (*Fps*), and FPP cyclase (*Fpc*).

This is illustrated by the fact that transgenic plants overexpressing HMG-CoA reductase in tobacco did not affect isoprenoid biosynthesis in the plastid pathway but increased the sterol content (Chappell et al., 1995; Schaller et al., 1995). Using a biochemical approach, we have demonstrated that the plastidic and the cytosolic pathways for isoprenoid biosynthesis are interdependent, at least in elicited tissue. Although the data concerning elicited pepper pericarp is consistent with the cytosol being the sole site of IPP synthesis, we cannot exclude additional mechanisms of regulation affecting the partition of the carbon precursors.

Finally, from these data we conclude that the plastidic and the cytosolic pathways of isoprenoid biosynthesis do not respond equivalently to developmental and environmental signals. The specific activation of the plastid pathway demonstrated in this study is reminiscent of previous data indicating that mechanical injury accelerates the ripening of tomato fruits (Ulrich and Renac, 1950; Fleuriet and Macheix, 1975), which is characterized by a massive synthesis of lycopene. Therefore, it would be of great interest to identify and delineate the different components involved in this signal transduction pathway.

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LITERATURE CITED

Bouvier F, Hugueney P, d'Harlingue A, Kuntz A, Camara B (1994) Xanthophyll biosynthesis in chromoplast: isolation and molecular cloning of an enzyme catalyzing the conversion of 5,6-epoxycarotenoid into ketocarotenoid. Plant J **6:** 45–54

Brindle PA, Kuhn PJ, Threlfall DR (1988) Biosynthesis and metabolism of sesquiterpene phytoalexins and triterpenoids in potato cell suspension cultures. Phytochemistry **27:** 133–150

Camara B (1985) Carotene synthesis in *Capsicum* chromoplasts. Methods Enzymol **110:** 244–253

Camara B (1993) Plant phytoene synthase complex: component enzymes, immunology, and biogenesis. Methods Enzymol **214:** 352–365

Camara B, Hugueney P, Bouvier F, Kuntz M, Monéger R (1995) Biochemistry and molecular biology of chromoplast development. Int Rev Cytol **163:** 175–247

Campbell AD, Huysamer M, Stotz HU, Grevel LC, Labavitch JM (1990) Comparison of ripening processes in intact fruit and excised pericarp discs. Plant Physiol **94:** 1582–1589

Casey WM, Keesler GA, Parks LW (1992) Regulation of partitioned sterol biosynthesis in *Saccharomyces cerevisiae*. J Bacteriol **174:** 7283–7288

Chappell J (1995) The biochemistry and molecular biology of isoprenoid metabolism. Plant Physiol **107:** 1–6

Chappell J, Wolf F, Proulx J, Cuellar R, Saunders C (1995) Is the reaction catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase a rate-limiting step for isoprenoid biosynthesis in plants? Plant Physiol **109:** 1337–1343

Choi D, Bostock RM, Adviushko S, Hildebrand DF (1994) Lipid-derived signals that discriminate wound- and pathogen-responsive isoprenoid pathways in plants: methyljasmonate and the fungal elicitor arachidonic acid induce different 3-hydroxy-3-methylglutaryl-coenzyme A reductase genes and antimicrobial

isoprenoids in *Solanum tuberosum* L. Proc Natl Acad Sci USA **91:** 2329–2333

Coggins CW, Henning GG, Yokoyama H (1970) Lycopene accumulation induced by 2-(4-chlorophenylthio)triethylamine hydrochloride. Science **168:** 1589–1590

Delourme D, Lacroute F, Karst F (1994) Cloning of an *Arabidopsis thaliana* cDNA coding for farnesyl diphosphate synthase by functional complementation in yeast. Plant Mol Biol **26:** 1867–1873

Deruère J, Römer S, d'Harlingue A, Backhaus RA, Kuntz M, Camara B (1994) Fibril assembly and carotenoid over accumulation: a model for supramolecular lipoprotein structures. Plant Cell **6:** 119–133

Facchini PJ, Chappell J (1992) Gene family for an elicitor-induced sesquiterpene cyclase in tobacco. Proc Natl Acad Sci USA **89:** 11088–11092

Fleuriet A, Macheix JJ (1975) Relations entre la maturation accélérée de fruits blessés et leur teneur en composés phénoliques. Colloq Int Cent Natl Rech Sci **238:** 147–152

Gillaspy G, Ben-David H, Gruissem W (1993) Fruits: a developmental perspective. Plant Cell **5:** 1439–1451

Gray JC (1987) Control of isoprenoid biosynthesis in higher plants. Adv Bot Res **14:** 25–91

Green TR, Baisted DJ (1972) Development of the activities of enzymes of the isoprenoid pathway during early stages of pea-seed germination. Biochem J **130:** 983–995

Guedes MEM, Kuc J, Hammerschmidt R, Bostock R (1982) Accumulation of six sesquiterpenoid phytoalexins in tobacco leaves infiltrated with *Pseudomonas lachrymans*. Phytochemistry **21:** 2987–2988

Heintze A, Riedel A, Aydogdu S, Schultz G (1994) Formation of chloroplast isoprenoids from pyruvate and acetate by chloroplasts from young spinach plants. Evidence for a mevalonate pathway in immature chloroplasts. Plant Physiol Biochem **32:** 791–797

Hugueney P, Camara B (1990) Purification and characterization of farnesyl pyrophosphate synthase from *Capsicum annuum*. FEBS Lett **273:** 235–238

Hugueney P, Römer S, Kuntz M, Camara B (1992) Characterization and molecular cloning of a flavoprotein catalyzing the synthesis of phytofluene and ζ-carotene in *Capsicum annuum* chromoplasts. Eur J Biochem **209:** 399–407

Kleinig H (1989) The role of plastids in isoprenoid biosynthesis. Annu Rev Plant Physiol Plant Mol Biol **40:** 39–59

Koyama T, Fuji H, Ogura K (1985) Enzymatic hydrolysis of polyprenyl pyrophosphates. Methods Enzymol **110:** 153–155

Kuc J (1982) Phytoalexins from the Solanaceae. *In* JA Bailey, JW Mansfield, eds, Phytoalexins. Blackie & Son, Glasgow, Scotland, pp 81–133

Kuntz M, Römer S, Suire C, Hugueney P, Weil JH, Schantz R, Camara B (1992) Identification of a cDNA for the plastid located geranylgeranyl pyrophosphate synthase from *Capsicum annuum*: correlative increase in enzyme activity and transcript level during fruit ripening. Plant J **2:** 25–34

McCaskill D, Croteau R (1995) Monoterpene and sesquiterpene biosynthesis in glandular trichomes of peppermint (*Mentha* × *piperita*) rely exclusively on plastid-derived isopentenyl diphosphate. Planta **197:** 49–56

Roby D, Marco Y, Esquerre-Tugaye MT (1988) Ribulose 1,5-bisphosphate carboxylase/oxygenase gene expression in melon plants infected with *Colletotrichum lagenarium*. Activity level and rate of synthesis of mRNAs coding for the large and small subunits. Physiol Mol Pathol **32:** 411–424

Römer S, Hugueney P, Bouvier F, Camara B, Kuntz A (1993) Expression of the genes encoding the early carotenoid biosynthetic enzymes in *Capsicum annuum*. Biochem Biophys Res Commun **196:** 1414–1421

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Schaller H, Grausem B, Benveniste P, Chye ML, Tan CT, Song YH, Chua NH (1995) Expression of the *Hevea brasiliensis* (H.B.K.) Müll. Arg. 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 in tobacco results in sterol overproduction. Plant Physiol **109:** 761–770

Shinka T, Ogura K, Seto S (1974) Farnesyl pyrophosphate and geranylgeranyl pyrophosphate synthetases during *Cucurbita pepo* germination. Phytochemistry **13:** 2103–2106

Threlfall DR, Whitehead IM (1988) Co-ordinated inhibition of squalene synthetase and induction of enzymes of sesquiterpenoid phytoalexin biosynthesis in cultures of *Nicotiana tabacum*. Phytochemistry **27:** 2565–2580

Tjamos EC, Kuc JA (1982) Inhibition of steroid glycoalkaloid accumulation by arachidonic and eicosa pentaenoic acids. Science **217:** 542–544

Treharne KJ, Mercer EI, Goodwin TW (1966) Incorporation of [^{14}C] carbon dioxide and [2-^{14}C] mevalonic acid into terpenoids of higher plants during chloroplast development. Biochem J **99:** 239–245

Ulrich R, Renac J (1950) Le métabolisme des fruits de Tomate et son altération sous l'effet des blessures. CR Acad Sci Paris **230:** 567–569

Verwoerd TC, Dekker BMM, Hoekema A (1989) A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Research **17:** 2362

Vögeli U, Chappell J (1988) Induction of sesquiterpene cyclase and suppression of squalene synthetase activities in plant cell cultures treated with fungal elicitors. Plant Physiol **88:** 1291–1296

Walters DR, Ayres PG (1984) Ribulose bisphosphate carboxylase protein and enzymes of CO_2 assimilation in barley infected by powdery mildew (*Erysiphe graminis hordei*). Phytopathol Z **109:** 208–218

Ward ER, Scott JK, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl-Goy P, Métraux JP, Ryals JA (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3:** 1085–1094

Watson DG, Brooks CJW (1984) Formation of capsidiol in *Capsicum annuum* fruits in response to non-specific elicitors. Physiol Plant Pathol **24:** 331–337

Weissenborn DL, Denbow CJ, Laine M, Lang SS, Zhenbiao Y, Yu X, Cramer CL (1995) HMG-CoA reductase and terpenoid phytoalexins: molecular specialization within a complex pathway. Physiol Plant **93:** 393–400

West CA, Dudley MW, Dueber MT (1979) Regulation of terpenoid biosynthesis in higher plants. Rec Adv Phytochem **13:** 163–217

Wright R, Basson M, D'Ari L, Rine J (1988) Increased amounts of HMG-CoA reductase induce "karmellae": a proliferation of stacked membrane pairs surrounding the yeast nucleus. J Cell Biol **107:** 101–114

Yang Z, Park H, Lacy GH, Cramer CL (1991) Differential activation of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes by wounding and pathogen challenge. Plant Cell **3:** 397–407

Zhang H, Scholl R, Browse J, Sommerville C (1988) Double stranded DNA sequencing as a choice for DNA sequencing. Nucleic Acids Res **16:** 1220

Zook MN, Kuc JA (1991) Induction of sesquiterpene cyclase and suppression of squalene synthetase activity in elicitor-treated or fungal-infected potato tuber tissue. Physiol Mol Plant Pathol **39:** 377–390