Curr Genet (1997) 31: 38–47 © Springer-Verlag 1997

ORIGINAL PAPER

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# 3-Hydroxy-3-methylglutaryl-CoA reductase gene of *Gibberella fujikuroi*: isolation and characterization

Received: 23 December 1995 / Accepted: 2 August 1996

**Abstract** 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR) is the first specific enzyme of the isoprenoid pathway, which leads to several classes of primary and secondary metabolites such as sterols, quinones, carotenoids and gibberellins. The structural gene of HMG-CoA reductase was isolated from the ascomycetous fungus *Gibberella fujikuroi*. Additionally, the most conserved region of this gene was also isolated from another plant pathogenic fungus, *Sphaceloma manihoticola*. Both ascomycetous fungi use the plant hormone gibberellin to induce an elongation of infected host plants, and in the case of *S. manihoticola* of plant tumors. Sequence analysis revealed a high degree of similarity between the deduced amino-acid sequences in the C-terminal catalytic domains of all known HMG-CoA reductases, but the highest degree was found between the sequences of both analysed ascomycetes. In contrast to *Saccharomyces cerevisiae*, *Ustilago maydis* and plants, *G. fujikuroi* and *S. manihoticola* possess only a single copy of this gene, although the product of HMGR (mevalonate) is the precursor for essential sterol and quinone biosynthesis and secondary metabolites such as gibberellins. RNA-blot and hybridization experiments showed that gene expression is not influenced by either glucose or ammonium excess.

**Key words** 3-hydroxy-3-methylglutaryl-CoA reductase · Isoprenoids · Gibberellins · *Gibberella* · *Sphaceloma*

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Communicated by O. C. Yoder

## Introduction

*Gibberella fujikuroi* is a rice pathogenic fungus producing high levels of gibberellins, a family of diterpenoid plant hormones. The identification of gibberellin as a plant growth regulator is an example of the interaction between a pathogen and its host plant. When the fungus attacks rice seedlings, an early disease symptom is the super-elongation of the shoots ("bakanae" disease). In 1935 Yabuta announced the isolation of a growth stimulant from the culture fluid of the fungus. The substance was called "gibberellin" according to the bakanae fungus. Only in 1956, however, gibberellins were found in extracts of higher plants (Radley 1956) and then defined as natural plant hormones, controlling such diverse processes as seed germination, cell elongation and division, flowering and fruit development. Extensive biochemical studies on endogenous gibberellin (GA) intermediates in GA-responsive plant dwarf mutants and GA-deficient mutants of *G. fujikuroi* have allowed the determination of the GA biosynthetic pathway (Bearder 1983; Graebe 1987). As diterpenes, the gibberellins are formed in the isoprenoid pathway, starting from mevalonic acid which is converted via isopentenyl, dimethylallyl, geranyl and farnesyl pyrophosphates to geranylgeranylpyrophosphate, being an important shared intermediate. Mevalonic acid is produced by the key enyzme 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34) from 3-hydroxy-3-methylglutaryl-CoA.

The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) gene has been isolated from several organisms such as *Saccharomyces cervisiae* (Basson et al. 1988), *Mesocricetus aureatus* (Chin et al. 1984; Skalnik and Simoni 1985), *Drosophila melanogaster* (Gertler et al. 1988), *Arabidopsis thaliana* (Learned and Fink 1989), *Hevea brasiliensis* (Chye et al. 1991 a, b) and *Ustilago maydis* (Croxen et al. 1994) because of its importance for sterols, and secondary metabolite biosynthesis. Whereas in animals only one HMGR gene copy was found, two ore more HMGR gene copies could be isolated from plants. For *H. brasiliensis* it was demonstrated that different HMGR

genes are differently regulated by ethylene (Chye et al. 1991 b). In the plant pathogenic basidiomycete *U. maydis* two HMGR isoenzymes were found (Croxen et al. 1994).

In mammals the synthesis and activity of this rate-limiting enzyme is regulated by negative feed back on steady state levels of HMGR mRNA as well as on the level of enzyme activity. For regulation at both levels, two products of the terpenoid pathway are necessary: a sterol (the so called low-density lipoprotein) and, second, a non-sterolic product which so far has not be determined exactly. Regulation of HMGR activity seems to be different in animals and plants. All structural genes of HMGR which have been analyzed to-date are highly conserved with respect to their structure and function. The enzymes concerned all possess two domains: the $NH_2$-terminal region encompasses the membrane-bound region whereas the COOH-terminal region of the protein is located in the cytoplasm and contains the biologically active domain. The N-terminal membrane anchor and the C-terminal catalytic domain are separated by a non-homologous linker region.

In mammals experimental evidence suggests that membrane attachment of HMGR is a prerequisite for the regulation of enzyme activity (Gil et al. 1985; Luskey and Stevens 1985). It is not known if such a regulation mode exists also in plants, in which the membrane-bound region is reduced from seven to two transmembrane domains.

Seven transmembrane domains are also assumed in yeast HMG-CoA reductases though it still has to be determined if HMG-CoA reductases have the same structure in filamentous fungi and if the structural similarity to mammalian proteins results in a similar regulation.

In the present study, we report the isolation and analysis of the complete genomic sequence of HMGR gene of the *G. fujikuroi* wild-type strain m567. The deduced amino-acid sequence was compared to HMGR sequences from other organisms. Furthermore, the most conserved region of the gene was isolated from the cassava pathogen *Sphaceloma manihoticola* which is well known as a producer of gibberellin $GA_4$. The HMGR gene copy number was estimated for both fungi. RNA-blot experiments showed that the HMGR gene is not the target for C-and N-catabolite repression of gibberellin biosynthesis.

## Materials and methods

*Strains and culture conditions. G. fujikuroi* m567 is a wild-type rice isolate which was provided by the Culture Collection for Fungi, Weimar (Germany). Strain *G. fujikuroi* MBC31 is a methyl-benzimidazol-2-yl carbamate-treated derivative of m567 with a reduced DNA content and a reduced GA-production level (B. Tudzynski, unpublished).Wild-type strain *S. manihoticola* Lu949, which was isolated from Cassava, was provided by W. Rademacher, BASF. Stock cultures of the strains were maintained on potato-saccharose agar (10% potatoes, 2% saccharose, 0.7% $CaCO_3$, 2% agar).

*RNA-blot and hybridization.* Cultivation for transcription analysis under inducing conditions was carried out in a $GA_3$ production medium [6% plant oil, 0.05% $(NH_4)_2SO_4$, 1.5% corn-steep liquor, 0.1% $KH_2PO_4$, pH 5.5]. For cultivation under GA-repressing conditions 10% glucose, 0.05% $(NH_4)_2SO_4$, 1.5% corn-steep liquor (Sigma), 0.1% $KH_2PO_4$, pH 5.5, was used. For the analysis of transcription level under N-excess or limitation and C-excess or limitation, a minimal medium with 1% ammonium, or without ammonium, and 10% glucose, or without glucose, respectively, was used. RNA was isolated by the urea / LiCl procedure (Chambers and Russo 1986). Total RNA was size-fractionated by electrophoresis on formaldehyde agarose gels (Church and Gilbert 1984) and downward blotted onto Hybond-N filters (Amersham, UK) (Sambrook et al. 1989). Pre-hybridization and hybridization were carried out at 60°C in 1% BSA fraction V, 1 mM EDTA, 7% SDS, 0.25 M $NaHPO_4$, pH 7.2. For hybridization a radioactively labeled fragment of the *G. fujikuroi* HMG-CoA reductase gene was used as the probe. Filters were washed twice with washing buffer 1 (0.5% BSA fraction V, 1 mM EDTA, 5% SDS, 40 mM $NaHPO_4$, pH 7.2) at room temperature, once with washing buffer 1 at 60°C for 15 min, and finally twice with washing buffer 2 (1 mM EDTA, 1% SDS, 40 mM $NaHPO_4$, pH 7.2) for 2 min at room temperature. The *gpd* gene of *Claviceps purpurea* was used as a control for RNA transfer (Jungehülsing et al. 1994).

*Library screening.* Plaques from a *G. fujikuroi* m567 genomic DNA library in phage EMBL3 were transferred onto nylon filters (Sambrook et al. 1989). A 123-bp PCR fragment derived from the genomic DNA of *G. fujikuro*i was used to probe the nylon membranes under conditions of high stringency (Sambrook et al. 1989). Phages yielding a positive signal on duplicate filters were purified, restriction-analysed, and probed with the PCR fragment. Hybridizing fragments were isolated and cloned into pUC vectors. Recombinant plasmids were propagated in *E. coli* DH5$\alpha$.

*Preparation of G. fujikuroi genomic DNA and phage DNA. G. fujikuroi* genomic DNA and the DNA of lambda phage were isolated according to the standard protocols of Cenis (1992) and Sambrook et al. (1989), respectively.

*DNA-sequencing and sequence analysis.* Fragments of genomic DNA clones, carrying parts of the HMGR-gene, were subcloned into pUC19. Sequencing with appropriate primers was performed for both DNA strains, using a Pharmacia T7-Sequencing Kit. The sequence data analysis, including the construction of trees, was performed with the program "Husar" (Heidelberg).

*PCR.* PCR conditions were: 94°C for 4 min, 30 cycles of 54°C for 1 min, 72°C for 1.5 min, 94°C for 1 min. Reactions contained 1×Super *Taq* reaction buffer (HT Biotechnology Ltd), 200 mM dNTPs, 50 ng of each primer, 50 ng template DNA, 0.5 units of Super *Taq* polymerase (HT Biotechnology Ltd). In order to isolate the HMG-CoA reductase gene from *G. fujikuroi*, two oligonucleotides were synthesized using the standard eukaryotic nuclear gene codon assignments (Gurr et al. 1987):

HMG 1 5′-GAT/C GCA/T ATG GGG/T ATG AAT/C ATG AT-3′
3′-CTA/G CGT/A TAC CCC/A TAC TTA/G TAC TA-5′

HMG 2 5′-AAU/C UGG AUU/C GAG GGN CG-3′
3′-TTA/G ACC TAA/G CTC CCN GC-5′

Both primers were used as mixtures of both strands. Primer sequences correspond to two conserved regions within the HMG-CoA reductase proteins derived from following organisms: *S. cerevisiae* (Basson et al. 1988), *A. thaliana* (Learned and Fink 1989), *M. aureatus* (Chin et al. 1984) and *D. melanogaster* (Gertler et al. 1988). Another set of oligonucleotides was used for the isolation of a terminal region of the *S. manihoticola* HMGR gene by PCR:

HMG 4 5′-AAU/C UGG AUU/C GAG GGN CG-3′

HMG 3 5′-ATG CTG GGC AAG ATC CCT GG-3′

The oligonucleotide primer HMG3 was synthesized on the basis of the sequence data of the *G. fujikuroi* HMGR gene. Primer HMG 4 was synthesized according to the alignment of the HMGRs of *S. cerevisiae*, *A. thaliana*, *M. auratus* and *D. melanogaster*.

*Southern-blot analysis.* Digestion of the genomic DNA of *G. fujikuroi* with restriction enzymes and gel electrophoresis were done as de-

scribed by Sambrook et al. (1989). Electrophoresed digested DNA was transferred onto Hybond-N filters (Amersham, UK) using the vacuum blotting method and hybridized at high-stringency conditions (Sambrook et al. 1989). Hybridization probes were prepared by random oligonucleotide priming using a GIBCO random prime labeling kit and the GIBCO protocol. Blots were washed twice with 1×SSPE, 0.1% SDS at 65°C for 15 min each; the final wash was carried out in 0.1×SSPE, 0.1%SDS at 65°C for 30 min.

## Results

### Isolation of the *G. fujikuroi* HMG-CoA reductase (HMGR) gene

Two oligonucleotides were synthesized to isolate the HMGR gene from *G. fujikuroi*. These primers encode the following peptide sequences relating to two conserved regions within the HMGR proteins of different organisms (see Materials and methods): HMG 1, Asp Ala Met Gly Met Asn Met; HMG 2, Asn Trp Ile Glu Gly Arg. Amplification of a corresponding DNA fragment of the *G. fujikuroi* gene was accomplished by PCR using these primers with *G. fujikuroi* m567 genomic DNA as a template. A single amplified DNA product (123 bp) was obtained and employed as a homologous probe to identify genomic DNA clones from a *G. fujikuroi* m567 EMBL3 library. Approximately 40 000 recombinant clones were screened at high stringency. Two positive plaques, designated l18/9 and l24/1, were isolated and purified. The DNA obtained from both λ clones was digested with restriction endonucleases (*Eco*RI, *Sal*I, *Bam*HI, *Xho*I, *Hin*dIII, *Pst*I, *Xba*I and combinations of *Eco*RI/*Sal*I and *Sal*I/*Bam*HI) and analyzed by Southern hybridization using the PCR fragment as a probe. Restriction analysis revealed that both λ clones contain overlapping inserts. Five different strongly hybridizing fragments of these λ clones were chosen for cloning into pUC vectors (Table 1). The 7.5-kb *Eco*RI fragment derived from λ18/9 (designated pJ25) contains the 1.05-kb *Sal*I/*Bam*HI, as well as the 4.2-kb *Eco*RI/*Sal*I, fragment of λ24/1 (Fig. 1) and overlaps the 10.0-kb *Pst*I and 5.0-kb *Hin*dIII fragments of plasmids pJ15 and pSTA1101 (Table 1) at the 5′ and 3′ ends, respectively. The smallest cloned 1.05-kb *Sal*I/*Bam*HI fragment from λ24/1 codes for the main part of the catalytic domain of the HMGR of *G. fujikuroi*.

**Table 1** List of fragments chosen for cloning into pUC vectors

| No. | Plasmid | Insert size (kb) | Cloning vector | Original phage | Restriction fragment |
|---|---|---|---|---|---|
| 1 | pSTA1100 | 1.05 | pUC13 | λ24/1 | *Sal*I/*Bam*HI |
| 2 | pSTA1101 | 5.0 | pUC13 | λ18/9 | *Hin*dIII |
| 3 | pSTA1102 | 4.2 | pUC18 | λ24/1 | *Sal*I/*Eco*RI |
| 4 | pJ15 | 10.0 | pUC19 | λ24/1 | *Pst*I |
| 5 | pJ25 | 7.5 | pUC19 | λ18/9 | *Eco*RI |

For DNA sequencing, relevant regions of the pJ25 insert were subcloned and sequenced on both strands using universal and reverse primers as well as specific oligonucleotides. The nucleotide and deduced amino-acid sequence of the HMGR gene are shown in Fig. 2. This region contains the structural gene and regulatory regions of the HMGR of *G. fujikuroi*. The sequence starts 769 bp upstream of a putative initiation codon (ATG) and continues 978 bp beyond a stop codon (TAA). This region of 2928 bp encodes a protein of 976 amino acids with a molecular mass of 105 449 Da. The ORF is interrupted in the strongly conserved domain b2 (Liscum et al. 1985) by a predicted 47-bp intron at position 2531. This deduced intron possesses typical fungal 5′ and 3′ splicing sites as well as the internal lariat consensus sequence. The intron position between *G. fujikuroi* and *A. thaliana* HMGR gene 1, *H. brasiliensis* HMGR gene1 and HMGR gene 3 and *M. auratus* HMGR gene is not conserved. The other fungal HMGRs which have been analyzed do not possess introns.

The nucleotide sequence surrounding the supposed translation initiation site (Fig. 2 marked with +1) is comparable to the Kozak consensus sequence which is described for translation initiation in fungi: the –3 position is an adenine (Gurr et al. 1987; Unkles 1992). In the promotor region no CAAT motif could be found; a TATAAA box is located at position –166 (TATTAC). Five putative CT motifs were present at positions –95, –153, –434, –517 and –716. Such CT-rich sequences are particularily obvious in genes lacking TATAAA and CAAT motifs and in highly expressed genes, suggesting their role as possible promotor elements (Gurr et al. 1987). Putative binding sites (GATA) for a nitrogen regulatory protein (AREA, NIT2) were found at positions –171, –189, –190, –496, –631 and –633 (AREA: Kudla et al. 1990; NIT2: Fu and Marzluf 1990). Gap alignment analysis of the deduced amino-acid sequence of the *G. fujikuroi* HMGR gene with the *U. may-*

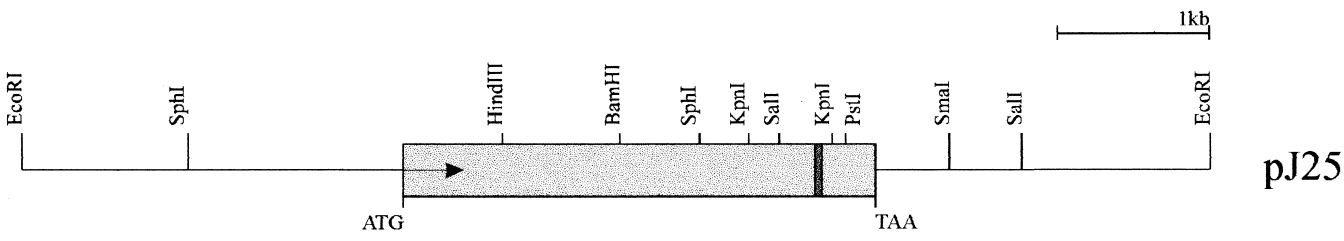



**Fig. 1** Physical map of plasmid pJ25 containing the *G. fujikuroi* HMGR gene. The *light dotted box* shows the position of the coding region and the dark dotted box indicates the position of the putative intron

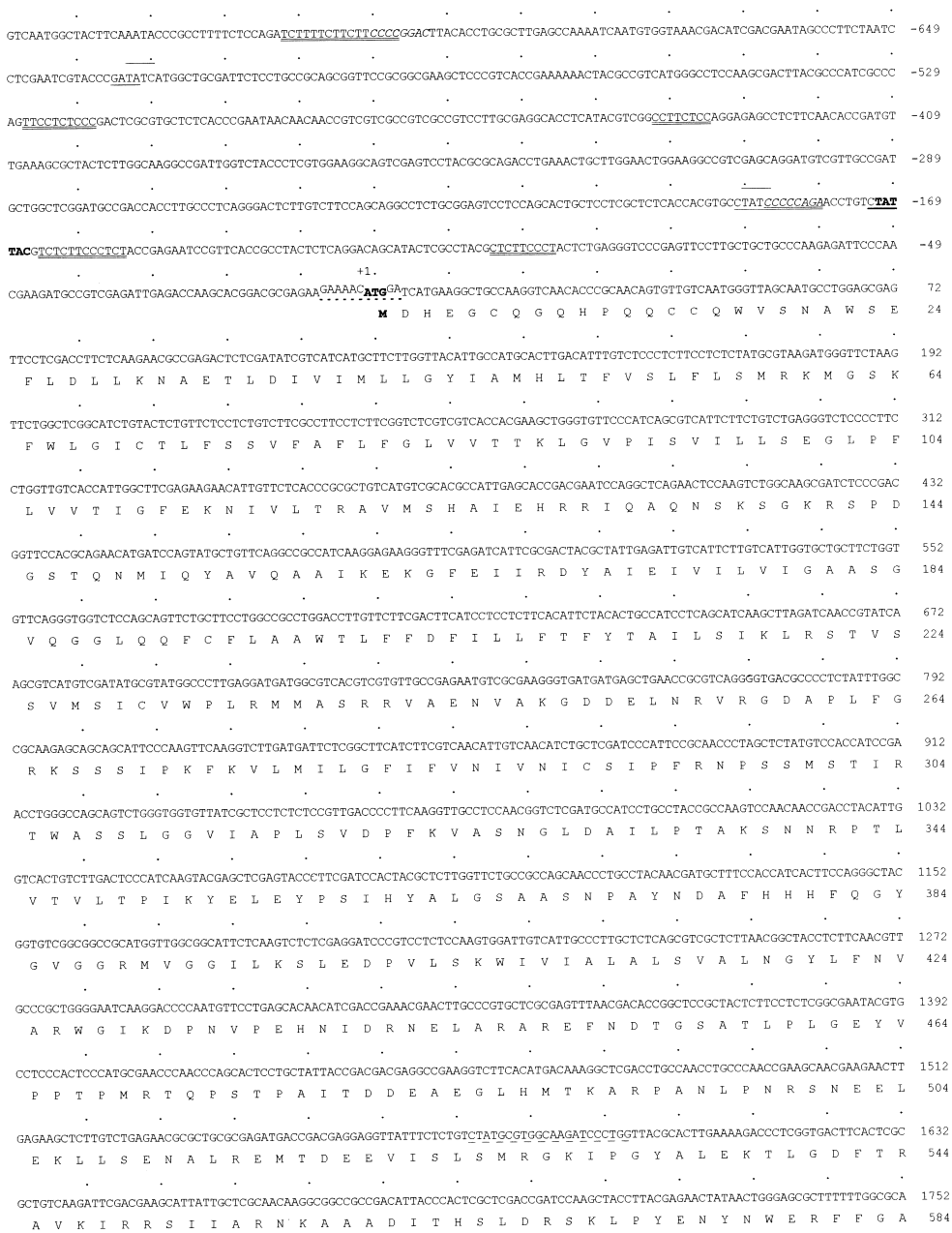


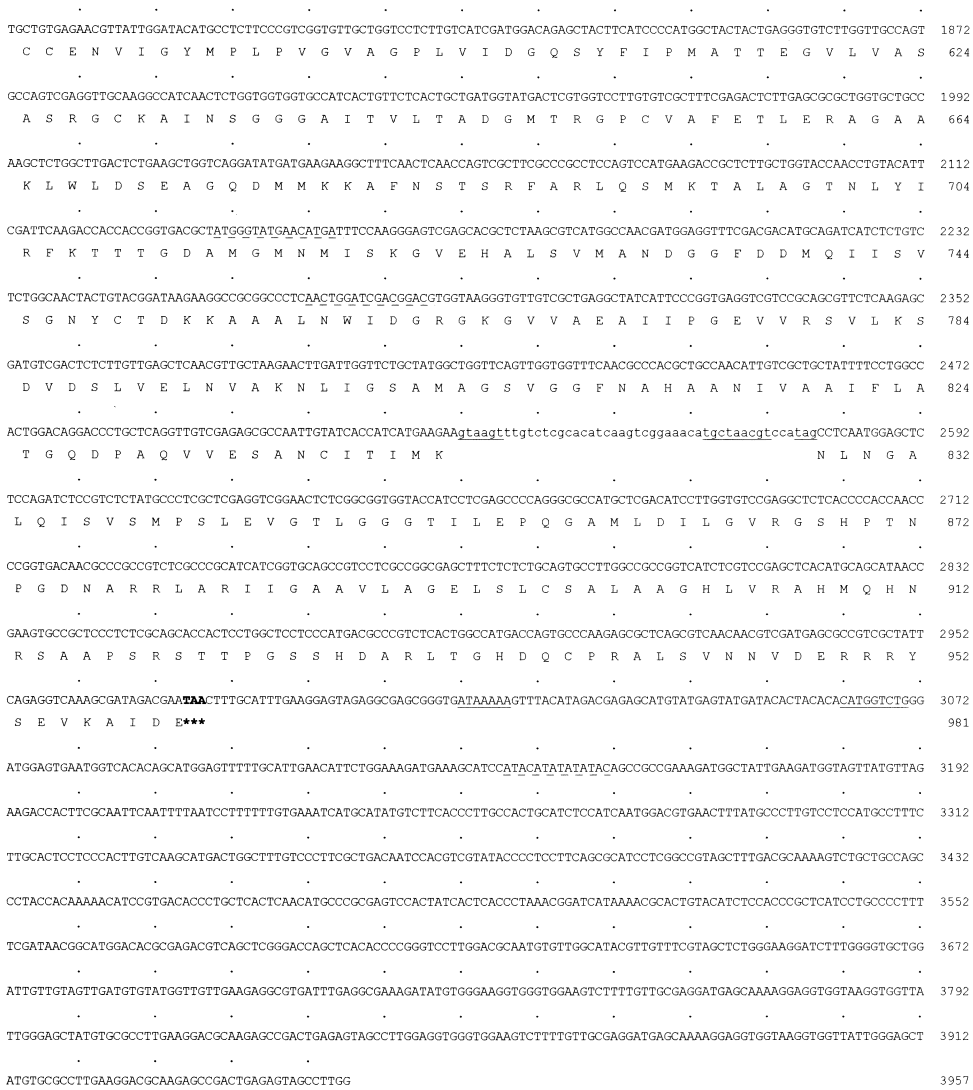
**Fig. 2** Nucleotide sequence of the HMGR gene of *G. fujikuroi* and its deduced amino-acid sequence (accession number X94307). The nucleotides are numbered relative to the putative translation start site ATG. The putative intron is shown in *lower-case letters*, its intron consensus sequences are marked by *underlined letters*, the Kozak-consensus sequence for the translation start point is marked by *dotted underlining*, the translation start point itself is written in *bold letters*. Promotor motifs are marked, putative TATA box (*bold letters*), pyrimidine-rich tracks (*double underlined*), putative GATA motifs (*under- and over-lined, respectively*) (Fu and Marzluf 1988; Kudla et al. 1990), a putative binding site for a CREA like protein (in *italics*). The stop codon in *bold* is marked by *three asterisks*, the putative polyadenylation signal is *underlined*. Primer sequences which were used for *S. manihoticola* PCR have *dotted underlines*

*dis* HMGR1 and the *S. cervisiae* HMGR1 and HMGR2 proteins revealed 64.3%, 64.1% and 65.8% overall similarity, repectively. This similarity extends from 76 up to 83% in the conserved C-terminal portion of HMGR gene product. The hydrophobicity plot of the *G. fujikuroi* protein revealed seven peaks of hydrophobicity within the N-terminal domain which may be transmembrane domains (data not shown). In *S. cerevisiae* HMGR1 and HMGR2, as well as in *U. maydis* HMGR1, eight hydrophobic regions have been identified, but only seven of them have been recognized as membrane-spanning domains (Sengstag et al. 1990).

## HMGR gene copy number

Previous experiments revealed that the mononucleate microconidia of the wild-type strain *G. fujikuroi* m567 are difficult to mutagenize. On the basis of the DNA content of strain m567 and its benomyl-treated derivative *G. fujikuroi* MBC31, it could be demonstrated that *G. fujikuroi* MBC31 possesses 50% of the DNA content of the wild-type strain m567. Additionally, *G. fujikuroi* MBC31 is characterized by a reduced $GA_3$ yield (tenfold less than the GA production of m567) and a tenfold increased rate of mutant production after the same UV treatment as m567

**Fig. 2** (continued)



(Bermudez-Torrez 1996). We conclude that the wild-type strain m567 is diploid or aneuploid but are unable to define whether the complete genome is doubled in DNA content or whether single chromosomes containing genes of the isoprenoid pathway are multicopy.

For gene copy number estimation of the HMGR gene, strains m567 and MBC31 were both analyzed. Genomic DNA was digested with several restriction enzymes and probed with the 1.05-kb *Bam*HI/*Sal*I fragment from $\lambda$24/1. Digestion resulted in one distinct hybridization band with the exception of *Sph*I, which cuts once in the 1.05-kb *Bam*HI/*Sal*I fragment that was used as probe (Fig. 3). Both the wild-type strain and its haploidized derivative showed the same hybridization pattern suggesting that if strain m567 is not haploid it contains two or more identical copies of the HMGR gene.

## Expression of the HMGR gene under different cultivation conditions

It is well known that gibberellin biosynthesis is inhibited by high amounts of glucose and ammonium (Brückner and Blechschmidt 1991; Brückner 1992), though the target genes involved are not known. To investigate whether expression of the HMGR gene is regulated at the transcriptional level, *G. fujikuroi* RNA was subjected to RNA-blot analysis. In a first experiment, expression of the HMGR gene was analyzed under conditions either inducing or repressing $GA_3$ production. *G. fuikuroi* m567 was incubated in a plant oil-production medium (avoiding glucose repression) with corn-steep liquor as a complex nitrogen source, and in a synthetic medium with glucose excess and ammonium, which reduces $GA_3$ amount about tenfold. After 3 days of growth, total RNAs were isolated from both mycelia and probed with the 4.2-kb *Eco*RI/*Sal*I fragment.

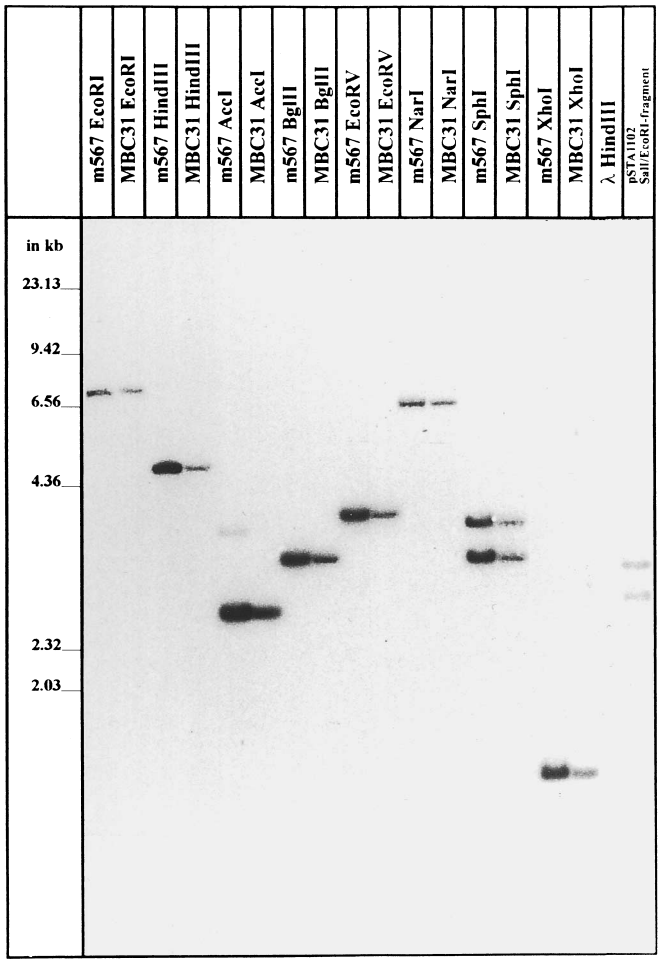
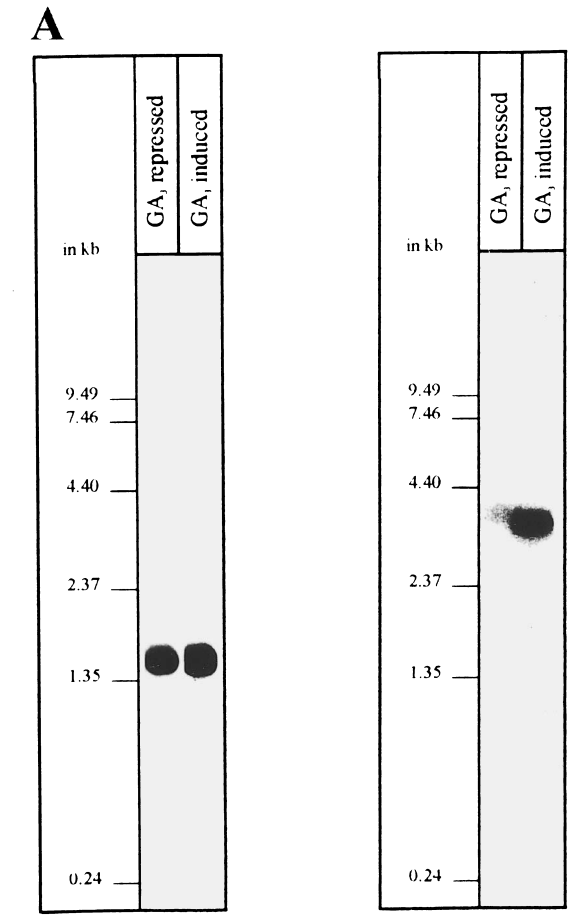



**Fig. 3** Copy number estimation for the HMG-CoA reductase gene of *G. fujikuroi* in the genome of the wild-type strain m567 and the haploidized derivative MBC31. Genomic DNA of both strains was digested with different restriction enzymes and probed with the 1.05-kb *Bam*HI/*Sal*I fragment of pJ25



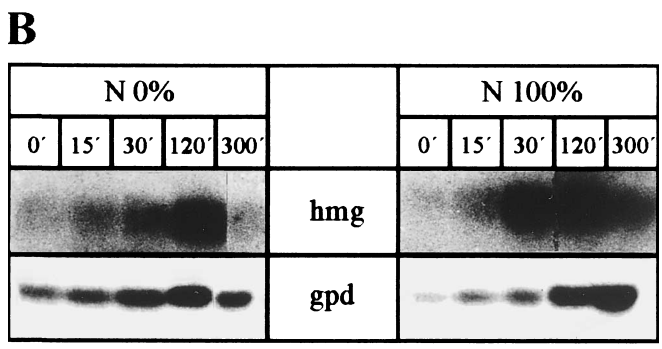




**Fig. 4A** RNA-blot analysis of the HMGR gene of *G. fujikuroi* m567 cultivated under conditions repressing $GA_3$ production (glucose excess) or stimulating $GA_3$ production (plant oil as carbon source, corn-steep liquor). The RNA was probed with the 4.2-kb *Eco*RI/*Sal*I fragment of pJ25. Left part shows the hybridization of the same total RNA with the *C. purpurea gpd* gene (Jungehülsing et al. 1994). **B** RNA-blot analysis of the HMGR gene of *G. fujikuroi* m567 under conditions of ammonium excess (N 100%) and starvation (N 0%), respectively. Total RNA was isolated after 0, 15, 30, 60, 120 and 300 min and probed with the 1.05-kb *Sal*I/*Bam*HI fragment of pJ25. Control hybridization was carried out with the *gpd* gene of *C. purpurea* (Jungehülsing et al. 1994)

This experiment revealed a transcript size of approximately 3.0 kb corresponding to the size of the HMGR gene (Fig. 4 A). As shown in Fig. 4, the HMGR gene mRNA level is much higher under $GA_3$-inducing conditions (plant oil and corn-steep liquor) than under $GA_3$ repressing conditions (glucose as carbon source, ammonium excess). To ascertain if the increase of HMGR mRNA level is induced by plant components (corn-steep liquor and plant oil) or is due to C- or N-de-repression, *G. fujikuroi* m567 was grown in a minimal medium with sucrose and additional supplements (vitamins and trace elements) for 6 days. After washing, the mycelium was transferred to minimal media either containing a glucose excess or else lacking glucose (data not shown). In a second experiment, the mycelium was transferred to synthetic media with an $NH_4^+$ excess or else lacking ammonia (Fig. 4 B). Total RNA was isolated from mycelia harvested at 0, 15, 30, 60, 120 and 300 min and probed with the *Sal*I/*Bam*HI fragment of pSTA1100. Under

all four conditions, with glucose or ammonia excess and glucose or ammonium starvation, no difference regarding the HMGR mRNA level could be detected between the different media. The HMGR transcript reached a maximum at 120 min; after that the transcript declined. This transient increase of transcript level could be the result of growth stimulation following the transfer into the new media after reaching the idiophase in pre-culture.

Therefore, the HMGR gene is expressed constitutively. The increased level of mRNA in plant-oil medium which is accompanied by an increased $GA_3$ level is not the result of C- or N-de-repression. $GA_3$ increase seems to be the consequence of a specific induction of the expression of the HMGR gene by plant components.

### Comparison to the HMGR encoding gene of *S. manihoticola*

The ascomycete *S. manihoticola* is known to be a pathogen of cassava (*Cassava esculenta*) causing an enormous internodal growth of young infected cassava plants and as well as the production of plant tumors as a result of gibberellin formation by the fungus. It has been shown that $GA_4$ is the active principle of the plant disease (Rademacher and Graebe 1979; Zeigler et al. 1980). In contrast to *G. fujikuroi*, *S. manihoticola* is not able to synthezise 1,2-dehydro- and 13-dehydroxy GAs like gibberellic acid $GA_3$ (Bearder 1983).

Nothing is known about the genetics and molecular genetics of GA synthesis in *S. manihoticola*. In order to compare the HMGR gene sequence data of the two GA-producing phytopathogenic ascomycetes, a part of the conserved C-terminal region of the *S. manihoticola* HMGR gene was isolated by PCR with the primer combination HMG 3/ HMG 4. The 840-bp *S. manihoticola* PCR fragment was subcloned into pCR-Script (Stratagene) and designated pSMHMG. This part of the *S. manihoticola* HMGR gene was sequenced and used for sequence comparisons.

In Table 2 the similarities of a 277 amino-acid fragment of the HMGR conserved region of *G. fujikuroi*, and other organisms, to the respective sequence of *S. manihoticola* are summarized. All ascomycetes share the greatest degree of similarity in this 277 amino-acid sequence, but the relationship between *G. fujikuroi* and *S. manihoticola* is the closest. This alignment was used for creating a phylogenetic dendrogram illustrating the evolutionary relationship of these HMG-CoA reductases (Fig. 5).

**Table 2** Comparison of the 277 amino acid HMGR PCR fragment of *S. manihoticola* (data bank accession number: X94308) with the homologous parts of some other HMGRs

| Organism | Numbers of aminoacids | Similarity (%) | Identity (%) |
|---|---|---|---|
| *S. manihoticola* | 1–277 | 100.0 | 100.0 |
| *G. fujikuroi* | 526–801 | 88.0 | 77.5 |
| *S. cerevisiae* HMGR1 | 626–897 | 80.6 | 70.5 |
| *S. cerevisiae* HMGR2 | 622–893 | 81.7 | 70.9 |
| *U. maydis* HMGR1 | 698–988 | 80.4 | 63.8 |
| *M. auratus* | 466–739 | 73.3 | 59.4 |
| *A. thaliana* HMGR1 | 173–446 | 70.0 | 53.2 |
| *H. brasiliensis* HMGR3 | 173–446 | 72.3 | 53.2 |
| *D. melanogaster* | 490–763 | 69.9 | 53.9 |
| *S. mansoni* | 471–748 | 63.9 | 42.7 |
| *H. volcanii* | 1–281 | 58.0 | 37.5 |
| *P. mevalonii* | 1–268 | 42.7 | 21.7 |

Estimation of HMGR gene copy number in *S. manihoticola* was carried out as described for *G. fujikuroi* m567. Genomic DNA of wild-type strain Lu949 was restricted with several enzymes and probed with the heterologous pSTA1100 *Sal*I/*Bam*HI fragment and the homologous PCR fragment (the 840-bp *Kpn*I/*Sal*I fragment of pSMHMG2). The two hybridization patterns were identical, indicating that *S. manihoticola*, like *G. fujikuroi*, possesses only one HMGR gene copy (data not shown).

## Discussion

In this study, we described the isolation and characterization of the *G.fujikuroi* HMGR gene and the isolation of a part of the *S.manihoticola* HMGR gene by PCR. Both *G. fujikuroi* and *S. manihoticola* contain only one HMGR gene, as indicated by Southern-blot analysis. This is in contrast to *S. cerevisiae* (Basson et al. 1986), *U. maydis* (Croxen et al. 1994) and some zygomycetes (Burmester and Czempinsky 1994) in which two structural genes are present. It is not possible to determine the exact number of homologous HMGR gene copies which should exist in *G. fujikuroi* wild-type strain m567. This isolate seems to possess more than a haploid genome according to DNA content measurement, mutation experiments, and the measurement of $GA_3$ yield, both for this wild-type strain m567 and for the benomyl-treated derivative MBC31 (Bermudez-Torrez 1996).

The 2928-bp open reading frame of the HMGR gene of *G. fujikuroi* is interrupted by a putative 47-bp-long intron in position 2591 whereas the HMGR genes of *S. cerevisiae* and *U. maydis* do not posses introns. A comparison with the introns of the *Arabidopsis thaliana* HMGR gene1, the *Hevea brasiliensis* HMGR gene1 and HMGR gene3, and the *Mesocricetus auratus* HMGR gene revealed that intron position is not conserved among the HMGR genes of these organisms. The HMGR gene of *G. fujikuroi* encodes a predicted protein of 976 amino-acids which is shortened in the N-terminus in comparison to the other fungal HMGRs. The *G. fujikuroi* HMGR exhibits a greater overall similarity to *U. maydis* HMGR1 (64.3%) and *S. cerevisiae* HMGR2 (65.8%) than to the product of the *S. cerevisiae* HMGR gene1 (63.8%). The sequenced C-terminal part of the *S. manihoticola* HMGR gene product reveals that the highest homology can be found between the two pathogenic ascomycetes *G. fujikuroi* and *S. manihoticola*. The similarity in this part of the C-terminus is about 83%. This high sequence similarity within the catalytic domain of the *G. fujikuroi* HMG-CoA reductase to *Ustilago* and yeast gene products and to HMG-CoA reductases from

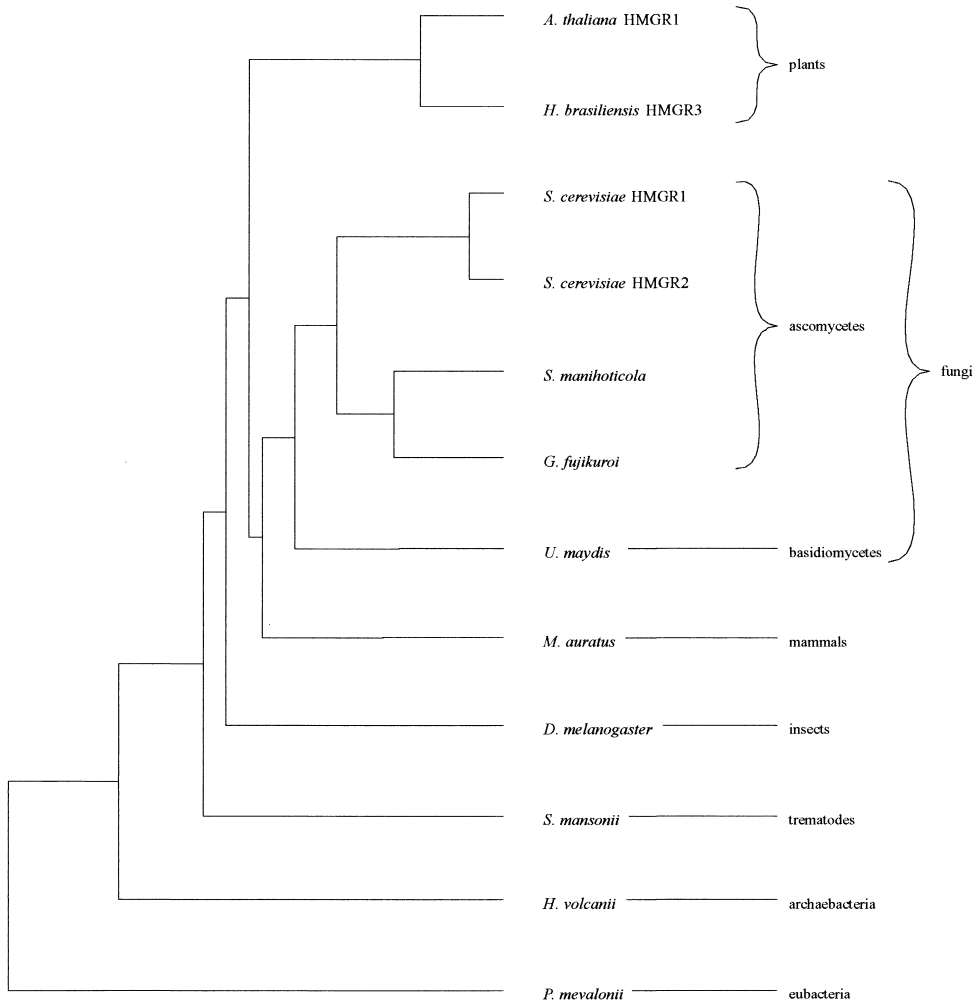



**Fig. 5** A phylogenetic tree based on the alignment of the amino-acid sequences shown in Table 2 was constructed using the program "Husar" (Heidelberg) (Sources of sequences: *S. cerevisiae* HMGR1, HMGR2: Basson et al. 1988; *U. maydis* HMGR: Croxen et al. 1994; *M. auratus* HMGR: Skalnik and Simoni 1985; *A. thaliana*: Learned and Fink 1989; Caelles et al. 1989; *H. brasiliensis* HMGR1 and HMGR3: Chye et al. 1991 b; *D. melanogaster* HMGR: Gertler et al. 1988; *S. mansoni* HMGR: Rajkovic et al. 1989; *H. volcanii* HMGR: Lam and Dollittle 1992; *P. mevalonii* HMGR: Beach and Rodwell 1989)

a wide range of other organisms indicates the high sequence conservation of this enzyme during evolution which may be associated with substrate and/or co-factor binding sites or catalytic activity.

The phylogenetic tree presented in Fig. 5 demonstrates that HMG-CoA reductase is helpful for analyzing the evolutionary relationships between different organisms. Protein structure and function are well conserved during evolution but the number of amino-acid exchanges which do not change the structure and the function of the enzyme give rise to a phylogenetic tree of general interest.

The N-terminal region of the *G. fujikuroi* HMG-CoA reductase gene contains seven putative hydrophobic domains using Kyte and Doolittle (1982) hydropathy plots (Sengstag et al. 1990). In *U. maydis* and *S. cerevisiae* eight hydrophobic regions were predicted but only seven have been recognized functionally as possible transmembrane domains (Sengstag et al. 1990). Besides anchoring into the endoplasmic reticulum, the membrane-spanning region has other functions. In mammalian cells, the membrane-bound portion of the protein is required for the sterol-regulated proteolytic degradation of the enzyme (Gil et al. 1985). The N-terminal region of the protein is also important in both *S. cervisiae* and mammalian cells for triggering increased membrane synthesis (Wright et al. 1988; Anderson et al. 1993).

Within the C-terminal domain some amino acids are functionally conserved and may play an important role in the structural conformation and/or in the catalytic properties of the enzyme. Two histidine and five cysteine residues are common to all HMGRs that have been analyzed (Caelles et al. 1989). Most of these residues are located within the more conserved region among the different HMG-CoA reductases, the so called b domains (b1 and b2) which have been postulated to be a part of the active site for hamster HMGR (Liscum et al. 1985). This region is especially rich in glycine and cysteine residues. Whereas the glycine residues are important in maintening the correct structure of the catalytic domains, the cysteine residues may play an important role in the catalytic process in addition to their function in structural maintenance (Dugan and Katiyar 1986). Amino acid residues which have already been shown to be important for catalysis in the HMGRs of *P. mevalonii* and hamster include the histidine

residue at position 924 (Darnay et al. 1992; Darney and Rodwell 1993) and the glutamate and aspartate residues at positions 618 (domain b1) and 828 (domain b2), respectively (Wang et al. 1990; Frimpong and Rodwell 1994). Of particular interest, with respect to the regulation of HMG-CoA reductase activity, is the serine residue at position 930. This residue is located close to the second catalytically important histidine residue at position 924. In eukaryotic HMGRs, this serine residue is known to be phosphorylated by AMP-activated proteine kinase, resulting in a loss of catalytic activity (Clark and Hardie 1990; Gillespie and Hardie 1992; Sato et al. 1993). Interestingly, neither of the *S. cerevisiae* HMGRs contain a serine residue at this position in the protein. The deduced amino-acid sequence of the C-terminal part of *S. manihoticola* HMGR possesses all conserved amino acids of domain b1 and all glycine and cysteine residues common to b1 and b2.

It is known that gibberellin biosynthesis is regulated via N- and C-catabolite repression (Brückner and Blechschmidt 1991; Brückner 1992). However, the target gene(s) for those regulation mechanisms are unknown.

The promotor region of 769 bp of the HMG-CoA reductase gene of *G. fujikuroi* was analyzed with respect to the known consensus sequences for DNA-binding proteins of C- and N-regulation. Five GATA motifs were found in the promotor region, three of them lay together in a 18-bp region next to the putative TATA box. Haas and Marzluf (1995) postulated that two or three such GATA elements must be located within a 100-bp region in order to be involved in N-metabolite regulation. A putative binding site for a glucose regulator protein was found 711 bp upstream of the translation initiation site, which is similar to the binding site for CREA of the amd S gene of *A. nidulans* (Davis et al. 1993): 5′-CCCCGGACT-3′.

The existence of such consensus sequences indicates the possible regulation of this primary metabolism gene. However, the expression analysis for the HMGR gene revealed an opposite result. Excess of glucose or ammonium does not have repressing effects on HMGR gene expression. Therefore, the HMGR gene is regulated like a housekeeping gene. Because of the importance of the gene for membrane synthesis and for cell growth, this result is not surprising. On the other hand, the HMGR gene seems to be induced by plant components, perhaps reflecting the close relationship between gibberellin biosynthesis and plant disease.

**Acknowledgements** This work was funded by a grant of the Deutsche Forschungsgesellschaft, Bonn-Bad Godesberg, Germany, (Grant number Br 1245/1-1) and the Biotechnology and Biological Science Res Council, UK, (Grant number GR/H/20/52).

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