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Differential Induction of 3-Hydroxy-3-methylglutaryl CoA Reductase in Two Cotton Species Following Inoculation with *Verticillium*

Oscar Joost[1], Graciela Bianchini[2], Alois A. Bell[3], C. R. Benedict[2], and Clint W. Magill[1]

[1]Department of Plant Pathology and Microbiology, Texas A&M University College Station 77843, [2]Department of Biochemistry and Biophysics, Texas A&M University, College Station 77843, [3]USDA, ARS, Southern Crops Research Laboratory, College Station 77845, U.S.A.

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***Gossypium barbadense* cottons are typically more resistant to wilt pathogens than are *Gossypium hirsutum* cultivars. Both species make terpenoid phytoalexins in response to infection, implicating isoprenoid biosynthesis as a factor in resistance. Conserved regions in plant 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), the first enzyme in the terpene biosynthesis pathway, were used to design polymerase chain reaction primers for cloning a fragment of a cotton HMGR gene. The clone was used as a probe on Northern blots to show that induction of HMGR mRNA following introduction of *Verticillium dahliae* spores into the vascular system is much more rapid in Seabrook Sea Island, a resistant *G. barbadense* cotton, than it is in Rowden, a susceptible *G. hirsutum*. The amount of HMGR mRNA returned to near control levels in 4 days in the former variety but continued to accumulate in the latter. Specific enzyme activity of HMGR also increased more rapidly in stele extracts of Seabrook Sea Island than in Rowden.**

Additional keyword: host defense.

Cotton plants make and store gossypol and related compounds in subepidermal pigment glands as a deterrent to insects and other herbivores. If conidia of *Verticillium dahliae* Kleb., the causal agent of Verticillium wilt, are introduced into the vascular system by stem puncture of young seedlings, gossypol and its biosynthetic precursors, including hemigossypol (HG), desoxyhemigossypol (dHG) and their 3-hydroxyl methyl ethers (MHG and dMHG), are also found in vascular extracts (Bell et al. 1993). Since low concentrations of several of these gossypol precursors inhibit the growth of *Verticillium* and other fungi, it is assumed that they are formed as part of an active host defense mechanism.

Like all isoprenoid compounds, gossypol and its precursors are derived from mevalonic acid (MVA), the product of the reaction catalyzed by 3-hydroxy-3-methylglutaryl CoA reductase (HMGR, EC. 1.1.1.34). Many other compounds, including carotenoids, gibberelins, abscisic acid, and the phytol tail of chlorophyll, that are essential for normal growth and development of plants are also made from condensing two or more isoprenoid units, making HMGR a key enzyme in plant metabolism.

Corresponding author:: Clint Magill; E-mail: c-magill@tamu.edu

Gossypium hirsutum L. cv. Rowden is an upland cotton variety that is very susceptible to *Verticillium dahliae.* It is severely stunted and wilted by nondefoliating strains of the pathogen and is completely defoliated by defoliating strains. *Gossypium barbadense* L. cv. Seabrook Sea Island 12B2 (SBSI) is a lower yielding Egyptian cotton, interspecifically compatible with *Gossypium hirsutum,* that has greater tolerance to *Verticillium.* It exhibits transient minor symptoms with nondefoliating strains and only partial defoliation and some stunting with defoliating strains.

Here we compare the induction of transcription and enzymatic activity of HMGR in control and inoculated seedlings of the two cotton varieties in order to further define the role of the terpenoid pathway as a natural defense mechanism.

RESULTS

Cloning of a cotton HMGR fragment.

Since prior attempts to use heterologous probes to quantify messages in cotton proved unsatisfactory, the first goal was to clone a portion of one or more cotton HMGR sequences. By aligning previously cloned HMGR sequences that were available in GenBank, it was possible to identify regions of high homology that were used to synthesize degenerate primers for polymerase chain reaction (PCR) amplification of the equivalent gene from cotton (Fig. 1). The PCR products using either *G. hirsutum* (Rowden) or *G. barbadense* (SBSI) DNA as target gave identical agarose gel electrophoresis patterns. After 28 cycles with an annealing temperature of 62°C, two products of about 350 and 400 bp were slightly more predominant than three larger fragments of 600 to 800 bases. The smallest band from the SBSI reaction was cut from the low melting point (LMP) agarose gel and cloned. The 313 bases sequenced for the HMGR clone are shown in Figure 1. The cloned PCR product includes the complement of a Primer 2 oligonucleotide at its 3′ terminus but does not include the upstream Primer 1, and is shorter than the size expected from the excised band. The cotton fragment begins about 52 bases downstream of the expected Primer 1 position based on the corresponding coding region of the HMGR gene from *Hevea brasiliensis* (Willd. ex Adr. Juss.) Müll. Arg.

(Chye et al. 1991). The cotton sequence includes portions of two exons corresponding to exons two and three of *H. brasiliensis* and an intervening intron of 92 bp. Nucleotides in the upstream exon are 77% identical with the sequence of HMGR from *Lycopersicon esculentum* L. and the 168 nucleotides of the downstream exon are 82% and 77% identical to *H. brasiliensis* and *Arabidopsis thaliana* (L.) Heynh. HMGR and even 71% identical to the mouse gene. When translated to a peptide sequence, the cotton probe shows over 90% amino acid identity with the two corresponding open reading frames of the *H. brasiliensis* gene (Fig. 2).

Genetic complexity.

The cotton HMGR fragment was used as a probe in a Southern blot of Rowden and SBSI genomic DNA digested with *Eco*RI and *Hin*dIII restriction enzymes. There are no *Eco*RI or *Hin*dIII recognition sites internal to the cotton HMGR probe, so each hybridizing fragment should represent one or more distinct HMGR genes. The SBSI/*Eco*RI lane had fragments of 6.9, 5.6, 4.7, 4.1, 3.8, 3.4, and 1.8 kb, with the two largest bands possibly representing doublets. SBSI DNA digested with *Hin*dIII revealed 7 fragments: 6.4, 4.85, 4.15, 2.85, 2.5, 2.4, and 1.0 kb. Digests of Rowden genomic DNA resulted in similar patterns of hybridization, with the exception of a 5.9 instead of a 6.4 kb fragment in the *Hin*dIII digest. No hybridization with *Verticillium dahliae* DNA was detected with this cotton HMGR probe.

HMGR mRNA induction in the disease interaction.

When compared with low levels of HMGR mRNA present in water-inoculated plants, there was a large increase in HMGR mRNA in the Seabrook plants within 10 h post-inoculation (p.i.) in response to both live and heat-killed conidia (Fig. 3). The level of expression was similar in all three fungal treatments (viable TS-2 and V-76 and heat-killed V-76 conidia). Hybridization of the same membranes to a probe for β-1,3 glucanase, which was constitutively expressed under all treatment conditions, verified that equivalent

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Primer 1:  5' AGY ACT GGY GAT GCW ATG GGR ATG A  3'

5' T TTG GAT TTC CTT CAA ACT GAT TTC CCT GAC ATG GAT GTC ATT
   GGC ATC TCT G

   GTGAGTTTCA  CTTTTTGACT  TGTAATTGTG  TTCTTTCTAT  GTTTTTACTG
   TTCTATATAG  GATTACTGAA  ATTTATGAAT  TTGCCCATAT  AG

    GA AAT TTT TGT TCC GAC AAA AAA CCA GCG GCT GTA AAT TGG ATT
   GAA GGA CGA GGC AAA TCT GTT GTT TGC GAA GCC ATC ATT AAG GGT
   GAT GTG ATT AAG AAG GTC TTG AAG ACA AGT GTG GAA TCT CTC GTT
   GAG CTT AAC ATG CTT AAG AAC CTT GCT GGC TCT G  3'

Primer 2: 3' TAC GAR TTY TTR GAA YGA CCR AGA C 5'

     R = Pur    W = A,T    Y = C,T
```

Fig. 1. Nucleotide sequences of upstream and downstream mixed oligonucleotide primers and the polymerase chain reaction product used as a 3-hydroxy-3-methylglutaryl CoA reductase probe. The underlined region shows the incorporation of the complement of a Primer 2 set member. IUPAR symbols are used in the primer sequences to indicate multiple bases randomly incorporated at that position. Open reading frames are shown as triplets and the intron region is shown as ten-mers.

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Cotton:          LDFLQTDFPDMDVIGISG
                   |   |  |          * * * * 92 base intron * *
Rubber plant:    LEFLQSDFSDMDVIGISG

       NFCSDKKPAAVNWIEGRGKSVVCEAIIKGDVIKKVLKTSVESLVELNMLKNLAGS
* * *                          || |  | |  | |
       NFCSDKKPAAVNWIEGRGKSVVCEAIIKEEVVKKVLKTNVASLVELNMLKNLAGS
```

Fig. 2. Comparison of the translated peptide sequences of 3-hydroxy-3-methylglutaryl CoA reductase genes from *Gossypium barbadense* and *Hevea brasiliensis*. Amino acid mismatches are indicated.

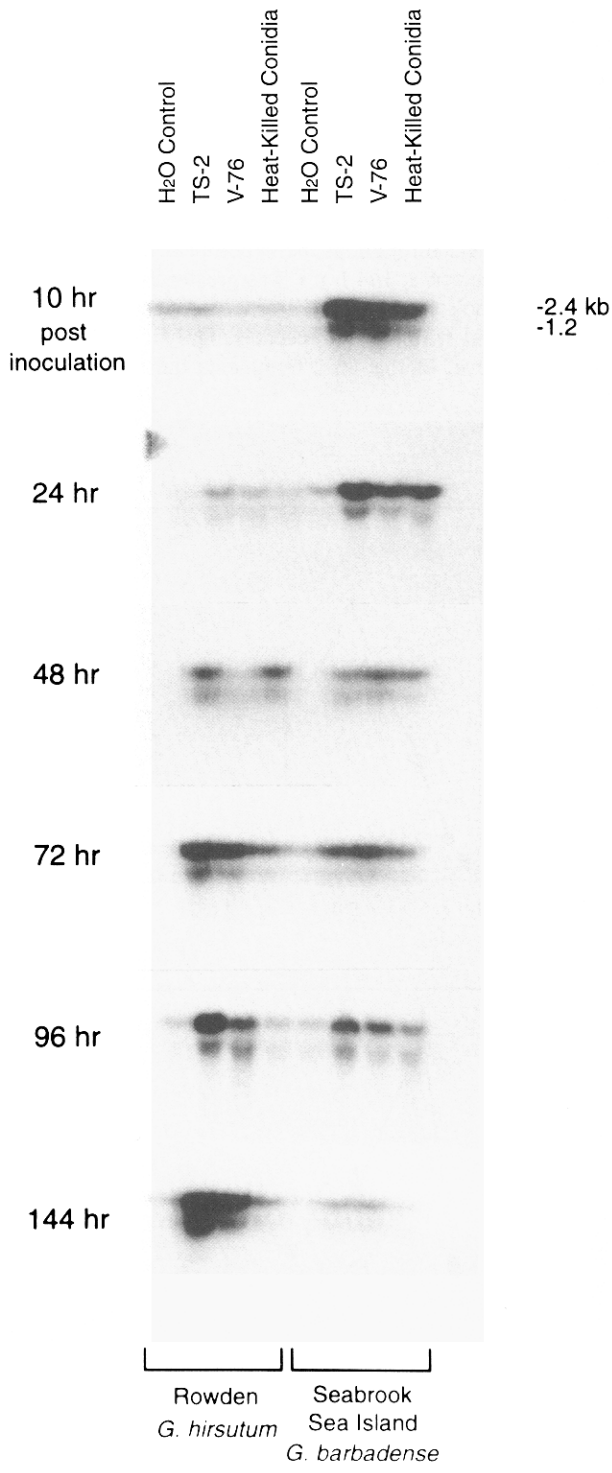

Fig. 3. 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) mRNA extracted from stele tissue of cotton cultivars Rowden and Seabrook Sea Island 12B2 (SBSI) after inoculation with conidia of *Verticillium dahliae*.

amounts of equal RNA were loaded in each lane. The highest level of HMGR mRNA was detected at the 10-h p.i. sampling. Decreased amounts of message, though still well above the level in control plants, were present in the 24- to 96-h p.i. samples. By 144 h the levels of expression in the treated plants returned nearly to that of the water control.

A more gradual increase was seen in HMGR expression in Rowden plants. A slight increase was seen at 24 h in treated plants, and while duplicate experiments gave somewhat variable results at 48 h, all plants at 72 h p.i. showed induced levels of HMGR mRNA in response to all fungal treatments. Heat-killed conidia elicited an initial response at 48 h that was as strong as that in either live fungal treatment but that then declined to control levels by the 96-h sampling. Rowden plants steadily increased the amount of message present in response to TS-2 throughout the experiment and the amount of message present at 144 h p.i. was greater than at any time in the Seabrook plants. HMGR message accumulation in Rowden treated with V-76 proceeded more slowly and never reached the level of the TS-2 treatments during this experiment.

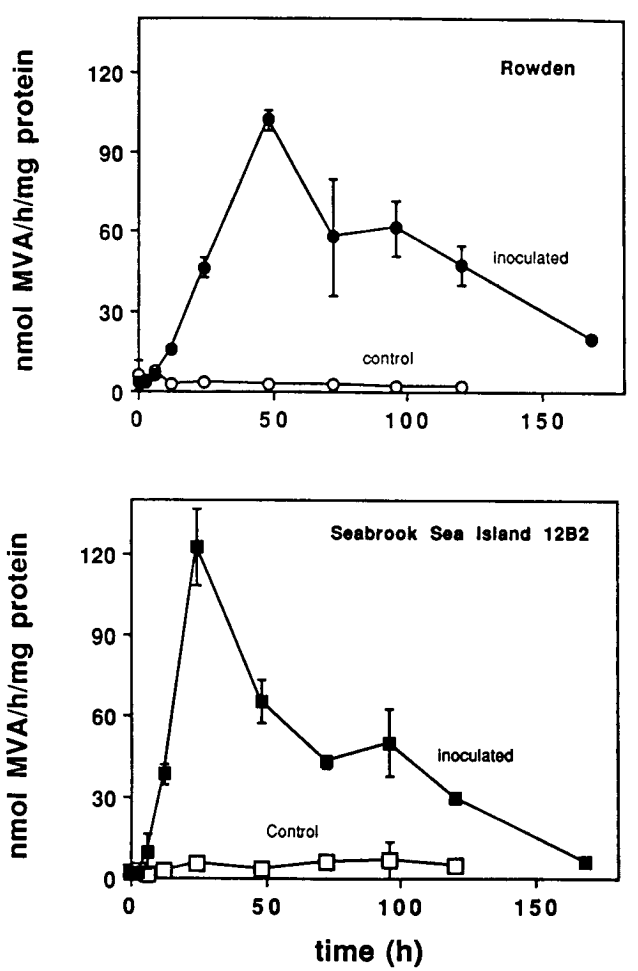

Fig. 4. Specific activity of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) in extracts from stele tissue of cotton cultivars Rowden and Seabrook Sea Island 12B2 (SBSI) after inoculation with conidia of *Verticillium dahliae*.

HMGR enzymatic activity.

Protein extracts from stele tissue of 6-week-old plants of both cultivars were made at selected times after inoculation with conidia of the V76 isolate of *Verticillium dahliae*, or with water injections in the control plants (Fig. 4). The enzyme activity of the control plants remained at very low levels throughout the experiment. After 24 h, the HMGR activity in inoculated SBSI plants had increased to 122.5 nmol MVA/h/mg protein, a >60-fold increase over the control and initial measurements. The activity declined rapidly, falling to half the peak over the next 24 h. By 7 days specific activity had returned to the low levels seen in control plants. The Rowden plants showed a similar pattern of peak and decline, but in plants of this cultivar the maximum peak of 102 nmol MVA/h/mg protein was not reached until 48 h p.i., and then activity declined more gradually. Similar peaks were obtained for each of the cotton cultivars using heat-killed conidia (data not shown). A test of HMGR activity in extracts of the fungus grown in culture revealed less than 1.0 nmol MVA/h/mg protein even when both the microsomal and soluble fractions were combined.

DISCUSSION

The availability of HMGR sequences from other plant species greatly facilitated the cloning of a segment of an HMGR from cotton. By identifying regions of high homology, it was possible to design mixed oligonucleotide primers that would consistently amplify several fragments of cotton DNA. Since one of these fragments was near the predicted size, it was selected for cloning. The clone that was sequenced turned out to be shorter than the amplification product and lacked one of the primers, possibly resulting from nuclease activity prior to blunt end ligation. However, based on the location of an intron and sequence comparisons to HMGRs from *Arabidopsis* (Learned and Fink 1989), potato (Stermer et al 1991), tomato (Park et al. 1992), and especially rubber plant (Chye et al. 1991), the clone is clearly from an HMGR gene. Nucleoside variation in the coding regions was mostly in silent third base "wobble" positions of the open reading frames, suggesting the clone was derived from an active copy rather than a pseudogene.

Southern hybridization using the cloned probe revealed seven to nine distinct members of an HMGR gene family in these two cotton cultivars. These numbers correspond well to the situation found in other plants (Stermer et al. 1994), especially when it is considered that cotton is a tetraploid. Mevalonate is required in the synthesis of numerous compounds essential to plant growth, including the hormones ABA, gibberellin, and cytokinin, as well as carotenoid pigments and the isoprenoid-derived phytol side chains required for chlorophyll function. The numerous copies provide adaptive flexibility in that separate regulatory mechanisms can permit differential expression during development and in response to different stresses. Evidence for differential expression of at least three HMGR isogenes has already been demonstrated in rubber plant (Chye et al. 1992), tomato (Cramer et al. 1993), and potato (Choi et al. 1992). The multiple copies of HMGR genes present in the genome may explain some of the extra copies of PCR products that were formed using our degenerate primers. Likewise, the low constitutive levels of HMGR mRNA often seen in water-inoculated plants probably reflect

the multiple pathways that require mevalonate and the continual need for its synthesis. Since the clone used as a hybridization probe includes highly conserved sequences, it would be expected to hybridize to HMGR messages in general. The primary transcript detected on Northern blots (Fig. 3) is approximately 2.4 kb, similar to the HMGR messages described for potato (Stermer et al. 1991) and *Arabidopsis* (Learned and Fink 1989).

Terpenoids are not normally found in the vascular systems of healthy cotton plants, but the sesquiterpenoid aldehydes and napthofurans that are the precursors of gossypol, hemigossypol, and their methyl ethers accumulate in stem xylem and the surrounding parenchyma cells upon infection by *Verticillium dahliae* (Mace et al. 1985). The most potent antifungal terpenoid based on in vitro assays is desoxyhemigossypol (dHG). It is effective at lower concentrations in inhibiting germination of *Verticillium* conidia and in killing conidia and mycelia (Bell et al. 1994). dHG is readily soluble in aqueous xylem fluid to effective concentrations and has been histochemically visualized in xylem vessels, paravascular parenchyma cells, and on the surface of mycelia and conidia in infected cotton steles (Mace et al. 1989). Prior observations by Stipanovic et al. (1988) suggesting that differences in phytoalexin responses contribute to the differential resistance of *G. hirsutum* and *G. barbadense* to *Verticillium* include the following: *Verticillium*-inoculated *G. barbadense* plants were found to have detectable amounts of dHG in xylem vessels within 18 h of inoculation and to produce effective fungistatic amounts of phytoalexin within 2 days, 24 to 48 h earlier than susceptible *G. hirsutum* plants. Resistant SBSI plants ceased to accumulate phytoalexin by 6 days p.i. while susceptible Rowden plants continued to increase phytoalexin for up to 2 weeks and eventually exceeded the resistant plants in total phytoalexin accumulation.

Patterns of HMGR mRNA and enzyme expression seen in this study are consistent with the above observations, and suggest that transcriptional regulation of one or more HMGR loci is critical to controlling accumulation of the terpenoid phytoalexins. The timing and rate of message accumulation and de novo enzyme synthesis correlate well with the previously described pattern of phytoalexin accumulation in *Verticillium*-infected stele tissues of SBSI cotton. The rapid increase of HMGR mRNA in SBSI plants that was detected at the 10-h p.i. sampling precedes the peak of enzymatic activity detected at the next sampling time (24 h). As previously suggested for enzymes in the pathway for phenylpropanoid-derived compounds (Bell et al. 1984), it seems likely that the rapid increase in HMGR activity leads to a burst of phytoalexin production that is sufficient to halt the spread of the pathogen and thus to confine the defense response to those cells that are initially affected.

Visible signs of germination of conidia of *V. dahliae* plated on suitable culture media are not detected before 18 to 24 h. Assuming that germination in planta follows the same time course, and since the initial response is equivalent for heat-killed conidia, SBSI plants must be able to recognize and respond to some conidial component. Vegetative compatibility group membership or pathogen virulence had no discernible effect on HMGR activation in SBSI plants. The *G. barbadense* genetic response to *Verticillium* conidia has several adaptive advantages as a strategy for control of terpenoid phytoalexin production. Secondary conidia traveling in the respiration stream are the first fungal cells to which any particular stele tissue will be exposed in the infection process and sensitivity to them allows for the most rapid and effective response. Since terpenoid phytoalexins may be as injurious to plant cells as they are to pathogen cells, the rapid decline in HMGR messages after sufficient enzyme has been produced to provide an effective antifungal accumulation of dHG protects the plant itself from unnecessary exposure to these compounds. However, the observation that some viable conidia persist for as long as 7 to 10 days in infected SBSI tissues (Mace et al. 1985) suggests that other, slower defense responses, such as tannin accumulation, may also be important in the ability of SBSI to overcome the pathogen and avoid wilting.

HMGR is also induced in Rowden, a susceptible *G. hirsutum* cultivar, following inoculation with viable *Verticillium* conidia, but within the first 24 h the amounts of mRNA and the enzymatic activity are much lower than in SBSI. If Rowden responds to the same signal as SBSI, its recognition or signal response pathway must be less efficient. However, since the conidia probably have sufficient time to germinate, it is also possible that all or part of the defense response in Rowden is triggered by a mycelial component, a fungal metabolite, or a hydrolytic enzyme. Heat-killed conidia may decompose in the xylem to release an inducer, and the limited quantity available from nongrowing cells could account for the transient kinetics of induction. Under this model, conidia from both TS-2 and V-76 could continue to be made and move through the vascular system ahead of the region of highest response and thus lead to transcription of HMGR mRNA in more and more cells. The less virulent nondefoliating TS-2 is a more effective elicitor of HMGR, and thus phytoalexin, and this may account for its reduced virulence. The defoliating members of VC1 may be more destructive on cotton because they are less effective elicitors. It seems likely that the response seen in Rowden in mRNA accumulation and in enzyme activity comes too late to prevent the spread of the pathogen, causing more cells to become involved in the defense response. Continued accumulation of mRNA without a continuous rise in HMGR specific activity in Rowden may reflect translational regulation due to high concentrations of an endproduct, relative increases in the levels of other soluble protein in the infected tissues, or even that tannins and other induced compounds accumulate to levels sufficient to inhibit HMGR activity.

In summary, these observations support earlier models suggesting that isoprenoid-derived phytoalexins are a key to defense against Verticillium wilt. Seabrook Sea Island is relatively resistant and the rapid increase in activity of HMGR can be expected to increase the pool of mevalonic acid needed for synthesis of isoprenoid compounds even before injected conidia would be expected to germinate, even if a less fungitoxic compound is the primary product. The delayed response seen in Rowden may permit the fungus to stay a step ahead of the host defense.

MATERIALS AND METHODS

Plant materials.

Seeds of Rowden and SBSI were pregerminated in paper rolls at 30°C for 40 h and then transferred to 16-ounce plastic

cups and grown in the greenhouse. After the expansion of six true leaves the plants were moved to environmental growth chambers with a lighted day temperature of 28°C and dark temperature of 22°C. Plants were allowed to equilibrate for at least 1 week at these conditions before inoculation.

Fungal strains and inoculum preparation.

Verticillium dahliae isolate Ts-2, isolated from diseased tomatoes, is a member of vegetative compatibility (VC) group II (Puhalla and Hummel 1983) and is a weak nondefoliating pathogen on cotton plants. *Verticillium dahliae* isolate V-76, isolated from cotton in Mexico, is a member of VC group I and is a virulent defoliator of cotton. Both fungi were grown on potato dextrose agar (PDA) plates at room temperature. For inoculum preparation, plates were flood inoculated with a conidial suspension, allowed to grow for 3 to 4 days, and then washed with sterile water to remove conidia. Conidia were washed once with sterile water and diluted to a concentration of 2 to 5 × 10^7 cells/ml. Heat-killed conidia were held at a temperature of 50°C for 15 min in a water bath and adjusted to a concentration of 2 to 5 × 10^8 cells/ml. An aliquot of this suspension was plated onto PDA plates to confirm nonviability.

Inoculation and plant tissue preparation.

Plants were stem inoculated immediately above the cotyledonary node with 50 µl of conidial suspension (or sterile water) injected into the stele (Bugbee and Presley 1967). The plants were returned to the growth chamber until sampling, at which time stems were removed 2 cm above the injection site, the bark peeled away, and approximately 1.5 g of stele material retained. Material not used immediately was frozen in LN_2 and stored at –70°C until use.

DNA isolation and Southern blotting.

Stele material was ground in LN_2 in a mortar and pestle with 5% polyvinylpyrrolidone (PVP) and crude DNA extracted by sequential salt and alcohol precipitation (Hughes and Galau 1988). *Verticillium dahliae* isolates TS-2 and V-76 were grown for 10 days in potato dextrose broth. Mycelia were harvested and genomic DNA isolated as described previously (Taylor and Natvig 1987). DNA was used directly or digested with *Eco*RI or *Hind*III for electrophoresis in 0.9% agarose before blotting to GeneScreen*Plus* (DuPont Co., Wilmington, DE). Blotting, probe preparation, and hybridization at 65°C followed standard protocols (Sambrook et al. 1989) or the guidelines of suppliers.

Plant RNA isolation and Northern blotting.

RNA was isolated from cotton stele as described previously (Hughes and Galau 1988), except that tissue was ground to powder in a mortar and pestle with LN_2 and then added to ice-cold buffer plus 1% PVPP-40. The material was also homogenized with a Polytron at medium speed for 3 min. Absorbance at 260 and 280 nm was determined spectophotometrically to determine purity and concentration of RNA. Thirty micrograms of total soluble RNA was loaded into each well of a 1% agarose, 0.7 M formaldehyde gel (Sambrook et al. 1989). The gel was washed twice for 10 min in water and blotted onto GeneScreen*Plus* by capillary action with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). RNA was UV linked to the membrane and the membrane baked at 80°C for 90 min. Hybridization was as described above. Molecular sizes were determined by preparing a blot with plant RNA and denatured DNA size markers. Results were confirmed by repeating the entire experiment using new sets of plants.

Primer selection and PCR amplification.

An HMGR sequence from potato was used to identify related sequences available in GenBank and EMBO files via the FASTA protocol (Devereux et al. 1984). The highest sequence similarity scores based on mRNA or complete gene queries always selected genes from other species encoding HMGR. Homologous plant sequences were transported to MacVector files and aligned by Pustell matrix analysis for pairwise graphic analysis. Conserved regions of very high homology bracketing approximately 300 bases of protein coding region were chosen as the basis for the pairs of PCR oligonucleotide primers. Degenerate forward (5′ AGY ACT GGY GAT GCW ATG GGR ATG A 3′) and reverse (3′ TAC GAR TTY TTR GAA YGA CCR AGA C 5′) (R = Pur; W = A, T; Y = C, T) primers were synthesized in the Texas A&M University Advanced DNA Technology Laboratory.

PCRs were carried out in a Perkin-Elmer Cetus (Norwalk, CT) thermal cycler using *Taq* DNA polymerase. Cotton DNA of Rowden and Seabrook cultivars from the isolations above was used as genomic target. Beginning at 50°C, annealing temperatures were increased by 3° in successive experiments to identify conditions under which the optimal amount of product of the predicted size would be made. Reactions were assembled on ice in a total volume of 25 µl containing 25 ng of genomic DNA and placed into a thermal cycler preheated to 94°C. After 7 min of denaturation at this temperature, reactions were run for 28 cycles of 1 min at 94°C, 1 min at annealing temperature, and 2 min at 72°C each, followed by one cycle of 7.5 min at 72°C. Total reactions were electrophoresed in 0.8% low melting point agarose with 0.5 µg of ethidium bromide per ml and bands of interest were excised for cloning.

Cloning of PCR products and sequencing.

The reagents and directions supplied with a SureClone ligation kit (Pharmacia, Uppsala, Sweden) were used to clone the PCR products into pUC18, using blunt end ligation. The size of the insert in plasmid DNA extracted from selected (white) colonies was tested by electrophoresis on 1.5% agarose gels following digestion of plasmid DNA with *Eco*RI and *Xba*I. Colonies that contained an insert of the predicted size were grown overnight in 5 ml of terrific broth (TB) medium (Gibco BRL, Gaithersburg, MD), and pure plasmid DNA extracted using MagicMinipreps (Promega, Madison, WI). Dideoxy sequencing (Sanger et al. 1977) using both forward and reverse M13 sequencing primers was conducted in the Texas A&M University Advanced DNA Technology Laboratory.

HMGR extraction and assays.

Approximately 1 g of stele tissue was ground in LN_2 and homogenized with 8 ml of grinding buffer (50 mM potassium-phosphate pH 7.5, 10 mM EDTA, 0.35 M sucrose, 20 mM 2-mercaptoethanol, 5% insoluble PVP). The protease

inhibitors E-64 (trans-epoxysuccinyl-L-leucylamino-[4 guanidino] butane), 5 μg/ml; leupeptin, 5 μg/ml, and Pefabloc SC (Boehringer Mannheim, Indianapolis, IN), 20 μg/ml were routinely added to the extraction buffer unless otherwise noted. The homogenate was filtered through cheesecloth and the filtrate centrifuged at 3,000 × *g* for 5 min; the supernate was centrifuged at 10,000 × *g* for 30 min and again at 105,000 × *g* for 1 h. The final microsomal pellet was resuspended in buffer containing 50 mM potassium-phosphate, pH 7.5, 30 mM dithiothreitol (DTT), and the protease inhibitors. Protein content was estimated using a commercial Coomassie blue assay, as directed by the manufacturer (Pierce, Rockford, IL).

HMGR activity was determined via a radiolabel assay modified from Russell (1985). The assay reaction was started by adding 50 μl of assay cocktail to 50 μl of the enzyme preparation (15 to 50 μg of protein). The combined mixture contained 50 mM potassium-phosphate (pH 7.5), 1 mg of bovine serum albumin (BSA) per ml, 15 mM EDTA, 12.7 nmol (62.5 nCi) of [14C]HMG-CoA (DuPont NEN, Wilmington, DE), and 2 mM NADPH. After 30 min incubation at 30°C, the reaction was stopped by adding 10 μl of 6 M HCl. The stopped reaction was incubated at room temperature for at least 30 min more to ensure complete conversion of MVA to mevalanolactone. The mixture was then centrifuged for 3 min at full speed in a microfuge and a 30-μl aliquot was applied to a thin layer plate of activated silica gel, Si250-19C, purchased from JT Baker (Phillipsburg, NJ) The plate was developed with chloroform/acetone (2:1, vol/vol). The region of the plate corresponding to a mevalanolactone standard was scraped off and the amount of ^{14}C determined by liquid scinitillation counting. The HMGR data shown are the means for two independent replicates of the experiment.

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