EXPRESSION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE IN MAIZE AS INFLUENCED BY LIGHT AND BLEACHING HERBICIDES

by

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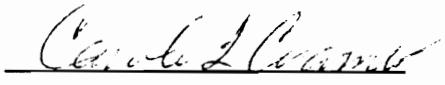

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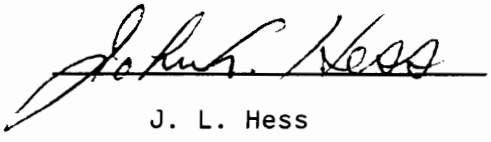

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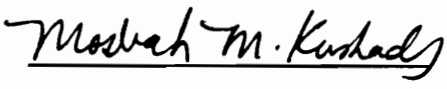

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(ABSTRACT)

The activity of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR, EC 1.1.1.34) is highly expressed in 4-day-old etiolated seedlings of normal ('DeKalb XL72AA'), dwarf ($\underline{d_5}$), and albino ($\underline{lw_3}$) maize (Zea mays L.). HMGR activity of maize seedlings appeared to be exclusively associated with the microsomal rather than the plastidic fraction of maize cells. Maize tissues with high meristematic activity such as germinating seeds, leaf bases, root tips, and the site of origin of lateral roots contained high levels of HMGR activity. The activity of HMGR extracted from leaf tips of normal, dwarf, and albino maize seedlings was regulated by light. HMGR activity from leaf tips of 4-day-old maize seedlings was inhibited significantly following exposure to strong light (600 $\mu mol/m^2/s$) for more than 10 h. In contrast, HMGR activity from leaf bases and root tips of maize was not inhibited by exposure to strong light. These results suggest that HMGR may play an important role in cell division and that light may regulate HMGR activity indirectly by increasing cell differentiation. Under conditions of strong light pretreatment with the bleaching herbicides clomazone, norflurazon, fluridone and acifluorfen stimulated by 4-to 7-fold the activity of HMGR extracted from 'Dekalb XL72AA'

maize seedlings. The *in vivo* activity of maize HMGR was not stimulated by any of the four bleaching herbicides when herbicide-treated maize seedlings were grown under continuous dark conditions. When maize seedlings were germinated and grown in weak light (40 $\mu mol/m^2/s$) intermittent with long dark period (6hr light and 18hr dark), the only herbicide that bleached maize seedlings and stimulated their HMGR activity was clomazome. None of the bleaching herbicides examined in this study exhibited any direct effects on the *in vitro* activity of HMGR extracted from etiolated maize seedlings. The above results suggest a possible interaction between mature chloroplasts and the nucleus of maize cells in regulating the expression of HMGR activity. A cDNA sequence from 'DeKalb XL72AA' maize was amplified by Polymerase-catalyzed chain reaction (PCR) procedures using two oligonucleotide primers that were based on conserved regions of the sequence of the HMGR gene from tomato (*Lycopersicon esculentum* Mill.). Sequencing analysis revealed that this cDNA sequence was exactly the same as that of the tomato HMGR gene. This indicates that the the cDNA might be that of tomato resulting from contamination of the PCR apparatus by tomato nucleic acids. Southern blot analysis, showed that this sequence hybridizes to both maize and tomato genomic DNAs under high stringency conditions. Use of this cDNA sequence as a probe for Northern blot analysis revealed a great discrepancy between the levels of HMGR mRNA and the expression of HMGR activity in maize. The nature of the isolated cDNA sequence is presently unclear. Further research is needed to clarify these results.

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LIST OF ABBREVIATIONS

Acifluorfen, 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid

BSA, bovine serum albumin

Clomazone, 2-(2-chlorophenyl) methyl-4,4-dimethyl-3-isoxazolidinone

CPP, copalyl pyrophosphate

EPTC, S-ethyl dipropylcarbamothioate

Fluridone, 1-methyl-3-phenyl-5-[3-(trifluoromethyl)-phenyl]-4(H)-pyridinone

GGPP, geranylgeranyl pyrophosphate

HMG-CoA, 3-hydroxy-3-methylglutaryl Coenzyme A

HMG-CoA reductase or HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase

Metolachlor, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide

MVA, mevalonate

Norflurazon, 4-chloro-5-(methylamino)-2-[3-(trifluoromethyl)-phenyl]-3(2H)-pyridazinone

PCR, polymerase-catalyzed chain reaction

PPFD, Photosynthetic photon flux density

TLC, thin layer chromatography

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CHAPTER I

INTRODUCTION AND OBJECTIVES

The enzyme 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR, EC 1.1.1.34) catalyzes the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate. Mevalonate is the precursor of all isoprenoid compounds produced by animals and plants (2, 9). Isoprenoid synthesis and in particular the biosynthesis of cholesterol in mammalian systems has been studied extensivelly in recent years. In fact, ten of the numerous investigations on the molecular structure, synthesis, and regulation of cholesterol have been awarded the Nobel Prize in Medicine and Chemistry (10). All this research has proven unequivocally that, in mammalian systems, HMGR is the rate-limiting enzyme for isoprenoid synthesis regulated by a mutivalent feedback inhibition by its end products (9, 32). Accumulation of cholesterol combined with LDL (low-density lipoproteins) or other end products results in a decrease of the HMGR protein and its mRNA (9).

Research on the function and regulation of plant HMGRs has been limited in spite of the fact that plant cells contain a myriad of isoprenoid compounds that play an important role in plant growth and development (2, 6, 19). For a long time, it was assumed that isoprenoid synthesis in plants and animals followed the same route. It is currently recognized, however, that plant isoprenoid synthesis is a highly compartmentalized process. For example, the phytol group of chlorophylls, the side chain of plastoquinone, and carotenoids are synthesized in plastids; phytosterols are synthesized in cytosol

whereas the side chain of ubiquinone is synthesized in mitochondria (19, 21). In turn, it has been suggested that plant HMGRs exist as different isozymes that may be localized in plastids, mitochondria, and the cytosol (8, 19, 21, 23). However, isoprenoid synthesis in plastids is further complicated by the reported inability of plastids to incorporate [^{14}C]acetate and [^{14}C]mevalonate into chlorophylls and carotenoids (18, 21, 31, 41). Mevinolin, a specific inhibitor of HMGR, when applied *in vivo* to radish seedlings it inhibited only their growth while chlorophyll and carotenoid syntheses were unaffected (4, 40). Thus, the subcellular localization and compartmentation of plant HMGR activity is still an unresolved question.

The expression of HMGR activity has been documented in a number of plants including mainly dicotyledonous species such as peas, sweet potato, tobacco, radish, pepper, carrot, soybean, spinach, sunflower, potato and the Hevea rubber plant (*Hevea brasiliensis* L.) (2, 6). Assays of HMGR activity in monocotyledonous plants are surprisingly limited. As of today, barley is the only monocotyledonous plant whose HMGR activity has been studied to any degree (1). According to a very recent review summarizing the enzymology of mevalonate biosynthesis in plants (6), studies on the expression of HMGR in maize, a major crop of agronomic importance, have not been published.

The activity of plant HMGR is known to be regulated by light (8, 46). Etiolated pea seedlings have high HMGR activity, but after exposure to light, their HMGR activity drops rapidly (8). It is well known that when etiolated seedlings turn green they synthesize large amounts of chlorophyll and carotenoid pigments (16, 18, 28). During

this time, however, their HMGR activity decreases considerably. It has also been observed that immature tomato fruits have high HMGR activity. However, when the fruit turns red and lycopene synthesis is at a maximum HMGR activity is considerably lower (33). It is obvious then, that the relationship between plant isoprenoid synthesis and HMGR activity is not always clear.

In addition to light, plant HMGR activity has been shown to be regulated by phytohormones (3, 8, 37), phytochrome (8, 46), endogenous protein factors (30), and fungal infection (42). Grumbach and Bach (20) reported that SAN 6706, a methylated derivative of the bleaching herbicide norflurazon, stimulated the activity of HMGR associated with the plastidic fraction of radish seedlings.

Terpenoid biosynthesis has long been established as a target site involved in the phytotoxic action of several herbicides (45). Herbicidal inhibitors of carotenoid pigments include phenylpyridazinone (e.g norflurazon), aminotriazole (e.g. amitrole), pyridinone (e.g. fluridone) and isoxazolidinone (e.g. clomazone) derivatives (38). Norflurazon and fluridone interfere with carotenoid biosynthesis by inhibiting mainly the desaturation reactions of phytoene (38). Clomazone has been reported to interfere with chloroplast development (14) and to bleach sensitive plants by inhibiting two key enzymes regulating the formation of geranylgeranyl pyrophosphate (GGPP) from isopentenyl pyrophosphate (39). Two recent reports, however, have disputed these previous results and the exact mechanism of the bleaching action of clomazone is still unknown (27, 43). Inhibition of gibberellic acid precursor biosynthesis by chloroacetanilide (e.g. metolachlor) and

carbamothioate (e.g. EPTC) herbicides has been reported (45). In addition, the herbicidal activity of clomazone appears to be related, at least partially, to an inhibition of gibberellin biosynthesis (14, 47). Finally, diphenyl ether herbicides (e.g. acifluorfen-methyl) have been shown to interfere with tetrapyrole synthesis in greening chloroplasts of sensitive species by inhibiting the enzyme protoporphyrinogen oxidase which catalyzes the conversion of protoporphyrinogen IX to protoporphyrin IX (29).

During the last decade, research on the expression and regulation of mammalian HMGR has been greatly enhanced following the molecular cloning of the HMGR gene from Chinese hamster (12, 13). From the full length cDNA clone of this gene, the amino acid sequence of hamster HMGR was deduced and the actual molecular weight of the HMGR protein was determined to be 97-kDa (12). Based on the predicted amino acid sequence, it was determined that the the hydrophobic amino-terminal region of the HMGR protein spans the endoplasmic reticulum seven times (12). Directed-site mutagenesis experiments showed that the membrane spanning region plays an important role in the degradation of the HMGR protein (10). Genomic DNA sequences showed that the transcription of the HMGR gene is under the control of a special promotor which lacks a TATA or CCAAT box and initiates transcription from multiple sites (36). The promotor contains a site to which a regulatory element may bind and this mechanism is believed to be involved in the regulation of the transcription of the HMGR gene by cholesterol (34).

HMGR genes of eukaryotic organisms are highly conserved. Supporting evidence is provided by the successful use of a cDNA probe from

hamster HMGR gene for isolating HMGR genes from human cells, yeast and Drosophila (7, 17, 26). The membrane-spanning, amino-terminal region was shown to be the most highly conserved region of HMGR protein among these species, but the carboxyl-terminal catalytic site was also conserved (26).

The successful use of cDNA probes from hamster and yeast HMGR genes for the isolation of HMGR genes from several plant species such as Arabidopsis (11, 24), radish (15), and tomato (33, 35) has been reported. Synthetic oligonucleotide sequences based on the conserved catalytic site of other species, have also been used for the isolation of an HMGR gene from tomato (33). The amino acid sequence of HMGR predicted from the cDNA of the Arabidopsis HMGR gene was drastically different from that reported for mammalian HMGRs in the membrane spanning region, since its amino-terminal end had only two membrane spanning regions instead of seven (11, 15).

In spite of repeated efforts, purification of HMGR from plants has proven difficult because of the low content of the enzyme in plants and its strong binding to the membranes of the endoplasmic reticulum. Although partial purification of radish HMGR has been reported (5, 22), reports on the production of specific antibodies for plant HMGR are currently unavailable. Recent advances in molecular biology techniques, however, have offered some solutions to the perennial problem of plant HMGR purification. Ferrer et al (15) reported recently that by cloning a cDNA sequence coding for radish HMGR into the vector pET-8c and expressing it in Escherischia coli, large amounts of pure radish HMGR could be produced.

OBJECTIVES OF DISSERTATION RESEARCH

The major goal of this research was to document the occurence of HMGR in maize and to study its regulation by light, genetic variability and bleaching herbicides. Specific objectives were as follows:

1. Document the expression and subcellular localization of HMGR activity in maize and determine the developmental expression and tissue specificity of HMGR activity in maize.

2. Evaluate the potential regulation of HMGR activity by genetic variability by investigating its expression in a normal 'DeKalb XL72AA' maize hybrid, a dwarf ($\underline{d_5}$, gibberellin-deficient) and an albino ($\underline{lw_3}$, chlorophyll- and carotenoid-deficient) mutant of maize.

3. Investigate the light-induced regulation of HMGR activity in maize.

4. Examine the potential effects of selected herbicides interfering with isoprenoid synthesis on the activity of maize HMGR. Herbicides selected for these experiments included the bleaching herbicides clomazone, norflurazon, fluridone and acifluorfen and the growth retarding herbicides EPTC and metolachlor.

5. Use two oligonucleotide primers from the conserved region of tomato HMGR gene and employ polymerase-catalyzed chain reaction (PCR) procedures to amplify an HMGR sequence from maize cDNA.

6. Use this amplified sequence for determining the number of HMGR genes in maize and for studying the developmental regulation of maize HMGR at the mRNA level.

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CHAPTER II

EXPRESSION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE ACTIVITY IN MAIZE

INTRODUCTION

3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR; EC 1.1.1.34) catalyzes the major rate-limiting reaction in animal and plant isoprenoid biosynthesis which is the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate (3, 12). In turn, mevalonate serves as the precursor of many isoprenoid compounds produced by both animals and plants (reviewed in 5, 9). Brown and Goldstein 1980). Because of its critical role in cholesterol biosynthesis and the modification of proteins (9, 20, 22), HMGR has been studied extensively in mammalian systems. By comparison, research on the expression, function, and regulation of plant HMGRs has been rather limited in spite of the diverse array of biologically important isoprenoid compounds produced by plants.

The expression of HMGR activity has been documented in a number of plants including mainly dicotyledonous species such as peas, sweet potato, tobacco, radish, pepper, carrot, soybean, spinach, sunflower, potato, and the Hevea rubber tree (Hevea brasiliensis L.) (reviewed in 3, 5). Assays of HMGR activity in monocotyledonous plants are surprisingly limited. As of today, barley is the only monocotyledonous plant whose HMGR activity has been studied to any degree (2). According to a very recent review summarizing the enzymology of mevalonate biosynthesis in plants (5), studies on the activity of HMGR in maize, a major crop of agronomic importance, have not been published.

Plant isoprenoid synthesis is a highly compartmentalized process. For example, the phytol group of chlorophylls, the side chain of plastoquinone, and carotenoid pigments are synthesized in plastids; and phytosterols are synthesized in cytosol, while the side chain of ubiquinone is synthesized in mitochondria (12, 15). Consequently, it has been suggested that plant HMGRs may exist as specialized isozyme forms localized in the cytosol, plastids, and mitochondria (4, 7, 16, 23, 26). Although the compartmentation of plant HMGR activity is still an unresolved and controversial question, the primary subcellular localization of plant HMGR appears to be the membrane of the endoplasmic reticulum (5, 12).

The activity of plant HMGR is known to be regulated by light (8, 26). Etiolated pea seedlings have high HMGR activity, but after exposure to light, their HMGR activity drops down rapidly (8). It is well known that when etiolated seedlings turn green they synthesize large amounts of chlorophyll and carotenoids (10, 11, 12). During this time, however, their HMGR activity is substantially lower than that of etiolated tissue. It is obvious then, that the relationship between plant isoprenoid synthesis and HMGR activity is not always clear.

The objectives of the present study were to a) document the expression and subcellular localization of HMGR activity in maize; b) determine the developmental expression and tissue specificity of HMGR activity in maize; and c) investigate the effect of light on HMGR activity in maize. In addition to the normal 'DeKalb XL72AA' maize hybrid, a dwarf ($\underline{d}_5$, gibberellin-deficient) and an albino ($\underline{lw}_3$,

chlorophyll- and carotenoid-deficient) mutant of maize were included in some of the aforementioned studies for comparative purposes.

MATERIALS AND METHODS

Chemicals. Radiolabeled HMG-CoA (Hydroxy-3-methylglutaryl coenzyme A, DL-3-glutaryl-3-^{14}C; sp. act. 58.0 mCi/mmol) was purchased from DuPont's New England Nuclear Research Products (Boston, Massachussetts). The Coomasie protein determination kit, which employs the method of Bradford (6), was purchased from Pierce (Rockford, Illinois). All other reagents were purchased from Sigma Chemical Company (St. Louis, Missouri).

Plant material. Seeds of the normal 'DeKalb XL72AA' maize hybrid were provided by DeKalb AgResearch, Inc. (DeKalb, Illinois). Seeds of dwarf (d_5, catalog number 17-7110) and albino (lw_3, catalog number 17-7100) maize mutants were purchased from Carolina Biological supply Company, Burlington, North Carolina.

Studies on the subcellular localization, developmental expression and tissue specificity of maize HMGR activity were conducted with seed, leaf, or root tissues of normal 'Dekalb XL72AA' maize. Maize seeds were placed in petri dishes (9-cm dia.), covered with vermiculite and germinated in a growth chamber at 30 ^{o}C. Etiolated seedlings were grown in complete dark for four or more days. Maize leaf and root tips used for HMGR extraction were 1 cm long, whereas leaf base tissues included 0.5 cm long segments cut immediately above the first node of maize shoots. In the study of the developmental expression of HMGR activity in germinating maize seeds, seed tissue excluding leaf or root tissues was assayed for HMGR activity at selected time intervals after seed imbibition and the initiation of germination.

Effect of light on HMGR activity. Etiolated and light-grown seedlings of the three genetic lines ('Dekalb XL72AA', dwarf, and albino) of maize were used in these studies. Light-treated maize seedlings were germinated and grown for four or more days in a chamber with a 24-h photoperiod provided by a mixture of low pressure sodium, cool-white fluorescent, and incandescent lamps. The photosynthetic photon flux density (PPFD) was 600 $\mu mol/m^2/s$ in the band from 400 to 700 nm. The same fluence rate was used for the exposure of etiolated maize seedlings to light. For the exposure of maize roots to light, vermiculite was washed at the desirable time period and the seedlings were placed in petri dishes containing wet filter papers.

HMGR extraction. Crude enzyme extracts were isolated following the procedure of Russell (21) with modifications. All steps were carried out at 0-4 ^oC unless noted otherwise. Leaf or root tissues (0.5-2 g) were frozen in liquid nitrogen and ground with a mortar and pestle after adding 6 ml of a homogenizing buffer containing 10 mM Tris-HCl (pH 7.0), 0.35 M sucrose, 30 mM EDTA, 10 mM 2-mercaptoethanol, 0.1% BSA and 3.5% (w/v) polyvinylpolypyrrolidone. The homogenate was sqeezed through a layer of Miracloth and the filtrate centrifuged in a swinging bucket rotor at 500 g for 3 min to remove cell debris and nuclei. The supernatant was then centrifuged in a swinging bucket rotor at 3,500 g for 3 min to pellet plastids. The plastids were ruptured following suspension in a medium containing 10 mM MOPS buffer (pH 7.5); 10 mM KCl; 25 mM EDTA; and 10 mM dithiotheitol. The rupture medium was used in a ratio of 2 ml per gram of original tissue. Plastidic membranes were collected by centrifugation and used for assaying

plastid HMGR activity following suspension in a medium containing 0.25 M potassium phosphate monobasic (pH 7.8) and 25 mM dithiotheitol. The plastid membrane was suspended in this medium at a ratio of 30 µl suspension medium per gram of original tissue. The supernatant of the 3,500 x g centrifugation was then centrifuged at 12,000 x g for 15 min to pellet mitochondria and chloroplasts and avoid contamination of the microsomal fraction. The supernatant of this centrifugation was then centrifuged at 105,000 g for 1 h to pellet microsomal membranes which were suspended in a medium containing 0.2 M potassium phosphate monobasic (pH 6.9) and 25 mM dithiotheitol. The microsomal membrane pellet was suspended in its suspension medium in a ratio of 0.15 ml suspension medium per gram original tissue.

Protein assay. Protein content of the membrane preparations was measured by using the Pierce Coomassie Protein Assay Reagent which employs the Bradford (6) method for protein determination with Bovine serum albumin (BSA) as a standard.

HMGR assay. HMGR activity was determined by a modified radioassay method described by Russell (21). The enzyme assay mixture was prepared on ice in 1.5 ml microcentrifuge tubes. The mixture consisted of 1 µl 0.2 M dithiotheitol, 1 µl 160 mM NADPH, 5 µl enzyme extract (microsomal, pH 6.9 or plastidic, pH 7.8), 7 µl 0.3% BSA solution and 6 µl of radioactive DL-[3-^{14}C] HMG-CoA containing 12 nCi in 0.204 nmol. The reaction mixture was incubated for 30 min in a water bath at 30 ^{o}C. The reaction was stopped by adding 2 µl 6 M HCl and 2 µl 1 M mevalonate solution. The mevalonate solution was prepared by mixing 2 M mevalonate-lactone and 0.1 M KOH in 1:1 (v/v) ratio. After mix-

ing, the reactants were incubated at room temperature for 1 h for mevalonate lactonization to be completed. Then, the reactants were centrifuged at about 10,000 g for 3 min to pellet membrane fragments. The supernatant was then chromatographed by thin-layer chromatographic (TLC) analysis by applying 10 µl of supernatant on Whatman LK5OF silica gel plates (20 x 20 cm). The plates were developed in 50 ml of diethylether-acetone (3:1 v/v) for 15 min and analyzed by X-ray autoradiography as illustrated in Fig. 1. Radioactive spots corresponding to the substrate HMG-CoA (Rf=0.0) and the product mevalonate (Rf=0.9) were scraped and analyzed by liquid scintillation spectrometry. Specific activity of HMGR was calculated after correcting for counting efficiency and recovery of MVA-lactone from the TLC plate, and was expressed as nmol of mevalonate formed per mg protein per hour.

Each treatment was duplicated and each experiment was repeated at least two times. Variability differences in the levels of HMGR activity between experiments were observed, but the trends were similar in all experiments.

RESULTS

Expression and subcellular localization of HMGR activity in maize. Data in Figs. 1 and 2 demonstrate that leaf tips of 4-day-old etiolated maize seedlings possess high HMGR activity which appears to be associated exclusively with microsomal membranes. The microsomal HMGR activity of maize required NADPH as a co-factor (Fig. 1). NADH, however, was not a co-factor of HMGR extracted from maize microsomal membranes. The activity of maize microsomal HMGR was stable for at least 8 hr at 25 ^{o}C or for 3 months when stored at -20 ^{o}C. The *in vitro* activity of microsomal HMGR extracted from maize was not inhibited by light following exposures as long as 8 h. Because of this finding, all HMGR assays reported in this paper were conducted under regular room light conditions. These results are in contrast to previous studies reporting that HMGR activity from peas is very sensitive to light (21). Similar to previously published reports (5), the activity of maize microsomal HMGR was shown to be inhibited strongly by mevinolin (see Chapter III).

Crude extracts of plastidic membranes from shoots or roots of either etiolated or green 4-day-old maize seedlings were capable of converting radiolabeled HMG-CoA to a product that is different from mevalonate (Fig. 2). The identity of this product is currently unknown. NADPH was not necessary for the conversion of radiolabeled HMG-CoA to this product by crude extracts of maize plastidic membranes. Crude extracts from mitochondrial membranes (suspended pellets following centrifugation at 12,000 x *g*) were also incapable of converting radioalebeled HMG-CoA to mevalonate.

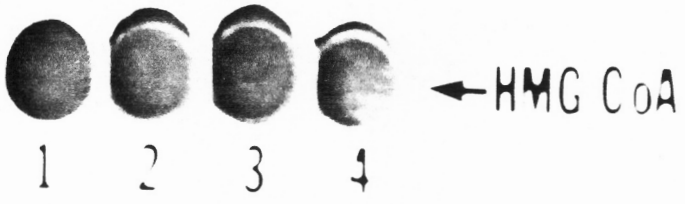

Fig. 1. Autoradiograph demonstrating the dependence of HMGR activity on NADPH. HMGR was measured with microsomal membranes isolated from leaf tips of 4-day-old etiolated maize seedlings. Radiolabeled substarte (HMG-CoA) is shown on the bottom of the autoradiograph. The product mevalonate (MVA) is shown on the top. Lane 1 represents HMG-CoA alone; Lane 2 represents HMG-CoA + crude HMGR; Lane 3 represents HMG-CoA + NADPH; Lane 4 represents HMG-CoA + crude HMGR + NADPH.

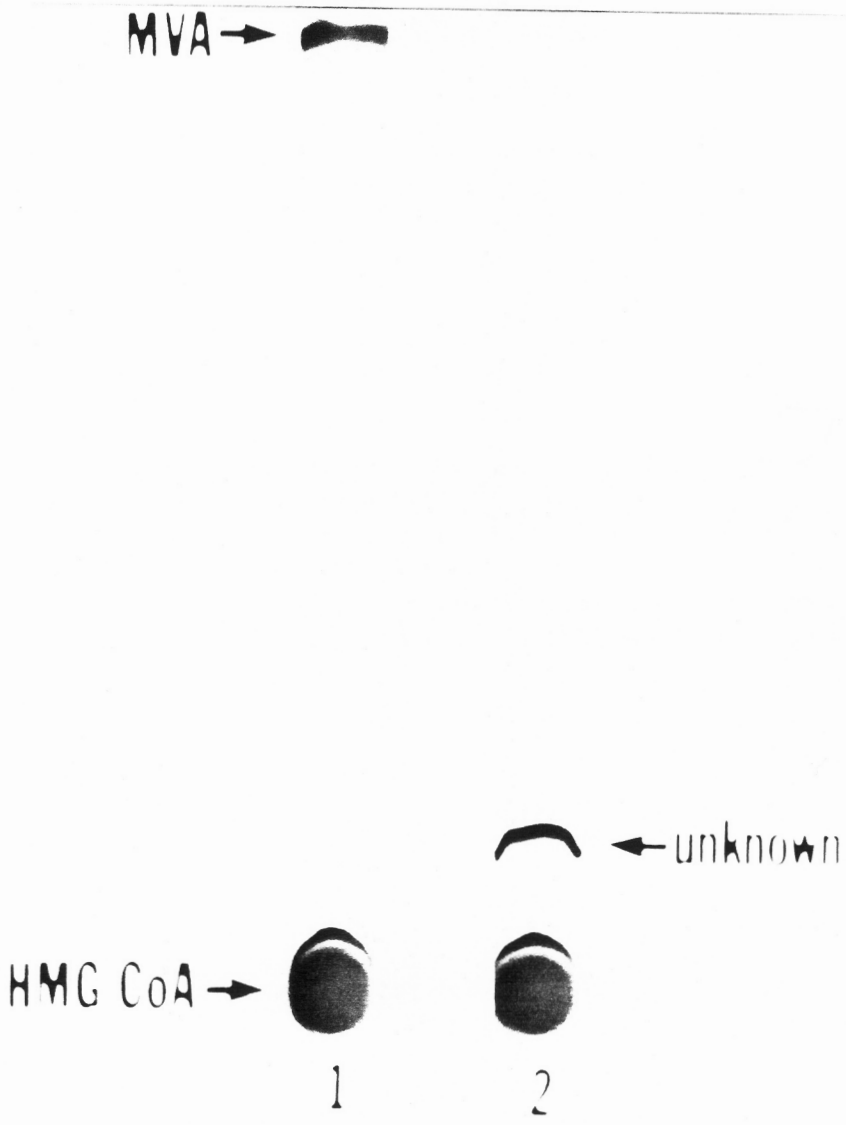

Fig. 2. Autoradiograph demonstrating the radioassay used to measure HMGR activity in subcellular membrane fractions isolated from leaf tips of 4-day-old etiolated maize seedlings. Radiolabeled substrate (HMG-CoA) was incubated with crude HMGR extracted from microsomal (Lane 1) and plastidic (Lane 2) membranes. Mevalonate (MVA), the product of the reaction catalyzed by HMGR, was formed only in the presence of microsomal membranes.

Developmental expression and tissue specificity of HMGR activity in maize. Microsomal HMGR activity is expressed in dry seeds of 'DeKalb XL72AA' maize, but at very low levels (Fig. 3). Following imbibition of the seeds with water and the initiation of germination, there is an increase in HMGR activity, reaching a maximum around 24 h. Microsomal HMGR activity remains high in germinating maize seeds up to 48 h and then decreases reaching a minimum at 72 h following the initiation of germination (Fig. 3). This drop of HMGR activity in maize seeds during the period of 48 to 72 h following the initiation of germination, coincides with the emergence of shoots and roots which were removed from the seed samples. Roots and shoots possess typically greater microsomal HMGR activity than germinating maize seeds.

Table 1 presents the results of a study assaying the expression of microsomal HMGR activity in different zones of roots of 4-day-old etiolated seedlings of 'DeKalb XL72AA' maize. It is evident that root zones with high meristematic activity such as the root tip (0-1 cm) and the site of origin of lateral roots (approximately 8 cm from root tip) possess relatively high HMGR activity. Microsomal HMGR activity in the root elongation and root hair zone (1-4 cm) is about half of that found in root tips or in the site of origin of lateral roots (Table 1). The results of studies examining the expression of HMGR activity in leaf tissues of maize are discussed in the next section.

Regulation of maize HMGR activity by light. A comparison of the activity of microsomal HMGR extracted from leaf tissues of 4-day-old etiolated and light-grown seedlings of 'DeKalb XL72AA', dwarf and albino maize illustrates the strong regulatory effect of light on this

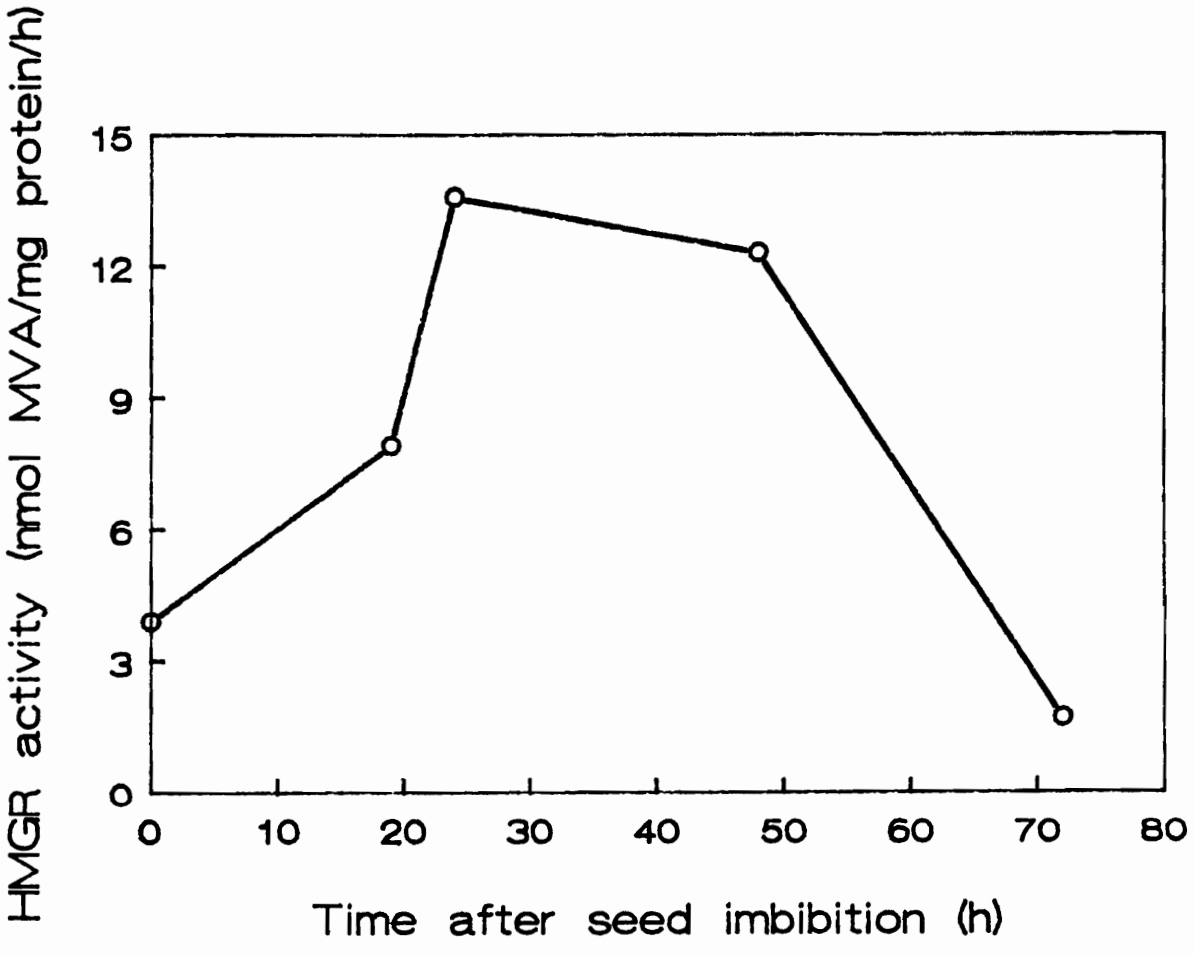

Fig. 3. Developmental expression of microsomal HMGR activity in germinating seeds of 'DeKalb XL72AA' maize at selected time intervals after seed imbibition and initiation of germination.

enzyme (Fig. 4). The dwarf ($\underline{d_5}$) mutant of maize is a single gene mutant blocked between copalyl pyrophosphate (CPP) and ent-kaurene, a key step in gibberellin (GA) biosynthesis catalyzed by ent-kaurene b synthetase (14). Seedlings of this dwarf maize mutant lack this enzyme and their ability to synthesize ent-kaurene from MVA, GGPP, and CPP is one-fifth of that of normal maize lines (14). The albino mutant of maize contains a lethal recessive gene for albinism ($\underline{lw_3}$) and is unable to synthesize chlorophyll and carotenoid pigments.

In etiolated seedlings of the three maize lines, microsomal HMGR was extracted from leaf tips, excluding the coleoptile. Expanded leaves, excluding the leaf base, were used for HMGR extraction from light-grown seedlings of the three maize genotypes. Microsomal HMGR activity was significantly lower when extracted from leaf tissues of light-grown seedlings of all three genetic lines of maize (Fig. 4). The ratio of etiolated to light-grown HMGR activity was about 3 in albino, 4 in normal ('DeKalb XL72AA'), and 5 in dwarf maize.

Table 2 presents the results of a time-course study examining the inhibitory effect of light on the activity of maize microsomal HMGR. HMGR was extracted from leaf tips of 4-day-old etiolated seedlings of 'DeKalb XL72AA' maize at various time intervals following their exposure to strong light (600 $\mu mol/m^2/s$) and the initiation of the greening process. It is evident that while the inhibitory effect of light on HMGR activity of maize is dramatic, it is expressed slowly requiring times of continuous exposure greater than 10 h (Table 2). Short term exposure of etiolated maize seedlings to light (15 min pulse with strong light) did not inhibit the activity of microsomal

TABLE 1

Expression of microsomal HMGR activity in maize root tissues. HMGR was extracted from root sections of 4-day-old etiolated maize seedlings. Values are means of 2 replications.

Root zone	Distance from root tip (cm)	HMGR activity (nmol MVA/mg protein/h)
Root tip	0 - 1	59
Root elongation	1 - 2	28
& root hair	2 - 3	23
zones	3 - 4	24
Site of origin of lateral roots	8 - 9	56

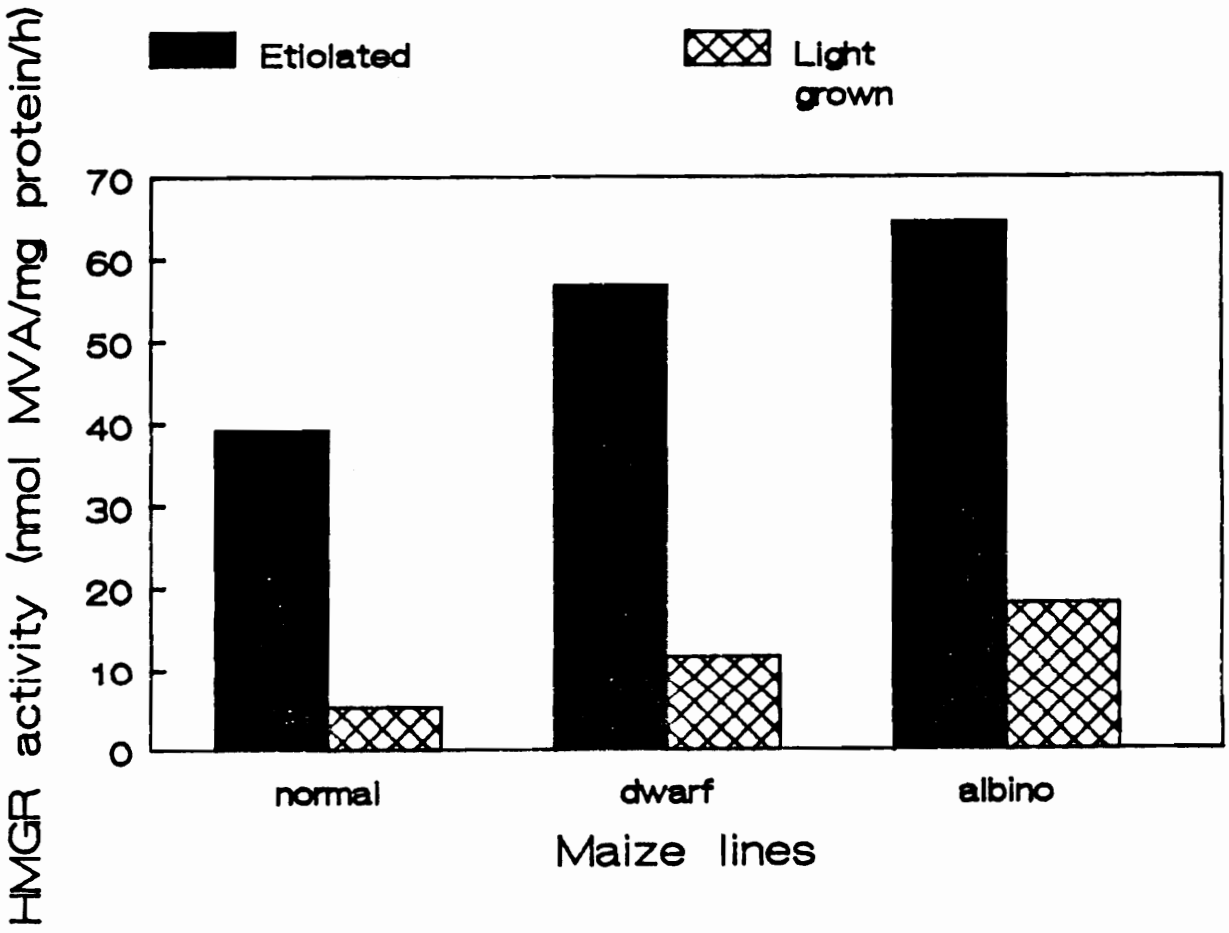

Fig. 4. Expression of microsomal HMGR activity in leaf tissues of 4-day-old etiolated and light-grown seedlings of normal ('DeKalb XL72AA'), dwarf ($\underline{d}_5$) and albino ($\underline{lw}_3$) maize.

TABLE 2

Light-induced regulation of microsomal HMGR activity extracted from leaf tips of 4-day-old maize seedlings. HMGR activity was assayed at selected time intervals after exposure of these seedlings to strong light (600 $\mu mol/m^2/s$). Values are means of two replications.

Duration of exposure to strong light (h)	HMGR activity (nmol MVA/mg protein/h)
0	42
2	43
4	50
6	41
8	47
10	50
26	5
45	4

HMGR extracted from leaf tips. Etiolated maize seedlings exposed to light turned greener during the time periods of 10-26 h. The activity of microsomal HMGR extracted from maize seedlings exposed to light for this period (10 - 26 h) was variable because of the variable degree of greening in the leaves of these seedlings. These results demonstrate a negative correlation between plastid-associated isoprenoid synthesis and microsomal HMGR activity since at a time of high rates of chlorophyll and carotenoid biosynthesis by these maize seedlings, the activity of microsomal HMGR decreases considerably.

Data in Fig. 5 illustrate the effects of light on the activity of maize microsomal HMGR extracted from leaf tips as well as from leaf bases of 3.5-and 5-day-old seedlings of 'DeKalb XL72AA' maize. HMGR activity is expressed highly in leaf bases of both etiolated and light-grown maize seedlings. By comparison, microsomal HMGR activity is expressed highly in leaf tips of 3.5-day-old etiolated seedlings, but it drops by more than 50% in leaf tips of 5-day-old etiolated seedlings of 'DeKalb XL72AA' maize. HMGR activity extracted from leaf tips of 3.5-or 5-day-old maize seedlings grown in the light is considerably lower than that of etiolated seedlings.

Microsomal HMGR activity extracted from tips of maize roots exposed to light was insensitive to any inhibitory effects induced by light, following exposure times of up to 60 h (Fig. 6). Thus, with respect to light-induced regulation, microsomal HMGR activity extracted from root tips appeared to behave similar to that extracted from leaf bases and opposite to that extracted from leaf tips.

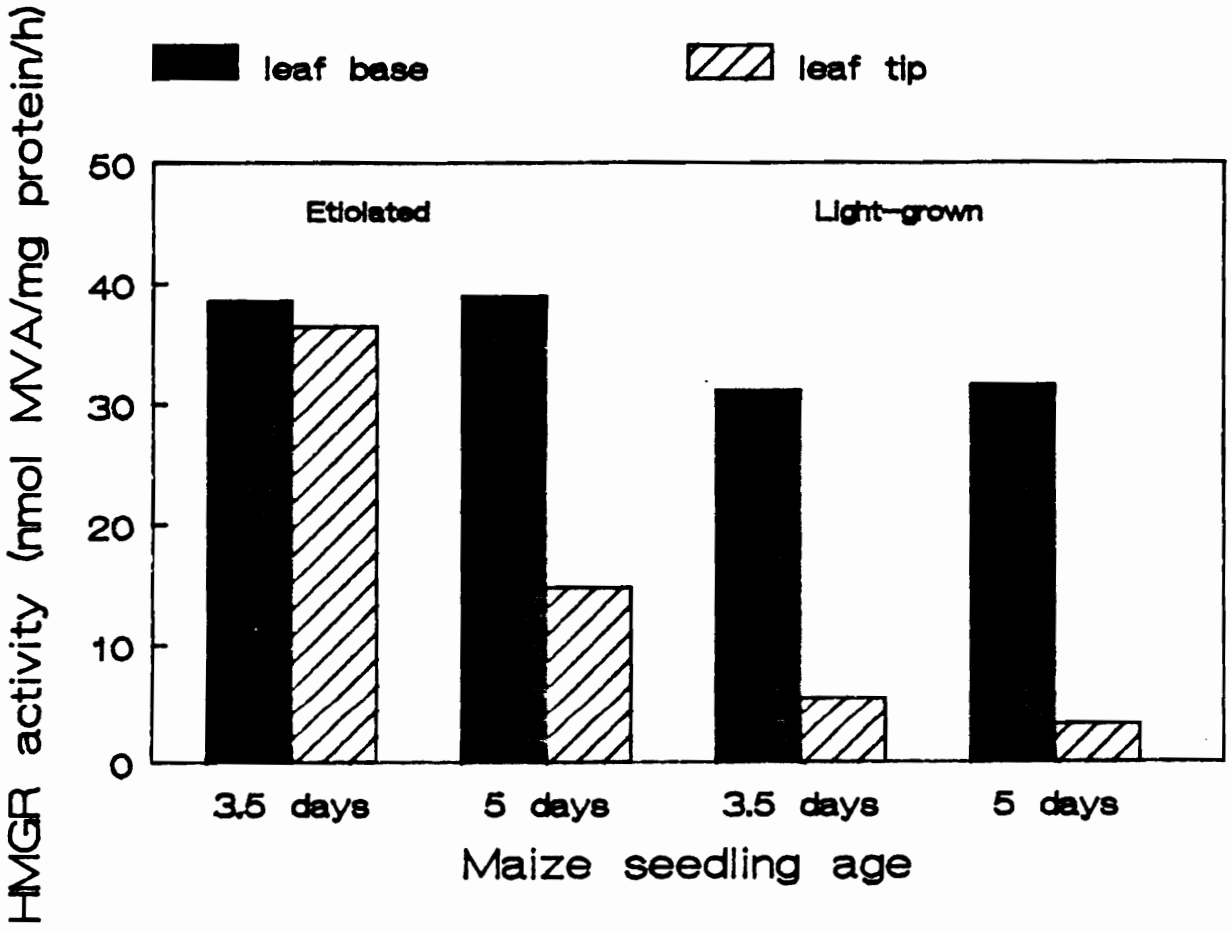

Fig. 5. Expression of microsomal HMGR activity in leaf bases and leaf tips of etiolated or light-grown maize seedlings. HMGR activity was assayed at 3.5 and 5 days after the initiation of seed germination.

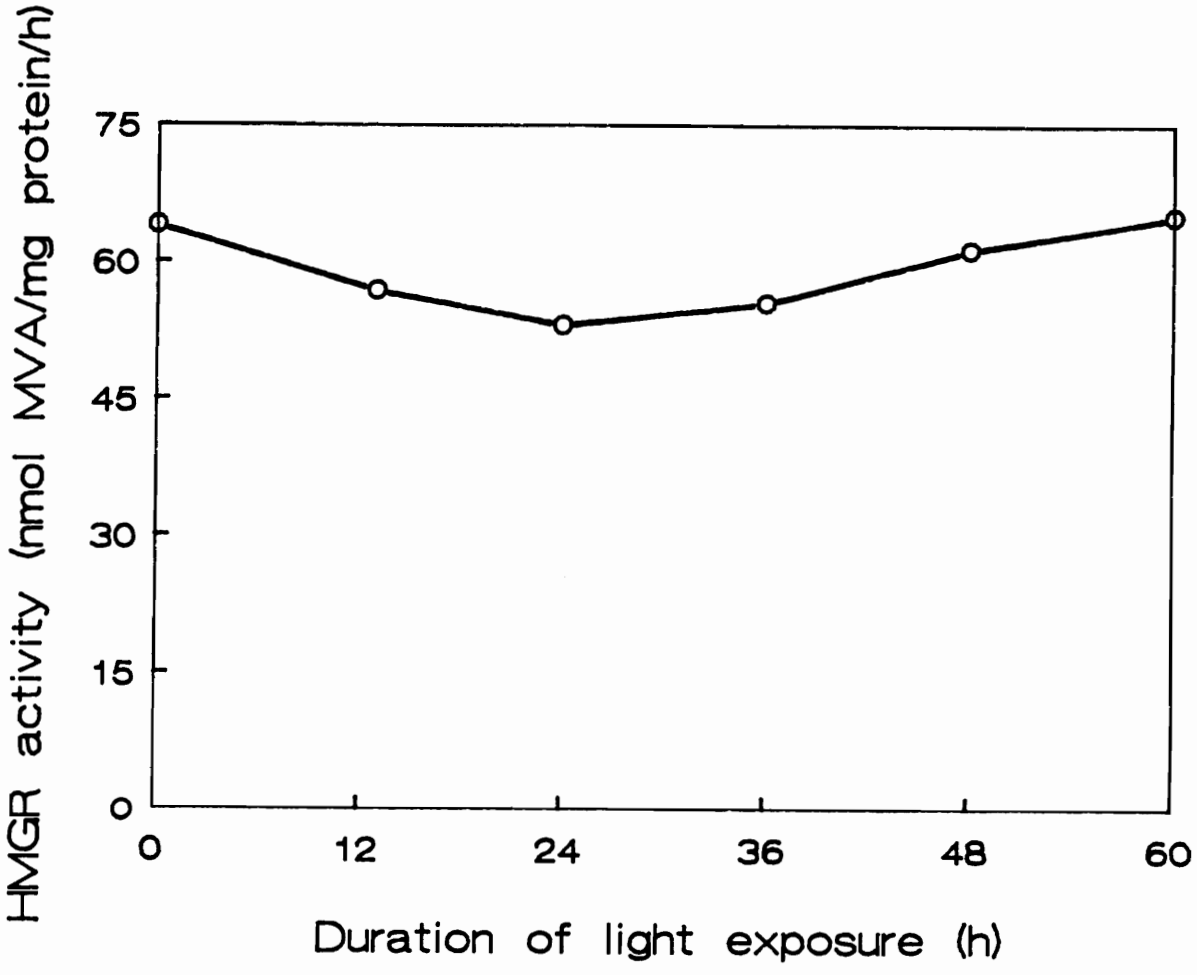

Fig. 6. Effect of light on the expression of microsomal HMGR activity extracted from root tips of 4-day-old etiolated maize seedlings. HMGR activity was assayed at selected time intervals following exposure of maize roots to strong light.

DISCUSSION

The results of the present study demonstrate for the first time the expression of microsomal HMGR activity in maize. Thus, in addition to barley (2), maize becomes the second monocotyledonous plant species with reported HMGR activity. The isolation and characterization of cDNA and genomic DNA fragments encoding wheat (Triticum aestivum L.) HMGR has been reported recently by Aoyagi et al. (1). However, specific assays of HMGR activity extracted from wheat were not included in that study.

Maize tissues with high meristematic activity such as germinating seeds, leaf bases, root tips, and the site of origin of lateral roots contained high levels of HMGR activity (Table 1 and 2, Fig. 3). HMGR activity of maize seedlings appeared to be exclusively associated with membranes of the endoplasmic reticulum (microsomal fraction) rather than membranes of the plastidic fraction of maize cells (Fig. 2). Similar to previously published reports on HMGRs from other plant species (21), maize microsomal HMGR activity was strongly dependent on NADPH (Fig. 1). The subcellular localization of plant HMGR has been a controversial issue for a long time (5, 12, 15). In preliminary studies with soybean seedlings, we were able to obtain good HMGR activity from plastidic membranes (Ji and Hatzios, unpublished results). In the present study, however, we did not detect any HMGR activity in maize plastidic membranes in spite of repeated efforts. The conversion of radiolabeled HMG-CoA to an unknown metabolite by crude enzyme extracts of maize plastidic membranes (Fig. 2) is interesting and needs to be invetsigated further in the future.

Microsomal HMGR activity was highly expressed in 4-day-old etiolated seedlings of normal ('DeKalb XL72AA'), gibberellin-deficient dwarf (d_5), and chlorophyll- and carotenoid-deficient albino (lw_3) maize (Fig. 4). Isoprenoid derivatives such as giberrellins, carotenoids, and the phytol group of chlorophylls are synthesized in the plastids of plant cells (12, 15). The high expression of microsomal HMGR activity in the dwarf and albino mutants of maize demonstrates again the lack of any correlation between microsomal HMGR activity and plastid-associated isoprenoid synthesis. Thus, it is likely that the microsomal HMGR which is highly expressed in maize may be related with the sterol biosynthesis and membrane biogenesis rather than plastidic isoprenoid synthesis.

Similar to previous studies with HMGR from dicotyledonous plants (8, 26), the activity of microsomal HMGR extracted from leaf tips of normal, dwarf, and albino maize seedlings is regulated by light (Fig. 4). However, the initiation time of the light-induced inhibition of maize HMGR activity was greater than that reported in studies on the light regulation of pea HMGR activity (8). Microsomal HMGR activity from leaf tips of 4-day-old maize seedlings was inhibited significantly only after exposure to strong light for periods greater than 10 h (Table 2). By comparison, microsomal HMGR activity from leaf bases and root tips of maize was insensitive to the effects of strong light (Figs. 5 and 6). These results emphasize again the unclear relationship between plastidic isoprenoid synthesis and microsomal HMGR activity. As mentioned earlier, HMGR activity would be expected to be high when chlorophyll and carotenoid biosyntheses are at a maximum

such as when etiolated seedlings are exposed to light and turn green. A similar situation has been reported by Narita and Gruissem (18), who studied the expression of HMGR activity in tomato fruits. Immature tomato fruits (0.2-0.4 cm-dia.) have high HMGR activity, but when these tomato fruits turn red, a developmental stage characterized by an enhanced synthesis of carotenoids such as lycopene, their HMGR activity drops significantly.

To explain this obvious discrepancy between isoprenoid synthesis and HMGR activity, Wong et al. (26) proposed that plastidic HMGR activity, which has been demonstrated in peas, is under a positive phytochrome control. However, compared to microsomal HMGR, the activity of plastidic HMGR is low and may not be enough to support the high rates of chlorophyll and carotenoid synthesis occurring when etiolated pea seedlings turn green. Brooker and Russell (8) have also proposed the involvement of a phytochrome effect in the regulation of pea HMGR activity by light. In our studies the effect of light on microsomal HMGR apears to be indirect and irreversible. The activity of microsomal HMGR extracted from light-grown 4-day old maize seedlings is low and it does not increase following the placement of these seedlings under conditions of continuous dark for 12 h. These results suggest that the light-induced regulation of maize microsomal HMGR activity may be related to the the direct effects of light on organellar differentiation such as the conversion of etioplasts to chloroplasts taking place in maturing seedlings growing under light conditions.

Our data indicate that microsomal HMGR activity is highly expressed in meristematic tissues of maize. A positive correlation

between rapidly dividing cells and HMGR activity has also been demonstrated in animal research. Rapidly proliferating cells such as brain cells of young animals and intestine cells have high HMGR activity, while kidney cells, which undergo a slow turnover, have very low HMGR activity (25). By using synchronized cell cultures, Quesney-Huneeus et al. (19) have shown that HMGR activity increases just before the S phase and that treatement with the HMGR inhibitor, compactin, during this phase blocks the synthesis of DNA by these cell cultures. This compactin-induced inhibition of cell DNA synthesis could be reversed by mevalonate but not by cholesterol treatment (19). Using an HMG-CoA synthase mutant (Mev-1), Sinensky and Logel (24) further showed that several polypeptides can be labeled with radiolabeled mevalonate. Upon mevalonate starvation, these peptides disappeared and their turnover rate followed closely the rate of decreased DNA synthesis by these Mev-1 mutants.

Recent research with mammalian tissues has shown that all pro-oncogenic or ongogenic ras proteins are polyisoprenylated (13). This finding coupled with the documented increase of HMGR activity in rapidly dividing mammalian cells suggests that mevalonate derivatives may play a very important role in cell division. The potential involvement of HMGR in mammalian cell development is currently the subject of extensive research. The possible role of plant HMGR in cell division and plant growth remains to be investigated.

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CHAPTER III

STIMULATION OF MAIZE 3-HYDROXY-3-METHYLGLUTARYL-COENZYME A REDUCTASE ACTIVITY BY CLOMAZONE AND OTHER BLEACHING HERBICIDES

INTRODUCTION

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGR; EC 1.1.1.34) catalyzes the conversion of HMG-CoA to mevalonate. Mevalonate is the precursor of many isoprenoid compounds in animal and plant cells (1). In mammalian cells, HMGR is a rate-limiting enzyme for isoprenoid synthesis and it is under multivalent feedback regulation by its end products. Accumulation of cholestrol (combined with low-density lipoproteins) or other end products results in a decrease of the HMGR protein and its mRNA (2). Because of its crucial role in synthesis of cholesterol and prenyl moieties involved in modification of oncogenic ras proteins (3), HMGR has been studied extensively in mammalian systems.

HMGR activity has been assayed in a number of plants including primarily dicotyledonous species such as peas, sweet potato, tobacco, radish, pepper, carrot, soybean, spinach, sunflower, potato, and the Hevea rubber plant (Hevea brasiliensis) (1). With the exception of barley, very little is known about HMGR in monocotyledonous plants (1). Based on the currently available literature, HMGR has not been studied in maize, a major agronomic grass crop.

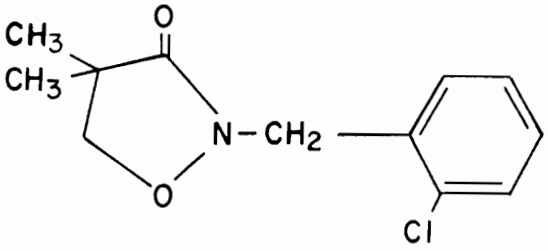
Research on the function and regulation of plant HMGRs has been limited in spite of the fact that plant cells contain a myriad of isoprenoids compounds (1). Plant isoprenoid synthesis is a highly com-

partmentalized process. For example, the phytol groups of chlorophylls, the side chain of plastoquinone, and carotenoids are synthesized in plastids; phytosterols are synthesized in cytosol and the side chain of ubiquinone is synthesized in mitochondria (4). In turn, it has been suggested that plant HMGRs exist as different isozymes that may be localized in plastids, mitochondria and the cytosol (5-10). At present, however, the compartmentation of plant HMGR activity is still an unresolved question (5). Plant HMGR activity has been shown to be regulated by phytohormones (11-13), phytochrome (8, 13), endogenous protein factors (14), and fungal infection (15). The results of research presented in Chapter II of this dissertation showed that HMGR activity is highly expressed in maize, it is compartmentalized in the microsomal membrane fraction and is regulated by light.

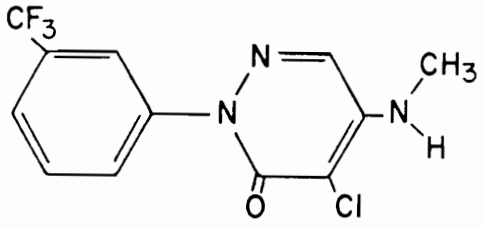
Terpenoid biosynthesis has long been established as a target site involved in the phytotoxic action of several herbicides (16). Herbicidal inhibitors of carotenoid pigments include phenylpyridazinone (e.g norflurazon), aminotriazole (e.g. amitrole), pyridinone (e.g. fluridone) and isoxazolidinone (e.g. clomazone) derivatives (17). Norflurazon and fluridone interfere with carotenoid biosynthesis by inhibiting mainly the desaturation reactions of phytoene, catalyzed by the enzyme phytoene desaturase (17). Clomazone has been reported to interfere with chloroplast development (19) and to bleach sensitive plants by inhibiting two key enzymes (prenyl transferase and isopentenyl isomerase) regulating the formation of geranylgeranyl pyrophosphate (GGPP) from isopentenyl pyrophosphate (18). Two recent reports, however, have disputed these previous results and the exact mechanism of

the bleaching action of clomazone is still unknonwn (20, 21). Inhibition of gibberellic acid precursor biosynthesis by chloroacetanilide (e.g. metolachlor) and carbamothioate (e.g. EPTC) herbicides has been reported (16). In addition, the herbicidal activity of clomazone appears to be related, at least partially, to an inhibition of gibberellin biosynthesis (18, 22). Finally, diphenyl ether herbicides (e.g. acifluorfen-methyl) have been shown to interfere with tetrapyrrole synthesis in greening chloroplasts of sensitive species by inhibiting the enzyme protoporphyrinogen oxidase which catalyzes the conversion of protoporphyrinogen IX to protoporphyrin IX (23). The chemical structures of the herbicides clomazone, norflurazon, fluridone, acifluorfen, EPTC, and metolachlor are shown in Figure 1.

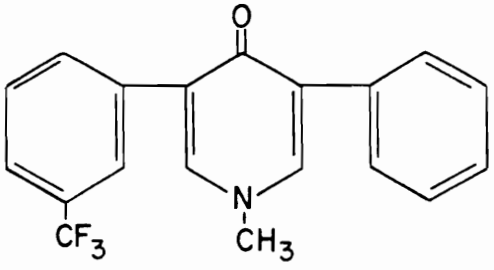
Grumbach and Bach (24) have reported that SAN 6706, a methylated derivative of the bleaching herbicide norflurazon, stimulated the activity of HMGR associated with the plastidic fraction of radish seedlings. Therefore, the main objective of the experiments reported in this study was to examine the potential effects of selected herbicides interfering with isoprenoid synthesis on the activity of maize HMGR.



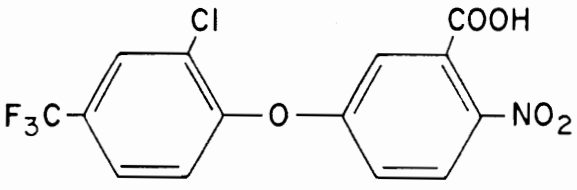
Clomazone



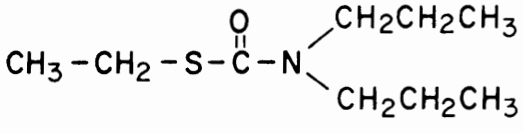
Norflurazon



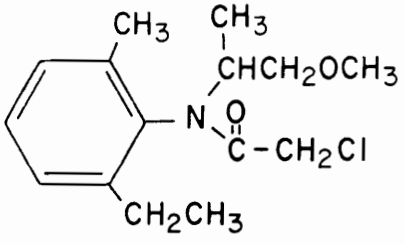
Fluridone



Acifluorfen



EPTC



Metolachlor

Figure 1. Chemical structures of the herbicides used in this study.

MATERIALS AND METHODS

Chemicals. Analytical grade samples with greater than 95% purity of the herbicides clomazone, norflurazon, fluridone, acifluorfen (Na-salt), EPTC, and metolachlor were obtained from the respective agrochemical companies that produce and market these herbicides (addresses are given in the acknowledgement section). Radiolabeled HMG-CoA (DL-3-glutaryl-3-^{14}C; sp. act. 58.0 mCi/mmol) was purchased from DuPont's New England Nuclear Research Products (Boston, Massachussetts). The Coomasie protein determination kit, which employs the Bradford method (25), was purchased from Pierce (Rockford, Illinois). Mevinolin was a gift from Dr. George Lacy (Virginia Polytechnic Institute and State University, Blacksburg, Virginia) and it was originally purchased from Sigma Chemical Company (St. Louis, Missouri). All other reagents were purchased from commercial sources.

Plant material and light treatments. Seeds of "Dekalb XL72AA" maize (Zea mays L.) were placed in petri dishes (9-cm dia.), covered with vermiculite and germinated in a growth chamber under dark conditions at 30 ^{o}C. The germinated maize seedlings were then grown for 5 days under variable light conditions depending on the needs of each experiment. Etiolated maize seedlings were grown in complete dark. Seedlings exposed to strong light intensity were grown in a chamber with a 24-hr photoperiod produced by a combination of low pressure sodium, cool-white fluorescent, and incandescent lamps. The photosynthetic photon flux density (PPFD) was 600 μmol/m^2/s in the band from 400 to 700 nm at a distance of 30 cm from the lamps. Seedlings exposed to weak light were grown in a chamber with a 6-hr photoperiod

for 5 conscutive cycles. The PPFD was 40 μmol/m^2/s.

Herbicide treatments. Application of the bleaching and growth retarding herbicides included soaking of the vermiculite content of the petri dishes with 50 ml of the respective herbicide solution at the time of planting. Three days after planting, the vermiculite in each petri dish was soaked again with 50 ml of herbicide solution. Following preliminary studies with a range of herbicide concentrations and based on the water solubility of the analytical grade standards of the tested herbicides the following concentrations were selected: Clomazone, acifluorfen-Na, EPTC, and metolachlor were all used at 100 μM. Because of their limited water solubility, norflurazon was used at 9 μM and fluridone was used at 3.6 μM. The effects of the bleaching and growth retarding herbicides on maize microsomal HMGR activity were tested under both in vivo and in vitro conditions. In the in vivo experiments, HMGR activity was assayed following its extraction from control or herbicide-treated plants grown under the different light conditions discussed earlier. In the in vitro experiment, HMGR was extracted from control etiolated 5-day-old maize seedlings and was assayed following the addition of the appropriate concentration of each of the bleaching herbicides and a 30-min incubation. Mevilonin, the fungal inhibitor of HMGR was used at 100 μM, only in the in vitro experiments of HMGR activity from maize.

HMGR extraction. Crude enzyme extracts were isolated following the procedure of Russell (26) with modifications. All steps were carried out at 0-4 ^{o}C unless noted otherwise. For etiolated seedlings, shoots above the first node were collected for enzyme extraction; for

light-treated seedlings, expanded leaves (excluding the leaf base) were collected for enzyme assay. Shoot or leaf tissues (0.5-2 g) were frozen in liquid nitrogen and ground with a mortar and pestle after adding 6 ml of a homogenizing buffer containing 10 mM Tris-HCl (pH 7.0), 0.35 M sucrose, 30 mM EDTA, 10 mM 2-mercaptoethanol, 0.1% BSA and 3.5% (w/v) polyvinylpolypyrrolidone. The homogenate was carefully squeezed through one layer of Miracloth and the filtrate was centrifuged in a swinging bucket rotor at 500 g for for 3 min to remove cell debris and nuclei. The supernatant was then centrifuged at 12,000 x g for 15 min to pellet mitochondria and chloroplast mebranes and avoid contamination of the microsomal fraction. The supernatant of this centrifugation was then centrifuged at 105,000 g for 1 hr to pellet microsomal membranes which were suspended in a medium containing 0.2 M potassium phosphate monobasic (pH 6.9) and 25 mM dithiothreitol. The microsomal membrane pellet was suspended in its suspension medium in a ratio of 0.15 ml suspension medium per gram original tissue.

Protein assay. Protein content of the membrane preparations was measured by using the Pierce Coomassie Protein Assay Reagent which employs the Bradford (25) method for protein determination with bovine serum albumin (BSA) as a standard.

HMGR assay. HMGR activity was determined by means of the radioassay method described by Russell (26) with modifications. The enzyme assay mixture was prepared on ice in 1.5 ml microcentrifuge tubes. The mixture consisted of 1 µl 0.2 M dithiothreitol, 1 µl 160 mM NADPH, 5 µl microsomal enzyme extract, 7 µl 0.3% BSA solution and 6 µl of radioactive DL-[3-^{14}C] HMG-CoA containing 12 nCi in 0.204 nmol. The

reaction mixture was incubated for 30 min in a water bath at 30 °C. The reaction was stopped by adding 2 µl 6 M HCl and 2 µl 1 M mevalonate solution. The mevalonate solution was prepared by mixing 2 M mevalonate-lactone and 0.1 M KOH in the ratio 1:1 (v/v). After mixing, the reaction mixture was incubated at room temperature for 1 hr for mevalonate lactonization to be completed. Then, the mixture was centrifuged at about 10,000 g for 3 min to pellet membrane fragments. The supernatant was then analyzed by thin-layer chromatographic (TLC) analysis by applying 10 µl of supernatant on Whatman LK5OF silica gel plates. The plates were developed in 50 ml of diethylether-acetone (3:1 v/v) for 15 min and analyzed by X-ray autoradiography. Radioactive spots corresponding to the substrate HMG-CoA (Rf=0.0) and the product mevalonate (Rf=0.9) were scraped and analyzed by liquid scintillation spectrometry.

Statistical analysis. Each treatment was replicated three times and each experiment was repeated at least two times. Variability differences in the levels of HMGR activity between experiments were observed, but the trends were similar in all experiments. Data presented are treatment means ± standard error of each mean, calculated from the three replications of representative experiments.

RESULTS

HMGR activity in maize. A comparison of the control values of maize microsomal HMGR activity shown in Tables 1 and 2 shows that HMGR activity obtained from leaf tissues of etiolated maize seedlings was about 10 times greater than that obtained from green maize seedlings grown under strong light. This illustrates the strong regulatory effect of light on the activity of plant HMGR which has been documented previously with maize (see Chapter II) and pea plants (8, 13). The exact mechanism of the regulation of plant HMGR activity by light is presently unknown. The potential involvement of a phytochrome effect in the light regulation of plant HMGR activity has been proposed (8, 13). Previous studies with broadleaved plants such as peas (8, 26), have also shown that plant HMGR activity is strongly dependent on the presence of NADPH. In parallel studies, we have confirmed that microsomal HMGR activity from etiolated as well as from green maize seedlings requires NADPH as a co-factor (27). Additional details on the occurrence, localization, and light regulation of maize HMGR activity were presented in Chapter II of this dissertation.

Influence of bleaching and growth retarding herbicides on the in vivo HMGR activity of maize grown under strong light conditions. Data in Table 1 show that specific concentrations of the bleaching herbicides clomazone, norflurazon, fluridone, and acifluorfen-Na stimulated greatly the activity of microsomal HMGR extracted from the expanded leaves of 5-day-old maize seedlings grown under strong light (PPFD = 600 $\mu mol/m^2/s$). Pretreatment of maize seedlings with 100 μM of clomazone and acifluorfen-Na caused a greater than 6-fold stimulation

of maize micorosomal HMGR activity. Norflurazon and fluridone, used at much lower micromolar concentrations, stimulated maize HMGR acti-

TABLE 1

Effect of Pretreatments with Selected Herbicides on the in vivo Activity of Microsomal HMGR from 5-day-old Maize Seedlings Grown in Strong Light[a]

Herbicide treatment	Concentration (μM)	HMGR activity[b] (nmol MVA/mg protein/hr)	Percent of control (%)
Control	0	3 ± 0.3	100
Clomazone	100	21 ± 1.5	620
Norflurazon	9	16 ± 0.7	470
Fluridone	3.6	20 ± 0.6	570
Acifluorfen	100	23 ± 0.5	640
EPTC	100	4 ± 0.3	120
Metolachlor	100	3 ± 0.3	100

[a] PPFD of 600 μmol/m^2/s for 24 hr.

[b] Mean values from three replications ± standard error.

TABLE 2

Effect of Pretreatments with Selected Bleaching Herbicides on the in vivo Activity of Microsomal HMGR from 5-day-old Maize Seedlings Grown under Continuous Dark.

Herbicide Treatment	Concentration (μM)	HMGR Activity[a] (nmol MVA/mg protein/hr)	Percent of control (%)
Control	0	37 ± 0.5	100
Clomazone	100	43 ± 1.6	116
Norflurazon	9	45 ± 0.2	120
Fluridone	3.6	46 ± 0.2	123
Acifluorfen	100	39 ± 0.6	105

[a] Mean values from three replications ± standard error.

vity by 4.7-fold and 5.7-fold, respectively (Table 1). These results are in agreement with those reported earlier by Grumbach and Bach (24). They showed that 10 μM of SAN-6706, a methylated derivative of the bleaching herbicide norflurazon, stimulated significantly the activity of HMGR extracted from radish seedlings. However, in contrast to our findings, SAN-6706 appeared to enhance HMGR activity associated with the plastidic rather than the microsomal fraction of radish cells (24).

Pretreatment of maize seedlings with 100 μM of the growth retarding herbicides EPTC and metolachlor did not cause any dramatic effects on the *in vivo* HMGR activity of maize (Table 1). Metolachlor had practically no effect on maize HMGR activity whereas EPTC caused a slight (20%) stimulation of HMGR activity under these conditions.

Effect of bleaching herbicides on the in vivo HMGR activity of etiolated maize seedlings. Data in Table 2 show the effects of pretreatments with the four bleaching herbicides on the *in vivo* microsomal HMGR activity extracted from etiolated 5-day-old maize seedlings grown under continuous darkness. A slight stimulation of HMGR activity ranging from 5 to 23% was observed with all bleaching herbicides tested. However, these stimulatory effects are far smaller than the ones caused by these herbicides on the *in vivo* activity of HMGR of maize seedlings grown under strong light (Tables 1 and 2). These results illustrate once more the importance of light in the stimulation of maize HMGR activity by these bleaching herbicides.

Effect of bleaching herbicides on the in vivo HMGR activity of maize grown under low light conditions. Data in Table 3 show the

TABLE 3

Effect of Pretreatments with Selected Bleaching Herbicides on the in vivo Activity of Microsomal HMGR from 5-day-old Maize Seedlings Grown in Weak Light[a]

Herbicide treatment	Concentration (μM)	HMGR Activity[b] (nmol MVA/mg protein/hr)	Percent of control (%)
Control	0	7 ± 0.4	100
Clomazone	100	27 ± 0.7	414
Norflurazon	9	8 ± 0.1	125
Fluridone	3.6	10 ± 0.4	150
Acifluorfen	100	6 ± 0.4	88

[a] PPFD of 40 mmol/m^2/s for 6 hr, followed by 18 hr of darkness.

[b] Mean values from three replications ± standard error.

TABLE 4

Effect of Selected Herbicides on the in vitro Activity of Microsomal HMGR Extracted from Etiolated 5-day-old Maize Seedlings.

Herbicide[a] treatment	Concentration (μM)	HMGR activity[b] (nmol MVA/mg protein/hr)	Percent of control (%)
Control	0	35 ± 1.7	100
Clomazone	100	33 ± 0.7	97
Norflurazon	9	33 ± 2.6	95
Fluridone	3.6	33 ± 0.2	96
Acifluorfen	100	33 ± 1.3	96
Mevinolin	100	2 ± 0.4	5

[a] Herbicides and mevinolin were added in vitro to the enzyme assay mixture.

[b] Mean values from three replications ± standard error.

effects of the four bleaching herbicides on the in vivo activity of microsomal HMGR extracted from 5-day-old maize seedlings exposed to 5 cycles of 6 hr of weak light (PPFD = 40 μmol/m^2/s), followed by 18 hr of continuous darkness. Under weak light, only clomazone could bleach effectively the maize seedlings and consequently clomazone was the only herbicide that stimulated maize HMGR activity in these experiments. A 4-fold increase of HMGR activity was observed in maize seedlings pretreated with clomazone (Table 3). Under the same conditions, norflurazon and fluridone could not bleach the first true leaf of maize, and they stimulated maize HMGR activity only slightly by 25 and 50% respectively (Table 3). Under these conditions of low light intermittent with long dark periods, acifluorfen-Na did not exhibit any bleaching effects and it did not stimulate the activity of maize HMGR. In contrast, a slight inhibitory effect of acifluorfen on maize HMGR activity was noticed under these conditions.

Influence of bleaching herbicides on the in vitro activity of maize HMGR. The effects of the bleaching and growth retarding herbicides on the activity of maize HMGR under in vitro conditions were conducted with crude extracts of microsomal HMGR obtained from leaf tissues of etiolated of 5-day-old maize seedlings. Mevinolin, a secondary metabolite isolated from the ascomycete Aspergillus terreus and a potent inhibitor of plant HMGR activity (9, 10) was also tested.

Data in Table 4 show that none of the four bleaching herbicides, which stimulated the in vivo HMGR activity of maize under strong light conditions (Table 1), had any effect on maize microsomal HMGR activity under in vitro conditions. In contrast to the bleaching herbicides,

mevinolin inhibited dramatically the *in vitro* activity of maize HMGR (Table 4). EPTC and metolachlor, the growth retarding herbicides, did not have any effect on the activity of maize HMGR under *in vitro* conditions. These results illustrate that the stimulatory effects of bleaching herbicides on the activity of maize HMGR under strong light conditions are indirect rather than direct.

DISCUSSION

As mentioned earlier, clomazone, norflurazon, fluridone, and acifluorfen exert their herbicidal activity by different mechanisms. However, all of them bleach effectively maize seedlings grown in strong light. Thus, the observed stimulation of the *in vivo* microsomal HMGR activity of maize pretreated with these four herbicides and grown under strong light conditions (Table 1), must be somewhat related to their bleaching activity. Amitrole, a well-known inhibitor of carotenoid synthesis did not stimulate the activity of maize HMGR in our experiments, probably because it bleaches maize only when applied postemergence. In our experiments, amitrole was used preemergence and it was not as effective in bleaching maize. The lack of any stimulatory effect of amitrole on microsomal or plastidic HMGR activity of radish has been reported also by Grumbach and Bach (24).

The relationship between plant HMGR activity and isoprenoid synthesis is not always clear. After fungal infections, the synthesis of isoprenoid compounds such as phytoalexins of potato has been shown to increase significantly following a concomitant increase in HMGR activity (15). In other instances, however, the correlation between HMGR activity and isoprenoid synthesis is not so evident (10, 29). The

significant drop in HMGR activity of etiolated plant seedlings after their exposure to light is such an example. Thus, while chlorophyll and carotenoid syntheses are increased significantly in these seedlings, HMGR activity is quite low (13, 28). Similarly, immature tomato fruits have a much higher HMGR activity than mature tomato fruits which are turning red and are very effective in synthesizing carotenoids (29).

Because bleaching herbicides inhibit the formation of carotenoids and induce the photodestruction of chlorophylls, the microsomal HMGR whose activity is stimulated in herbicide-treated maize seedlings, grown under strong light conditions, should be involved in the synthesis of isoprenoid products other than carotenoids and the phytol groups of chlorophylls. It is possible that treatment with bleaching herbicides may cause the loss of a repressor or a feedback inhibitor regulating the activity of maize microsomal HMGR under strong light conditions.

The activity of mammalian HMGR is known to undergo a multivalent feedback inhibition by its end-products (2). Thus, it may be postulated that the activity of plant HMGR may also be regulated by means of a feedback inhibition by its end products. Unfortunately, experimental evidence supporting the involvement of such a mechanism in the regulation of plant HMGRs is nonexistent. From the results of the present study it is obvious that a strong negative correlation exists between chlorophyll synthesis and microsomal HMGR activity. Green maize seedlings grown under strong light conditions had low HMGR activity, while etiolated and herbicide-bleached maize seedlings had high

HMGR activity. We have previously shown (27), that the activity of maize HMGR is localized in the microsomal rather than plastidic membranes. In addition, it has been demonstrated that the genes of plant HMGRs are nuclear- rather than chloroplast-encoded genes (29, 30). Therefore, it is possible that any regulatory effect of chlorophyll on microsomal HMGR activity of maize is indirect rather than direct. It is also possible that mature maize chloroplasts may contain some kind of an endogenous inhibitor which affects the transcription of HMGR genes and that bleaching herbicides may be releasing the effect of such an inhibitor on maize microsomal HMGR activity. Future work at the molecular biology level, will attempt to provide evidence in support or against these postulated mechanisms.

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CHAPTER IV

MOLECULAR STUDIES ON MAIZE 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE GENES AND GENE EXPRESSION

INTRODUCTION

3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR; EC 1.1.1.34) catalyzes the conversion of HMG-CoA to mevalonate. Mevalonate is the precursor of numerous important isoprenoid compounds produced in animal and plant cells (1, 4). Research with mammalian tissues has shown that HMGR is a rate-limiting enzyme in mammalian isoprenoid synthesis, mainly cholesterol synthesis, and it is under multivalent feedback regulation by its end products. Cholesterol combined with low density lipoproteins (LDL) or 25-hydroxy-cholesterol inhibit the transcription of mammalian HMGR genes while other non-sterol isoprenoid compounds inhibit the translation of mRNA coding for HMGR (4, 13, 16, 17, 21, 23). The degradation of HMGR protein in mammalian cells is known to be increased by both sterols and non-steroid metabolites (13, 23).

During the last decade, research on the expression and regulation of mammalian HMGR has been facilitated by the molecular cloning of the HMGR gene from Chinese hamster (7). From the full length cDNA clone of this gene, the amino acid sequence of hamster HMGR was deduced and the actual molecular weight of the HMGR protein was determined to be 97-kDa (6). Based on the predicted amino acid sequence, it was determined that the hydrophobic amino-terminus region of the HMGR protein spans the endoplasmic reticulum seven times (6). Directed-

site mutagenesis experiments showed that the membrane spanning region plays an important role in the degradation of the HMGR protein (13). Genomic DNA sequences showed that the transcription of the HMGR gene is under the control of an unusual promoter which lacks a TATA or CCAAT box and initiates transcription from multiple sites (25). The promoter contains a site to which a regulatory element may bind and this mechanism is believed to be involved in the transcriptional regulation of the HMGR gene by cholesterol (23).

HMGR genes of eukaryotic organisms are highly conserved. Supporting evidence is provided by the successful use of a cDNA probe from hamster HMGR gene for isolating HMGR genes from human cells, yeast, and Drosophila (2, 12, 17). The membrane-spanning, amino-terminus region was shown to be the most highly conserved region of HMGR protein among mammalian species, but the carboxy-terminus catalytic site seems to be highly conserved in all species (17).

The successful use of cDNA probes from hamster and yeast HMGR genes for the isolation of HMGR genes from several plant species such as Arabidopsis (5, 15), Hevea rubber tree (8), radish (11, 30), and tomato (24) has been reported. Synthetic oligonucleotide sequences based on the conserved catalytic site of other species, have also been used for the isolation of an HMGR gene from tomato (22). The amino acid sequence of HMGR predicted from the cDNA of the Arabidopsis HMGR gene was distinct from that reported for mammalian HMGRs, since its amino-terminus end has only one or two membrane spanning regions instead of seven (5, 15). The catalytic region, however, at the carboxy-terminus end of the Arabidopsis and mammalian HMGR genes

showed high sequence identity.

In the present study, we used polymerase-catalyzed chain reaction (PCR) procedures to amplify an HMGR sequence from maize cDNA, and we attempted to use this amplified sequence for determining the number of HMGR genes in maize and for studying the developmental regulation of maize HMGR at the mRNA level.

MATERIALS AND METHODS

Plant material. Three genetic lines of maize (Zea mays L.) and the 'EP-7' cultivar of tomato (Lycopersicon esculentum Mill.) were used in these experiments. The three maize lines studied included a normal 'DeKalb XL72AA' hybrid, a dwarf (d_5) mutant and an albino (lw_3) mutant. Seeds of 'Dekalb XL 72AA' were obtained from DeKalb AgResearch Inc., Sycamore, Illinois, whereas seeds of the dwarf (catalog # 17-7110) and albino (catalog # 17-7100) maize mutants were purchased from Carolina Biological Supply Co., Burlington, North Carolina. Seeds of the tomato 'EP-7' and tomato suspension cultures were provided by Dr. Carole L. Cramer (Virginia Polytechnic Institute and State University, Blacksburg, Virginia).

Maize seeds were placed in a small petri-dish (9-cm dia.), covered with vermiculite, and germinated in a growth chamber at 30 ^{o}C. Etiolated seedlings were produced from maize seeds germinated and grown in the dark. Green (light-grown) seedlings were produced from seeds germinated and grown under a 24-hr photoperiod with a photosynthetic photon flux density (PPFD) of 600 μmol/m^2/s. Maize leaf tissues including 1-cm long leaf tips or leaf bases cut at 0.5 cm above the first node were collected from 5-day-old etiolated or light-grown seedlings and used for the extraction of HMGR and of nucleic acids. Tomato plants were grown from seed under greenhouse conditions to the stage of flowering and fruit formation. Tomato tissues used for the extraction of HMGR and nucleic acids included leaves and fruits. Immature green tomato fruits (1-cm dia.) were used for the isolation of tomato DNA.

HMGR extraction and assay. Crude extracts of maize were prepared and assayed for microsomal HMGR activity as previously described (see Chapter II).

DNA extraction. Plant genomic DNA was extracted according to the methodology reported by Dellaporta et al. (9). One gram of leaf tissue was frozen in liquid nitrogen, ground to fine powder with a mortar and pestle and extracted with 15 ml of buffer containing 100 mM Tris (pH 8.0), 50 mM EDTA, 500 mM NaCl, and 10 mM β-mercaptoethanol. Following the addition of 1.0 ml of 20% SDS the slurry was then homogenized and incubated for 10 min at 65 °C. After this incubation, 5.0 ml of 5 M potassium acetate were added and the extract was incubated for 20 min at 0 °C to precipitate proteins and polysaccharides. Following a centrifugation at 25,000 x g for 20 min, the supernatant was poured into 10 ml of ice-cold isopropanol and stored for 30 min at -20 °C. DNA was pelleted by centrifugation at 20,000 x g for 15 min. The pellets were then suspended in 0.7 ml of 50 mM Tris (pH 8.0) and 10 mM EDTA. After the addition of 1/9 volume of 3 M sodium acetate the suspsension was mixed thoroughly and then 500 µl of ice-cold isopropanol were added to precipitate the DNA. The DNA was then collected by centrifugation for 30 sec in a microcentrifuge. The collected DNA was washed twice with 80% ethanol, dried, and then dissolved with 100 µl of distilled water. To eliminate the interference of contaminant RNA in the Southern blot experiments, the isolated DNA was treated with RNase A (Sigma Chemical Co., St. Louis, Missouri).

RNA extraction. RNA was isolated according to the methodology of De Vries et al. (29) with modifications. Plant tissue (0.4 g) was

frozen in liquid nitrogen and pulverized with a mortar and pestle. The tissue was then extracted with 1 ml of phenol and extraction medium mixed thoroughly in a 1:1 ratio. The extraction medium contained 100 mM LiCl, 1% SDS, 100 mM Tris-NaOH (pH 9.0) and 10 mM EDTA. The phenol solution contained 0.1% hydroxyquinoline. The mixture was vortexed and occasionally heated to 90 ^{o}C to make a sticky suspension. The test tubes were then shaken in a wrist shaker for 5 min. and then for another 15-30 min after the addition of 0.5 ml chloroform. The suspension was then transferred to a microcentrifuge tube, and centrifuged for 30 min at room temperature. The upper aqueous phase was then transferred to a new microcentrifuge tube, and following the addition of 0.5 ml chloroform it was shaken for another 15 min. The aqueous phase was transferred to a microcentrifuge tube and mixed with 1/3 volume of 8 M LiCl. After thorough mixing the tube was stored at 4 ^{o}C for 24 hr. RNA was collected by centrifugation for 30 min at 4 ^{o}C. The RNA pellets were washed once with 2 M LiCl and twice with 80% ethanol. The RNA was then dissolved in 135 µl distilled water. To eliminate any residual DNA, RNA was treated with 2 µl of RNase-free DNase (10 unit/µl, Stratagene, La Jolla, California), and incubated at 37 ^{o}C for 1 hr. Then 15 µl of 3 M sodium acetate were added and mixed well. RNA was then precipitated after the addition of 300 µl of cold 95% ethanol. The RNA was washed twice with cold 80% ethanol and was used in PCR and Northern blot analysis.

PCR-mediated Amplification. The methodology used is similar to that described by Mullis and Faloona (20). Two synthesized oligonucleotide primers, whose sequences were 5'-CGCAAGCTTGGTGATGCAATGGGAAT-

GAACATGGT-3' and 5'-ACTCTACGTTCGACTCAAGGTGGAGG-3', respectively, were provided by Dr. Cramer (Virginia Polytechnic Institute and State University, Blacksburg, Virginia). In tomato, these two sequences span 486 bp and include the catalytic site of HMGR carboxy-terminus, which is highly conserved among different species (22). The first primer also contains an additional sequence of 9 bases providing a Hind III site for subsequent subcloning. Single stranded cDNA was synthesized by mixing 15 μg RNA (isolated from etiolated or light-grown maize seedlings), 2 μl 10 mM oligo$(dT)_n$, 4 μl 10 mM dNTP mix, 4 μl 5x reverse transcriptase buffer, 1 μl reverse transcriptase (200 unit/μl GIBCO BRL, Life Technologies, Inc., Bethesda, Maryland). Following the addition of 20 μl of distilled water the mixture was incubated for 1 hr at 37 ^{o}C. Then 2 μl of RNase T_1 were added, and the mixture was incubated again for 1 hr at 37 ^{o}C. The synthesized cDNA was used directly for PCR amplification. The reaction mixture for PCR contained 10 μl 10x PCR buffer, 16 μl 1.25 mM dNTP mix, 40 pmol of each primer, the cDNA sythesized, 0.5 μl Taq DNA polymerase (5 unit/μl, Perkin Elmer Cetus, Norwalk, Connecticut) and water, in a final volume of 100 μl. The reactants were overlayed with mineral oil. PCR conditions used were as follows: number of PCR cycles was 42; denaturation temperature was 95 ^{o}C, 2 min; primer hybridization temperature was 58 ^{o}C, 3 min; polymerase reaction temperature was 72 ^{o}C, 3 min. The PCR apparatus used was an Ericomp twin block thermocycler (Ericomp, San Diego, California).

DNA fragment isolation and purification. DNA fragments from gels and solutions were purified with GENECLEAN^{TM} kit (BIO 101, La Jolla,

California).

Plasmid isolation. Plasmid DNA was isolated and purified according to a mini-prep method described by Kraft et al (14). Escherischia coli cells (DH5α) which harbor pBluescript SK- plasmids (Stratagene Cloning System, La Jolla, California) were cultured in 5 ml LB medium containing 100 μg/ml ampicillin. The cultures were then centrifuged in a microcentrifuge and the supernatant was discarded. The bacterial pellet was suspended in 100 μl ice-cold solution containing 50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl (pH 8.0). After 5 min at room temperature, 200 μl of freshly prepared 0.2 N NaOH and 1% SDS solution was added. The suspension was mixed well and incubated on ice for 5 min. Then a 150 μl of ice-cold solution of 7 ml glacial acetic acid, 3 ml distilled water, and 15 ml 5 M potassium acetate was added. The mixture was then centrifuged at 4 ^{o}C for 5 min and the supernatant was transferred to a new microcentrifuge tube. Two μl of RNase A (5 μg/μl Sigma Chemical Co., St. Louis, Missouri) were added and the solution was incubated at 37 ^{o}C for 30 min. After the addition of 400 μl of phenol/chloroform (1:1 saturated with TE buffer, TE buffer: 1 mM EDTA 10 mM Tris-HCl pH 8.0) the solution was vortexed and centrifuged for 2 min and the top aqueous layer was transferred to a new tube. One ml of ice-cold 95% ethanol was then added and the tube was kept at -70 ^{o}C for 30 min to precipate plasmid DNA. The tube was centrifuged at 4 ^{o}C for 5 min and the DNA pellet was washed twice with 80% ethanol. The pellet was then dissolved in 16.8 μl distilled water, mixed with 3.2 μl of 5 M NaCl and 20 μl of 13% PEG (polyethylene glycol MW 8000, Sigma Chemical Co., St. Louis, Missouri), and incubated on ice for 30

min. Finally, the solution was centrifuged at 4 °C for 10 min and the supernatant was discarded. The invisible plasmid pellet was rinsed twice with 80% ethanol, dried under vaccum and dissolved in 20 µl of distilled water.

Cloning of the PCR-amplified DNA fragment. PCR amplified DNA fragments were first cut with Hind III and then visualized by electrophoresis and staining with ethidium bromide on 1.4% agarose gels followed by purification with a GENECLEAN™ kit. The fragments were dissolved in 5 µl of distilled water. pBluscript SK- plasmids were cut with Hind III and Sma I and they were purified with GENECLEAN™ kit.

Mixtures of 2.5 µl (250 ng) vector, 5 µl (500 ng) amplified fragment, 0.5 µl 10 mM ATP, 0.5 µl T_4 DNA ligase (1 unit/ml, BRL, Bethesda, Maryland), and 2 µl 5x ligation buffer were incubated at 4 °C overnight.

Competent cells were prepared by transferring 0.25 ml of E. coli cells, cultured overnight, to a sterile centrifuge tube. After the addition of 25 ml LB medium (Luria-Bertani medium, 18) and a 2-hr incubation, the cultures were chilled on ice for 10 min, the cells were centrifuged at 4000 x g for 5 min. at 4 °C. The supernatant was discarded and the cells were suspended in 10 ml of 50 mM $CaCl_2$ solution. The assay tubes were then incubated on ice for 20 min. and centrifuged at 4000 x g for 5 min at 4 °C. Finally, the cells were suspended gently in 2 ml of 50 mM $CaCl_2$ and stored at 4 °C for 24 hr.

For transformation, 10 µl ligation mix and 600 µl competent cells were mixed in a sterile tube. The tube was incubated on ice for 40

min. Then the cells were heat-shocked at 42 °C for 1 min, and placed at room temperature. Transformed cells were plated on LB plates (18) containing 100 μg/ml ampicillin and 100 μg/ml X-gal (Sigma Chemical Company, St. Louis, Missouri). The plates were incubated at 37 °C for 24 hr.

DNA sequencing. The DNA Sequenase 2.0 kit was purchased from United States Biochemicals (Cleveland, Ohio). The methods for DNA sequencing were those of Kraft et al (14). Two μl of freshly prepared 2 N NaOH and 2 mM EDTA solution were added to 20 μl of previously prepared plasmid (500 ng), and incubated at room temperature for 5 min. Then, 5 μl H_2O, 3 μl primer (T_7 or T_3 primer), and 3 μl 3 M sodium acetate were added on ice. After the addition of 75 μl ice-cold 95% ethanol the tube was incubated at -20 °C for 20 min to precipate DNA and the mixture was centrifuged at 4 °C for 5 min. The supernatant was removed and the pellet was rinsed twice with 80% ethanol and then dried under vacuum. Then 2 μl of 5x Sequenase buffer and 8 μl water were added to the dry DNA and the sample was incubated at 37 °C for 30 min; this sample is called annealing mix.

To each annealing mix, 1.0 μl of 0.1 M dithiothreitol (DTT), 2.0 μl labeling mix dilution, 0.5 μl [^{35}S]dATP, and 2.0 μl Sequenase dilution were added and the labeling reaction was incubated at 37 °C for 5 min. The labeling mix was diluted with distilled water in a 1:5 ratio. The Sequenase enzyme was diluted with ice-cold TE buffer (pH 7.4) in a 1:8 ratio. Then 3.5 μl of the labeling reaction sample was transferred to four termination mix tubes each containing 2.5 μl ddGTP, ddATP, ddCTP, ddTTP, respectively and incubated at 37 °C for 5

min. A 4 µl of Stop Solution was added to each of the four tubes. Samples were then heated at 70 ^{o}C for 10 min. before loading 2.5 µl per lane on 8 % denaturing acrylamide gel for electrophoretic analysis.

The 8% acrylamide urea gels were prepared by dissolving 15.2 g acrylamide, 0.8 g N,N'-methylene bis-acrylamide, and 84 g urea in 40 ml 10x TBE buffer (TBE buffer: 0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.0), and brought to a final volume of 200 ml with distilled water. The acrylamide solution was degased under vacuum. Then, 50 µl of TEMED and 500 µl of fresh 10% ammonium persulfate were added immediately before making gel plate. The 40 cm gel plates were electrophoresed by using a power supply providing current of 1800 volts (40 mAmps).

Radiolabeling of DNA probe. The ^{32}P Random Primers DNA Labeling System (BRL, Bethesda, Maryland) was used. Twenty-three µl of unlabeled DNA probe (20 ng) was boiled for 5 min and then placed on ice immediately. Then 2 µl of dATP, dGTP, and dTTP were added, followed by the addition of 15 µl random primer solution, 5 µl ^{32}P CTP (50 µCi, NEN, Boston, MA), 1 µl Klenow DNA polymerase (6 unit/µl). The mixture was mixed thoroughly and kept overnight at room temperature. The labeled probe was further purified with GENECLEAN^{TM} kit.

Southern blotting. The method used is that described by Southern (27). NYTRAN membranes (pore size 0.2 µm) were purchased from Schleicher & Schuell, Inc. Keene, NH. Each lane was loaded with 5-10 µg of DNA. Prehybridization and hybridization were done in 6x SSC (1xSSC solution: 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS, 5x

Denhart's (Denhart solution: 0.02% BSA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 100 μg/ml denatured salmon testes DNA, at 68 ^{o}C for 12 and 24 hr respectively. Probe radioactivity was 1.5 x 10^{6} cpm. Washing was done at room temperature in 2x SSC, 0.5% SDS, for 15 min; 2xSSC, 0.1% SDS, for 15 min; 0.1x SSC, 0.1% SDS, for 60 min. Exposure time for autoradiography was 3-5 days.

Northern blotting. The method used was that of Thomas (28) and Maniatis (18). RNAs were denatured with formaldehyde and subjected to electrophoresis on a 2.2 M formaldehyde/1.4% agarose gel. 5 μg RNA was loaded in each lane. All remaining procedures were similar to those described in Southern blotting.

RESULTS AND DISCUSSION

HMGR enzyme activity is 5-10 times higher in etiolated maize seedlings than it is in light grown green seedlings (described in Chapter II). Maize RNA, isolated from etiolated or light grown 5-day-old seedlings, was used for cDNA synthesis and PCR amplification utilizing HMGR-specific oligonucleotides. In an initial experiment shown in Fig. 1, DNA of the expected size (approximately 0.5 kb) was generated from the etiolated but not from the light-grown cDNA. This suggested that etiolated seedlings contain higher levels of HMGR mRNAs which contribute to the HMGR activity. To address this possibility further and to generate maize-specific HMGR cDNA probe, I attempted to clone the PCR-generated fragment into bacterial plasmid vectors. Initial subcloning attempts were unsuccessful. A second PCR amplification, shown in Fig. 2, yielded somewhat different results. In this case, PCR product was generated in both etiolated an light-grown samples although greater amounts of product were evident in the etiolated sample.

PCR-product from the etiolated sample was digested with HindIII and ligated into pBluscript SK-digested with SmaI and HindIII. The recombinant plasmids were transformed into E. coli stain DH5α and bacteria harboring recombinant plasmids were selected based on ampicillin resistance and inability to metabolize the indicator dye (X-gal) to generate blue colonies. Eight white colonies of E. coli were recovered following these experiments. Electrophoretic analysis of plasmid DNA from these transformed colonies of E. coli showed that plasmids from all eight colonies contained the inserted fragment,

since their molecular weight was higher than that of the original Bluescript plasmid (Fig 3.). Subsequent digestion of the plasmid DNA from transformed colonies of E. coli with HindIII and XbaI restriction enzymes, followed by electrophoretic analysis demonstrated that the inserted fragment was indeed the correct size for the 0.5 kb fragment amplified by PCR (Fig. 4).

The next step was to sequence the inserted DNA fragment from both ends using the T_3 and T_7 primers. With T_3 primer, the sequencing result is:

5'-CCCCTGAGATGCAAGCTGAGTTCCACCTCCAACGGTACCAACCTCAATGGAAGGCATTGTA-
ACAGAAATATGGAGGTCCTTTGCCATCATTTACAGCCTCCATCATAGTGATGCAGTGCGAGCTCT-
CTATGTTCTG GCTGGATCCTGACCTGTGGCTATAAACACAGCTGAGACGATATTGCTGGCATGG-
GCATTGAAA-3'

Its complementary strand sequence should be as follows:

3'-GGGGACTCTACGTTCGACTCAAGGTGGAGGTTGCCATGGTTGGAGTTACCTTCCGTAACAT-
TGTCTTTATACCTCCAGGAAACGGTAGTAAATGTCGGAGGTAGTATCACTACGTCACGCTCGAGA-
GATACAAGACCCGACCTAGGACTGGACACCGATATTTGTGTCGACTCTGCTATAACGACCGTACC-
CGTAACTTT-5'

With T_7 primer, the sequencing result is:

5'-GTGATGCAATGGGAATGAACATGGTGTCCAAAGGTGTATACAAAATGTTCTTGATTACC-
TTCAGAATGAATATCCCGACATGGATGTCATCGGTATATCTGGGAACTTTTGCTCGGACAAG-
AAGCCAGCAGCAGTTAATTGGATCGAGGGCAGAGGAAAGTCTGTAGTTTGTGAGGCAATTAT-
CACAGAAGAGGT-3'

Comparison of this sequence with the tomato HMGR sequence reported by Park (24), revealed that the two sequences were identical. This finding indicates that, most likely, the maize cDNA sample was conta-

minated with a trace amount of tomato nucleic acid during PCR amplification. A major drawback of PCR-based DNA amplification is the extreme sensitivity of the technique and the potential for amplifying extremely low level of contaminating DNAs (10). Preferential amplification of tomato sequences rather than that of maize may have occurred for the following reasons: 1) the oligonucleotide primers were designed based on tomato HMGR (isogene HMG-2) sequences and thus would show stronger annealing to tomato sequences than maize, 2) the analogous tomato sequences had been amplified repeatedly in the laboratory where the PCR was performed providing the possibility for comtamination, and 3) amplification of tomato cDNA was carried out in adjacent tubes (Fig. 2) in the experiment used for subcloning. However, because this sequence represented the HMGR region most highly-conserved between species (2, 15, 24), I chose to test the efficacy of this amplified sequence as hybridization probe for maize DNA and RNA rather than persuing further PCR-amplification experiments.

Results of Southern blot analysis showed that the cloned cDNA fragment hybridized to genomic DNA from both maize and tomato under conditions of moderately high stringency (hybridization temperature of 68 ^{o}C). The 'EP-7' cultivar of tomato seems to have four copies of HMGR gene. Four fragments of 16 kb, 9 kb, 5.9 kb, and 4.4 kb sizes were detected in Hind III digestion, while in EcoRI digestion bands of 32 kb, 17 kb, 14 kb, and 1.6 kb sizes were detected (Fig. 5).

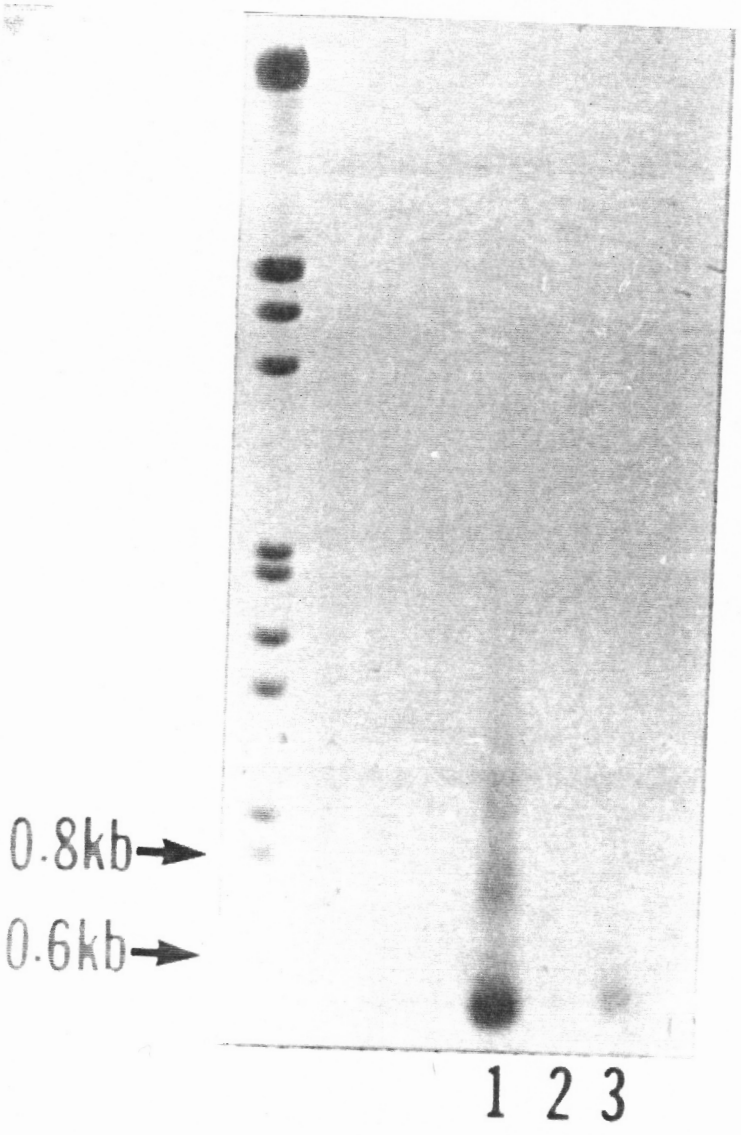


Fig. 1. PCR-mediated amplification of HMGR gene from cDNA of maize. PCR products were amplified from cDNA synthesized from mRNA of the following tissue: Lane 1, 'EP-7' tomato tissue culture; lane 2, leaf tissue of 5-day-old light-grown 'XL72AA' maize; lane 3, leaf tissue of 5-day old etiolated 'XL72AA' maize. PCR cycles: 37. Denaturation: 95 ^{o}C, 2min. Primer hybridization: 58 ^{o}C, 3min. Polymerase reaction: 72 ^{o}C, 3min.

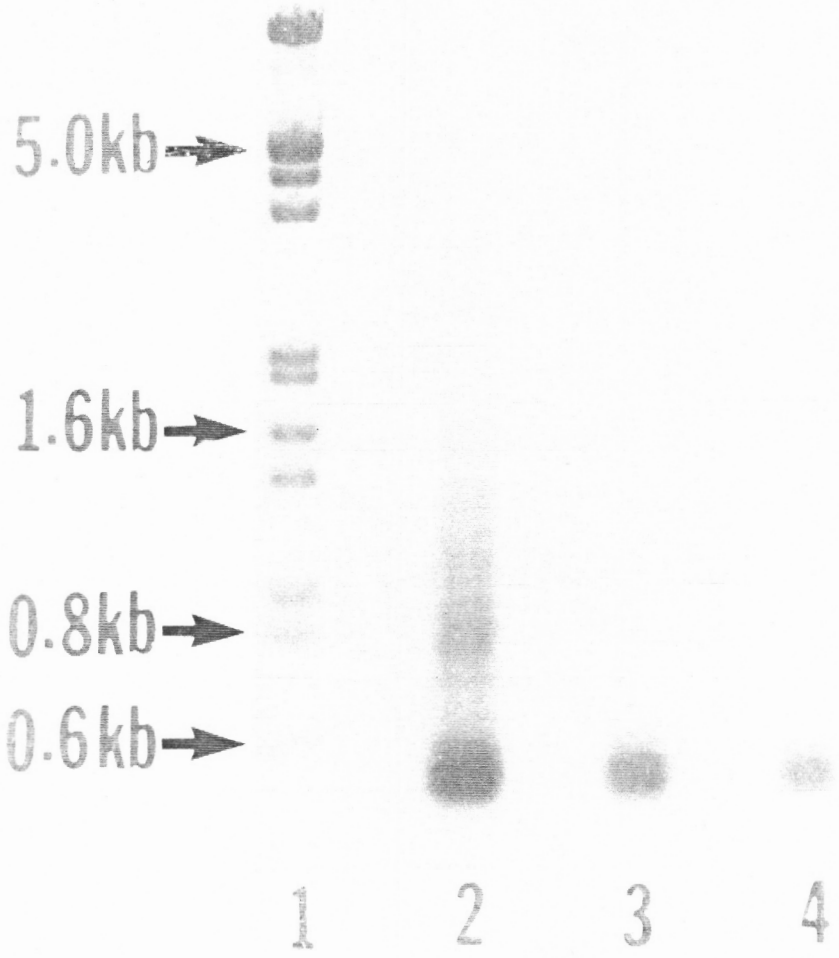


Fig. 2. PCR-mediated amplification of HMGR gene from cDNA of maize. Lane 1, Lambda DNA marker digested with HindIII and EcoRI. PCR products were amplified from cDNA synthesized from mRNA of the following tissue: lane 2, 'EP-7' tomato tissue culture; lane 3, leaf tissue of 5-day-old etiolated 'XL72AA' maize ; lane 4, leaf tissue of 5-day-old light-grown 'XL72AA' maize. PCR cycles: 42. Denaturation: 95 ^{o}C, 2 min. Primer hybridization: 58 ^{o}C, 3 min. Polymerase reaction: 72 ^{o}C, 3 min.

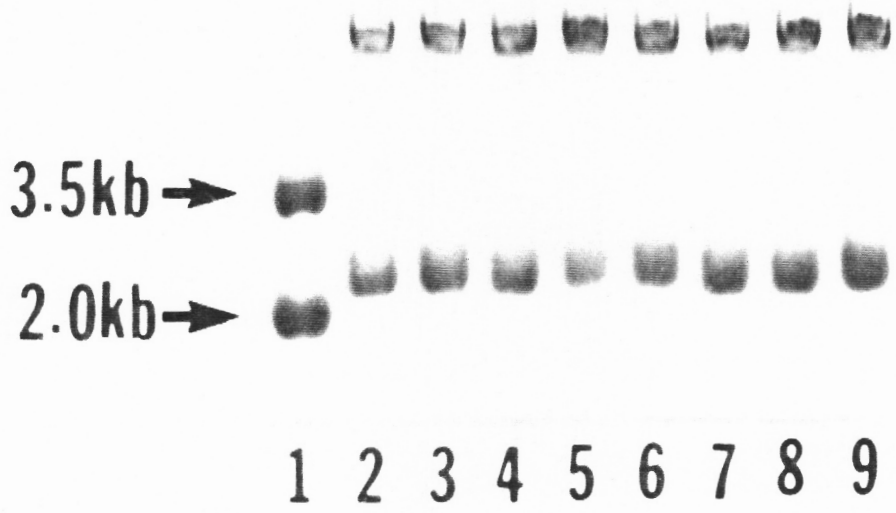


Fig. 3. PCR-amplified fragments cloned into Bluescript SK-plasmid. Lane 1, original Bluscript plasmid; lanes 2-9, plasmids with inserted PCR-amplified cDNA sequence from eight different colonies. Fragment sizes were calculated from lambda phage DNA digested with HindIII and EcoRI.

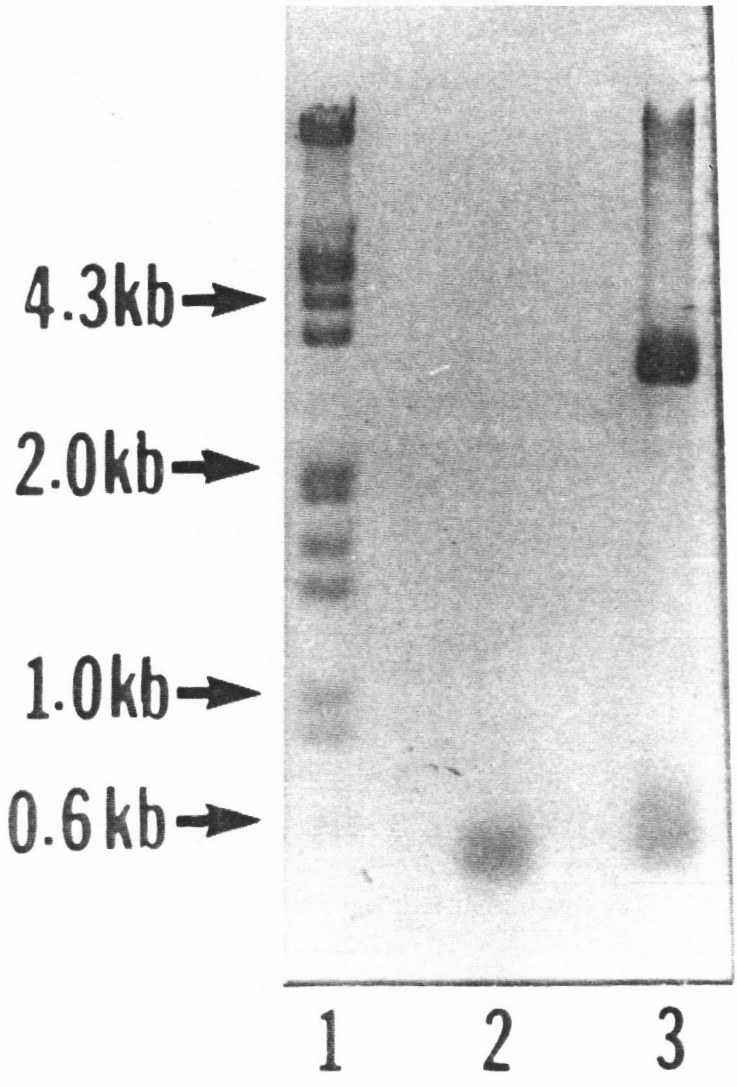


Fig. 4. Bluescript SK-plasmid cloned with the PCR-amplified cDNA fragment and digested with HindIII and XbaI. Lane 1, Lambda DNA marker; lane 2, PCR-amplified cDNA fragment (control); lane 3. Cloned Bluescript SK-plasmid following digestion with the restriction enzymes.

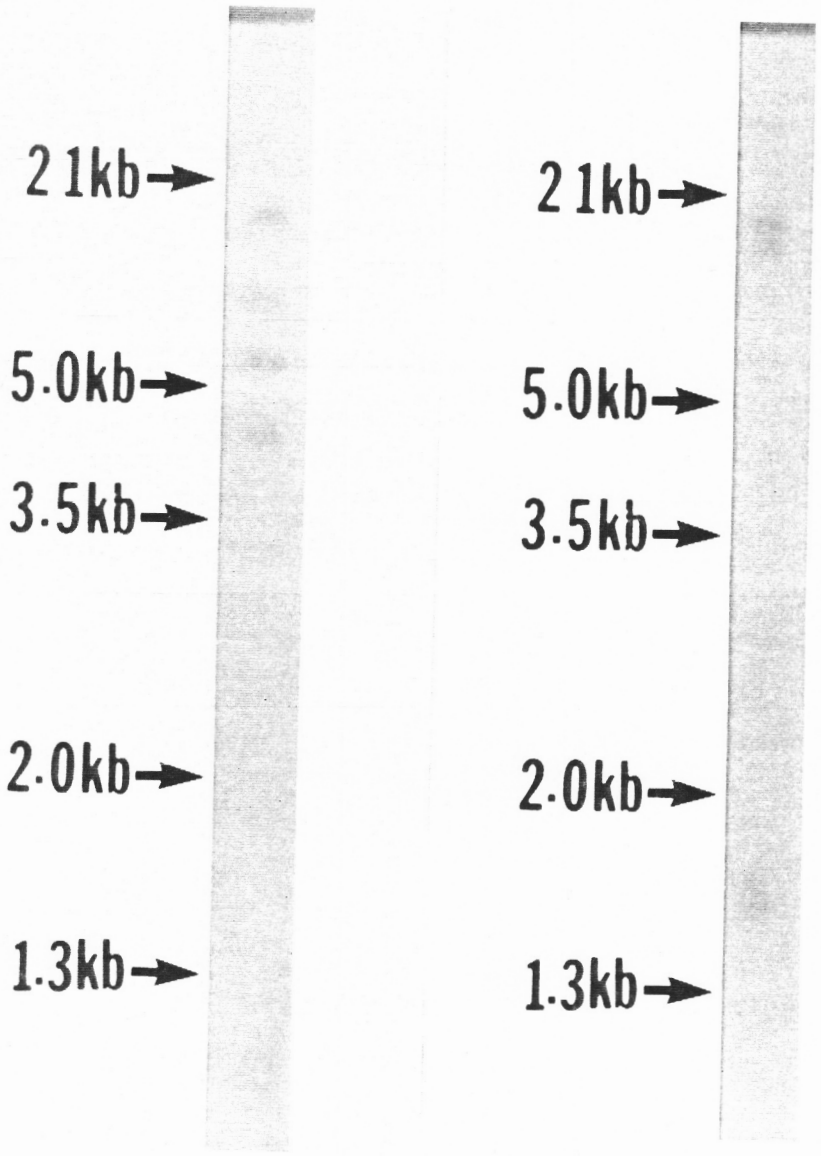


Fig. 5. Southern blot of tomato 'EP-7' genomic DNA following digestion with HindIII (left column) and EcoRI (right column). Hybridization temperature was 68 °C. All other details are given in the text.

Use of the cloned cDNA fragment for the analysis of three genetic lines of maize revealed the presence of restriction fragment length polymorphism (RFLP) of the HMGR gene. Digestion with HindIII revealed that all three lines of maize examined ('DeKalb XL72AA,' albino, dwarf) have a strong band at 3.6 kb, while only the 'Dekalb XL72AA' hybrid had another strong band at 2.9 kb (Fig. 6). Digestion with EcoR I showed that all three lines of maize had a 3.6 kb size band. However, the strongest band for the 'DeKalb XL72AA' maize hybrid had a size of 4.0 kb, while the albino and dwarf mutants of maize had a 4.1 kb band (Fig. 6).

Because this probe hybridizes to maize genomic DNA under high stringency conditions, it is appropriate to use it in Northern blot analysis for the detection of the levels of HMGR mRNA in maize. Unfortunately, in spite of many experiments we were unable to detect any levels of HMGR mRNA from leaf tissues of maize in which HMGR is known to be highly expressed (Fig. 7). Such tissues included leaf bases of etiolated and light-grown seedlings of maize as well as seedlings treated with the bleaching herbicides. As discussed in previous chapters of this dissertation these tissues were found to have high HMGR activity.

Narita and Gruissem (22) have reported that mRNA coding for HMGR and the activity of HMGR are highly expressed in young tomato fruit and this finding has been confirmed by other investigators (Ji and Cramer, unpublished results). Then it was expected that this probe should work in Northern blot analysis of tomato mRNA. The results of

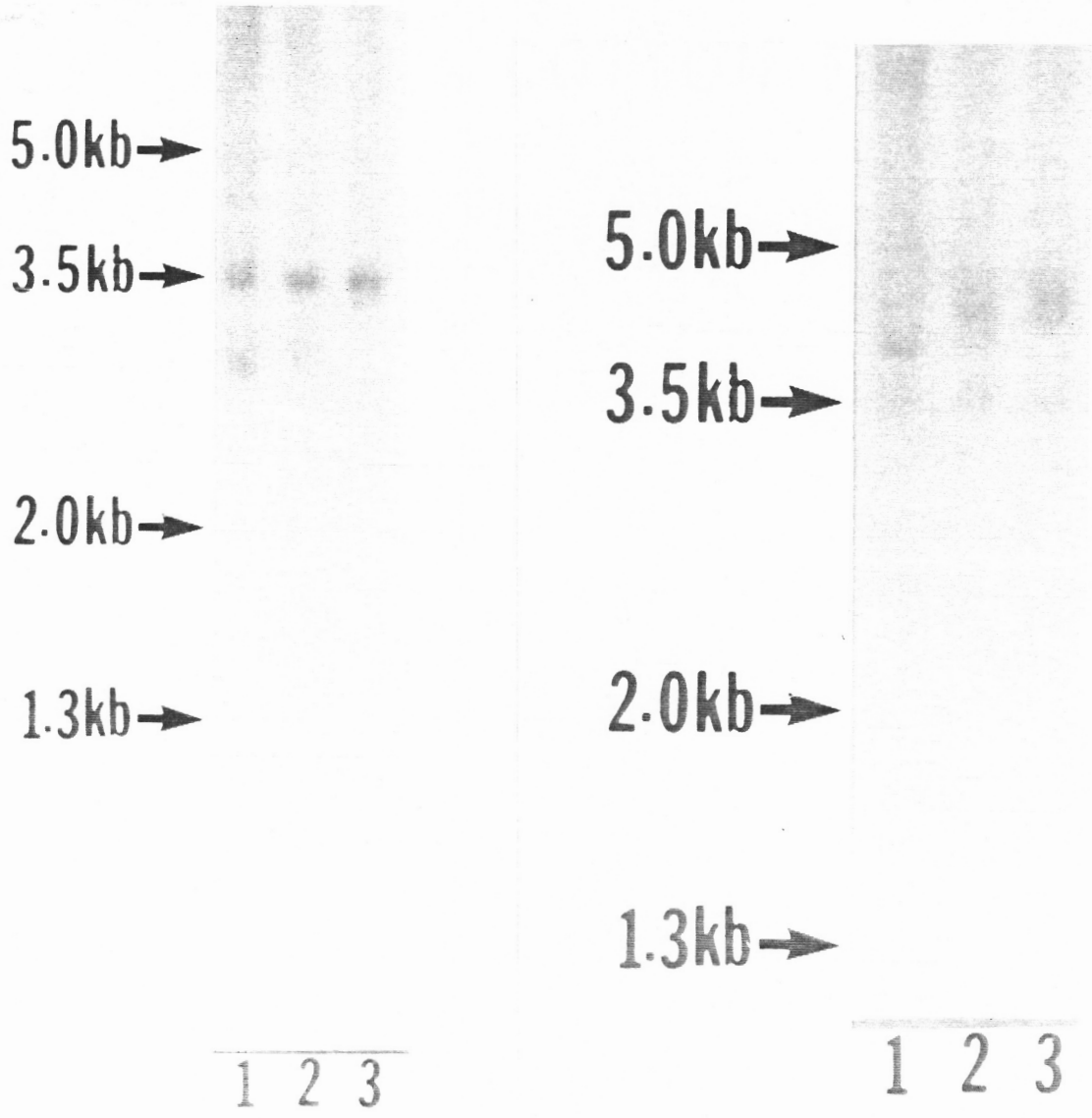


Fig. 6. Southern blot of maize genomic DNA following digestion with HindIII (left column) and EcoRI (right column). Lane 1, 'XL72AA' maize; lane 2, albino (lw_3) maize; lane 3, dwarf (d_5) maize. Reaction conditions were the same as in Fig. 5.

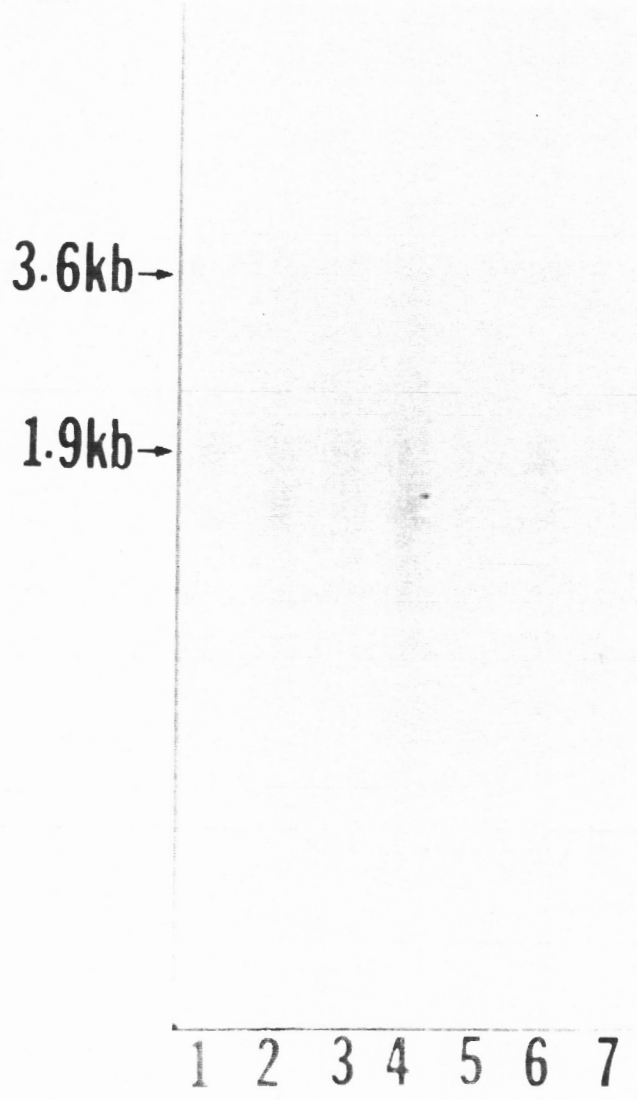
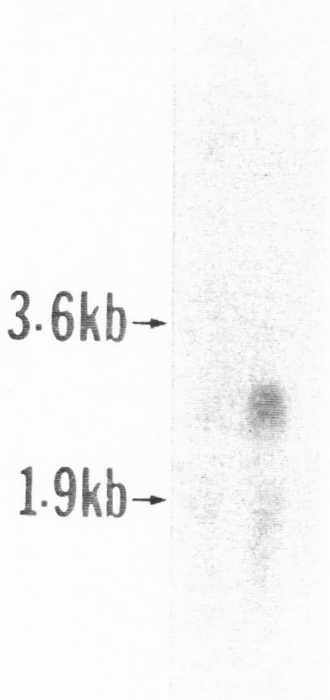


Fig. 7. Northern blot of maize RNA. Five μg total RNA isolated from the following tissues: Lane 1, leaf base of etiolated 'XL72AA' maize; lane 2, leaf tip of etiolated 'XL72AA' maize; lane 3, leaf base of light-grown 'XL72AA' maize; lane 4, leaf tip of light-grown 'XL72AA' maize; lane 5, leaf tissue of clomazone-treated 'XL72AA' maize; lane 6, leaf tissue of norflurazon-treated 'XL72AA' maize; lane 7, leaf tissue of fluridone-treated 'XL72AA' maize.

these experiments are shown in Fig. 8. It was surprising to find that this HMGR probe was not very efficient in detecting HMGR mRNA from immature tomato fruit (Fig. 8, Lane 1). However, the probe was much more efficient in detecting HMGR mRNA levels in tissue cultures of tomato pretreated with an elicitor for 24 hr (Fig. 8, lane 2). This was not unexpected since the HMGR probe sequenced by Park (24) was first isolated from elicitor-treated tissue cultures of tomato. The probe detected a 3.0 kb mRNA band which seems to be highly expressed in elicitor-treated tissue cultures of tomato. It is interesting to note, however, that the levels of HMGR activity in tomato tissue culture treated with elicitor were barely detectable (Table 1). Thus, a surprising discrepancy between HMGR activity and HMGR mRNA detected by the probe is evident. The next obvious question, of course, is to explain why maize HMGR mRNA cannot be detected by this probe?

Sequence comparisons indicate that this cDNA probe has 76.7 % identity to the published DNA sequence for the HMGR gene of Arabidopsis (5, 15). The Southern blot analysis further illustrate that this probe give similar hybridization patterns to the HMGR gene isolated from tomato by Narita and Gruissem (22). However, when the hybridization temperature is increased to 70 ^{o}C to enhance the stringency conditions, digestion with Hind III revealed that the strongest detected band had a size of 16 kb, while the 4.4 kb band had now disappeared (Fig. 9). It is worth noting here that the the 4.4 kb size band was the strongest band detected by Narita and Gruissem (22) under high strigency conditions.



1 2

Fig. 8. Nothern blot of 'EP-7' tomato RNA. Lane 1, immature tomato fruit; lane 2, tissue culture following a 24-hr treatment with elicitor.

TABLE 1

HMG-CoA reductase activity of different plant tissue.

Plant tissue	HMGR activity (nmol MVA/mg protein/h)
Etiolated maize leaf base	39
Etiolated maize leaf tip	15
Light-grown maize leaf base	31
Light-grown maize leaf tip	3
Clomazone-treated maize leaf	22
Norflurazon-treated maize leaf	17
Fluridone-treated maize leaf	20
Young tomato fruit	84
Elicitor-treated tissue culture	1

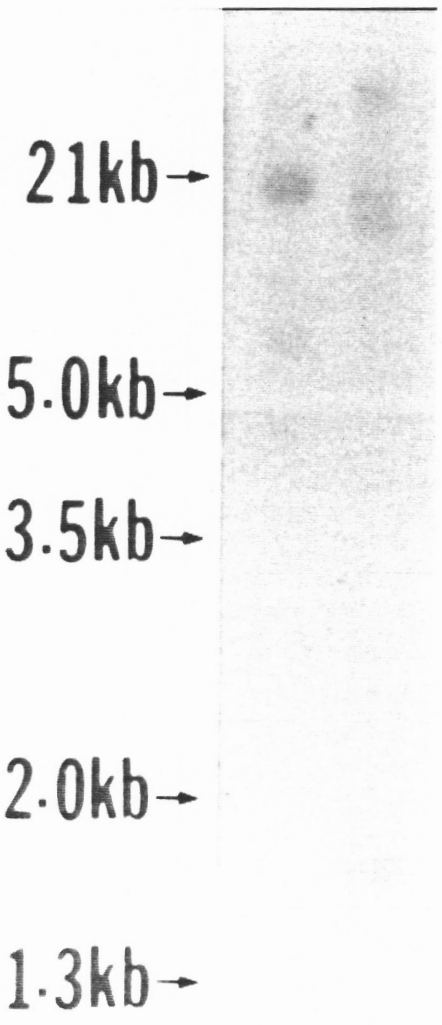


Fig. 9. Southern blot of 'EP-7' tomato genomic DNA following digestion with HindIII (left column) and EcoRI (right column). Hybridization temperature was 70 °C. All other details are specified in the text.

Inability to detect maize HMGR mRNA on Northern blots may indicate that elevated HMGR activity in etiolated or bleached seedlings is due to post-transcriptional regulation and not in mRNA levels. However, the initial PCR-amplification experiment (Fig. 1) suggested that etiolated seedlings did contain higher level of HMGR mRNA. An alternative explanation is that the cross-hybridization between the HMGR probe and maize HMGR mRNA may be insufficient for detection under the conditions used. In tomato, HMGR is encoded by a small gene family showing significant sequence divergence between members (Park and Cramer, unpublished). The HMGR cDNA sequence used in the experiments described here is analogous to tomato HMGR isogene HMG-2 which appears to be specifically induced during defence responses in potato and tomato (31). A distinct tomato isogene, HMG-1 was cloned by Narita and Gruissem (22) and is highly expressed in rapidly growing tissue such as immature fruit. Under stringent hybridization conditions, HMG-1 and HMG-2 cross-hybridize only weakly (Fig. 9. 22, 24, 31). This is consistent with the Northern hybridization results shown in Fig. 8; cross-hybridization to the immature tomato fruit HMGR is low although published results (22) and enzyme activity (Table 1) suggest that significant HMG-1 mRNA is present. In contrast, defense-elicitor treated tomato cells, shown to involve HMG-2 induction (24), show strong cross-hybridization to the HMGR cDNA probe.

It may be that maize HMGR mRNA is more closely related to HMG-1 and thus does not efficiently cross-hybridize to the HMGR cDNA probe used in the current experiments. To clarify these results, further analyses should be done using a) less-stringent hybridization condi-

tions, b) poly $(A)^+$ RNA, c) single-stranded RNA probes (RNA-RNA hybrids are more stable than RNA-DNA hybrids), or c) alternative hybridization probes (tomato HMG-1 or maize-specific HMGR probes as they become available).

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CHAPTER V

SUMMARY AND CONCLUSIONS

The major objectives of this research were to document the expression and the subcellular localization of maize HMGR and to find a correlation between HMGR activity and plant isoprenoid synthesis.

Because plastids synthesize several important isoprenoid compounds, such as the phytol group of chlorophylls, carotenoid pigments, and the side chain of plastoquinone, it was reasonable to check whether HMGR activity is expressed in maize plastids. It was found that plastidic membranes of maize do not possess any HMGR activity. These results are in agreement with the view proposed by Gray (6) and Kreuz and Kleinig (8) that isoprenoid synthesis in plant plastids begins with isopentenyl pyrophosphate (IPP) which is imported from the cytosol. According to this proposal, the HMGR-catalyzed conversion of mevalonate to HMG-CoA and the other early steps of the isoprenoid synthesis pathway leading to the formation of IPP take place only in the cytosol.

The results of this dissertation did not show any direct correlation between microsomal HMGR activity and isoprenoid synthesis in maize plastids. It was found that microsomal HMGR activity dropped considerably following the illumination of etiolated maize seedlings and the initiation of the greening process. Thus in maize seedlings, at a time when the synthesis of chlorophylls, carotenoids, and plastoquinone are at a maximum, microsomal HMGR activity is decreased. These results indicate that the source of plastid isoprenoid compounds is presently unknown. The existence of a pool of presynthesized

intermediates of the isoprenoid pathway in tomato plants has been suggested by Narita and Gruissem (10), but direct experimental evidence supporting this proposal is unavailable. Alternatively, it may be that these lower levels of HMGR are sufficient to provide the necessary precursors for plastid isoprenoids, but higher HMGR activities are required for sterol biosynthesis in rapidly dividing cells.

Substantial evidence has been provided in this research indicating that microsomal HMGR activity is highly expressed in meristematic tissues of maize seedlings such as the leaf base, root tips, the site of origin of lateral roots, and imbibed seeds before germination. The high expression of microsomal HMGR activity in meristematic tissues may be required for rapid membrane biogenesis and the modification of some crucial protein(s) (1, 7). Differentiated maize leaf tissues had low HMGR activity in both etiolated and light-grown seedlings. Light did not regulate the activity of microsomal HMGR in maize meristematic tissues. The regulation of microsomal HMGR activity of leaf tips of etiolated maize seedlings by light may be indirect resulting from a light-induced increase in cell differentiation.

The in vivo activity of microsomal HMGR from maize seedlings grown under conditions of strong light (600 $\mu mol/m^2/s$) following pretreatment with several bleaching herbicides was greatly stimulated. The bleaching herbicides tested in these experiments included clomazone, norflurazon, fluridone, and acifluorfen. These four herbicides are known to bleach susceptible plants by different mechanisms and they had no direct effect on the in vitro activity of microsomal HMGR extracted from maize. The stimulation of maize HMGR activity induced

by the four bleaching herbicides did not appear to be related to the inhibition of the synthesis of a specific end product. The stimulating effect induced by the four bleaching herbicides on maize microsomal HMGR activity was not observed when the herbicide-treated maize seedlings were grown under dark conditions. When herbicide-treated seedlings of maize were grown in a 6-hr photoperiod with weak light (40 $\mu mol/m^2/s$), the only herbicide that could bleach maize seedlings and stimulated their HMGR activity was clomazone.

The results of the experiments conducted with the bleaching herbicides support further the postulation that the light-induced regulation of maize microsomal HMGR activity may be indirect resulting from the direct influence of light on cell differentiation. Disruption of chloroplast formation, a well-defined differentiation process, by the bleaching herbicides alleviated the regulatory effect of light on the HMGR activity of herbicide-treated maize seedlings grown under stong light. It is possible that mature maize chloroplasts may contain some kind of an endogenous inhibitor which affects the transcription of HMGR genes as has been suggested for other genes of maize (2). Bleaching herbicides may be releasing the effect of such an inhibitor repressing the expression of microsomal HMGR gene in maize. Research with mammalian systems has demonstrated clearly that the ER membrane plays a critical role in the degradation of HMGR protein (6). Therefore it is also possible that chloroplast morphorgenesis may involve the use of degenerated ER membranes and that bleaching herbicides may disrupt such a process causing a delay in the degradation of HMGR protein.

The molecular biology aspects of this dissertation research were not as fruitful as were expected. This was primarily due to the use of an HMGR probe which is still uncharacterized. Although the DNA sequence of this probe is homologous to that of the HMGR gene from Arabidopsis, there is still no corresponding enzymology work to prove clearly that this probe is an HMGR gene. Moreover, corresponding enzymology research is lacking even for the two HMGR genes isolated and sequenced from Arabidopsis (3, 9). In the frame of these limitations, the results of this research showed that there is an obvious discrepancy between microsomal HMGR activity and HMGR mRNA levels detected with the isolated cDNA sequence believed to be an HMGR probe.

Further characterization of this cDNA probe similar to that reported recently for the HMGR gene from radish (4) is needed. It is suggested that future research on the characterization of this probe should include the following particular steps: 1) construction of a maize cDNA library; 2) use of the uncharacterized HMGR probe to isolate a full HMGR gene from the maize cDNA library; 3) expression of the HMGR gene in E. coli to see whether this gene is a real gene coding for active HMGR protein; 4) production of an antibody for the HMGR protein which is expressed in E. coli; and 5) testing to determine whether the antibody can inhibit maize HMGR activity in vitro.

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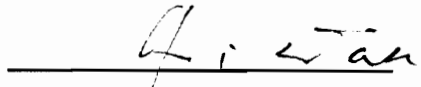

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