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Arabidopsis 3-hydroxy-3-methylglutaryl-CoA reductase is regulated at the post-translational level in response to alterations of the sphingolipid and the sterol biosynthetic pathways

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ABSTRACT

3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR, EC 1.1.1.34) catalyzes the major rate-limiting step in the mevalonate (MVA) pathway for isoprenoid biosynthesis. Its activity is regulated at different levels, from transcriptional to post-translational. Treatment of *Arabidopsis thaliana* plants with myriocin, a specific inhibitor of serine palmitoyltransferase (SPT), the first enzyme of sphingolipid biosynthesis, resulted in a concomitant reduction of both HMGR activity and the sterol content, which reveals regulatory cross-talk between these two lipid biosynthesis pathways. Myriocin-induced down-regulation of HMGR activity is exerted at the post-translational level, like the regulatory response of HMGR to enhancement or depletion of the flux through the sterol pathway.

1. Introduction

Lipid metabolism is a complex network of pathways, which needs to be regulated in a very precise and coordinated manner to maintain lipid homeostasis within cells. Studies in yeast and animal cells indicate that sterol metabolism and sphingolipid biosynthesis are regulated coordinately. In yeast, blocking sterol production at different steps in the biosynthetic pathway leads to reduced levels of particular sphingolipid species. Ceramide biosynthesis and hydroxylation of complex sphingolipids have been suggested as possible points of sterol-dependent regulation of sphingolipid biosynthesis (Swain et al., 2002). Studies using mammalian cells have shown that inhibition of *de novo* ceramide biosynthesis decreases levels of transcriptionally active sterol-regulatory element (SRE)-binding protein (SREBP) and SRE-mediated gene transcription. It has been hypothesized that ceramide biosynthesis contributes to the physiological processing of precursor SREBP to active mature SREBP (Worgall et al., 2004). The observation that depletion of the sterol pathway in leek seedlings also impairs the synthesis of the complex sphingolipids glucosylceramides (Hartmann et al., 2002) supports the view that sterol and sphingolipid metabolism is also coordinately regulated in plants. However, so far nothing is known about the effects of perturbation of sphingolipid biosynthesis on the plant mevalonate (MVA) pathway for isoprenoid production. The synthesis of MVA catalyzed by 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) is the main rate-limiting step in isoprenoid biosynthesis (Fig. 1). In yeast and mammals HMGR activity is tightly regulated at different levels, from transcriptional to post-translational (Goldstein and Brown, 1990; Hampton et al., 1996). In plants, much less is known about the regulatory mechanisms controlling HMGR activity. Studies have focused mainly on the effects of a variety of environmental and developmental factors on enzyme expression and activity, and have shown that regulation of HMGR is exerted mainly at the transcriptional level (Learned and Connolly, 1997; Stermer et al., 1994; Weissenborn et al., 1995). Recent reports indicated that internal perturbations of the metabolic flux through the sterol pathway also trigger a transcriptional up-regulatory response of HMGR; for example, in tobacco seeds overexpressing sterol C-24 methyltransferase (Holmberg et al., 2002) and in sterol-depleted tobacco BY-2 cells after treatment with squalestatin (Wentzinger et al., 2002), a highly specific competitive inhibitor of squalene synthase (SQS) (Bergstrom et al., 1993). SQS catalyzes the synthesis of squalene (Fig. 1), the first committed precursor to the sterol pathway. Mechanisms operating at levels other than transcriptional appear to control plant HMGR activity as well. For example, developmental and light-regulated post-translational control of HMGR levels has been demon-

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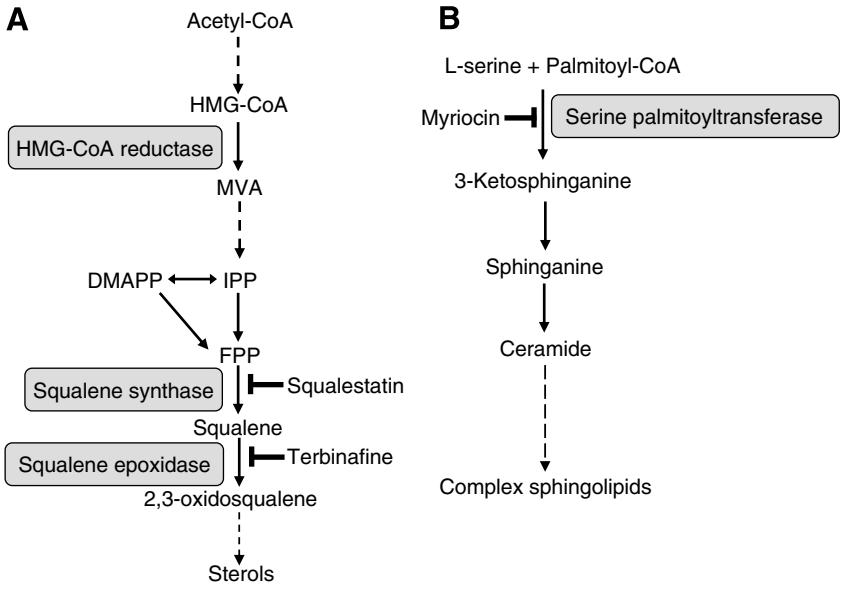

Fig. 1. Simplified scheme of the biosynthetic pathways for the production of sterols (A) and sphingolipids (B) in plants. The position of the reactions catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A (HMG–CoA) reductase, squalene synthase, squalene epoxidase and serine palmitoyltransferase is indicated. IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate. Dashed arrows represent multiple enzymatic steps. Major sterols are campesterol, stigmasterol, and sitosterol. Predominant complex sphingolipids are glycosilceramides and inositolphosphorylceramides.

strated in potato leaves (Korth et al., 2000) and treatment of tobacco BY-2 cells with terbinafine, a specific inhibitor of squalene epoxidase (SQE) (Ryder, 1992), the enzyme that catalyzes the conversion of squalene to 2,3-oxidosqualene (Fig. 1), triggers an increase in HMGR activity, even though it induces no changes in the HMGR transcript levels (Wentzinger et al., 2002). Interestingly, no similar studies using *Arabidopsis thaliana* as a plant system have been reported. The genome of *A. thaliana* contains two differentially expressed HMGR genes, *AtHMG1* and *AtHMG2*, (Enjuto et al., 1994) that encode three HMGR isoforms: HMGR1S (short isoform), HMGR1L (long isoform) and HMGR2. Isoforms HMGR1S and HMGR1L are both encoded by the *AtHMGR1* gene and are identical in sequence, except by an N-terminal extension of 50 amino acid residues in HMGR1L (Lumbreras et al., 1995). Although the different HMGR isoforms have the same structural organization, their subcellular localization (Leivar et al., 2005), the expression profiles of the corresponding genes (Enjuto et al., 1994), and the phenotypes of *A. thaliana hmg1* and *hmg2* T-DNA insertion mutants (Suzuki et al., 2004) are different. All these observations suggest that the different *A. thaliana* HMGR isoforms might have specialized functions in the isoprenoid biosynthetic pathway, although at present they remain to be established.

To gain greater insight into the regulation of the MVA pathway in plants, we investigated the effects on *A. thaliana* HMGR of depletion of sphingolipid biosynthesis as well as of enhancement or depletion of the MVA pathway itself. Our findings indicate that in all these metabolic settings HMGR activity is post-translationally regulated.

2. Results

2.1. Effects of myriocin on HMGR activity and sterol levels

To examine the effect of depletion of sphingolipid biosynthesis on the MVA pathway, we first measured HMGR activity in *A. thaliana* plants treated with myriocin, a toxic amino acid that is a specific and potent inhibitor of serine palmitoyltransferase (SPT) (Miyake et al., 1995), the first enzyme in *de novo* sphingolipid biosynthesis (Hanada, 2003) (Fig. 1). Actually, myriocin is known to inhibit plant sphingolipid biosynthesis as well (Spassieva et al., 2002). HMGR activity was assayed in extracts from plants grown for 9 days on germination medium (GM) plates supplemented with concentrations of myriocin ranging from 0 to 10 μM. Plants grown in the presence of myriocin were smaller than plants grown without the inhibitor (Fig. 2A), which is consistent with the phenotype seen in *A. thaliana* plants with a partial suppression of the expression of the LCB1 subunit of SPT (Chen et al., 2006). Myriocin also led to a significant concentration-dependent inhibition of HMGR activity, which at 10 μM myriocin decreased to ca. 55% of the activity in control plants (Fig. 2B). Addition of the same concentrations of myriocin to *in vitro* assays of HMGR activity did not alter enzyme activity (Fig. 2C), indicating that the inhibitory effect was not due to direct interaction of myriocin with HMGR. Further evidence of the indirect inhibitory effect of myriocin on the MVA pathway was obtained by sterol measurements in plants treated with 1 and 10 μM myriocin. In both cases, we detected a significant reduction in the levels of the three major sterols, namely campesterol, stigmasterol and sitosterol, which at 10 μM myriocin decreased by up to 40%, 30% and 25%, respectively, compared to the levels in untreated plants (Fig. 2D). Overall these results demonstrated that depletion of the sphingolipid pathway in *A. thaliana* leads to a concomitant reduction in HMGR activity, paralleled by a decrease in the sterol content.

2.2. Effects of myriocin on HMGR expression levels

To investigate whether the decrease in HMGR activity is due to transcriptional down-regulation of the *AtHMGR* genes, the steady state transcript levels of the two *A. thaliana* genes encoding HMGR (*AtHMG1* and *AtHMG2*) were measured in plants treated with 1 and 10 μM myriocin by quantitative real-time PCR (qRT-PCR), using primers that recognize specifically transcripts generated by each gene. Comparison of the mRNA levels of each

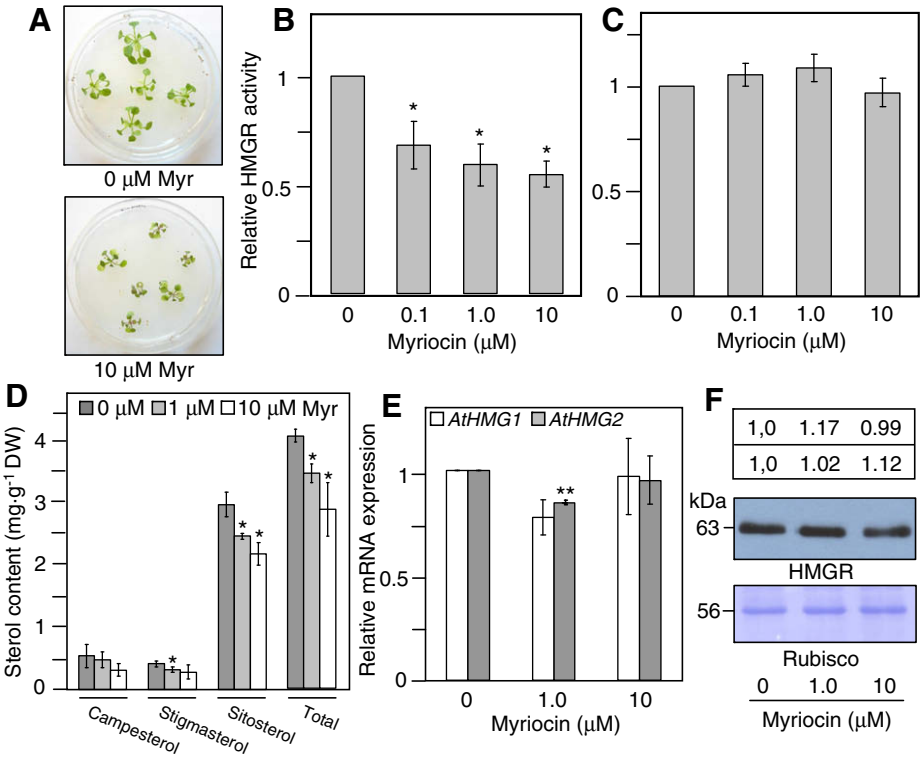

Fig. 2. Effects of myriocin on HMGR and sterol levels. (A) *A. thaliana* plants grown for 9 days on GM or GM with 10 μM myriocin. (B) HMGR activity in plants treated with the indicated concentrations of myriocin. (C) *In vitro* effect of myriocin on HMGR activity. For each concentration of myriocin, enzyme activities are expressed relative to the activity in the corresponding control samples without myriocin (set at 1). (D) Content of the individual sterols, campesterol, stigmasterol and sitosterol, and total content of these three sterol species. Sterol amounts are expressed in milligrams per gram of DW. (E) Steady state transcript levels of the *AtHMG* genes in plants treated with 1 and 10 μM myriocin. Transcript levels were normalized to the mRNA levels of the *AtGAPDH* gene and are expressed relative to the levels in untreated plants (set at 1). The mean values and s.d. shown in panels B to E were calculated from at least three independent experiments. Asterisks show the values that are significantly different ($^{*}p < 0.05$; $^{**}p < 0.005$) compared to their corresponding controls. (F) Western blot analysis of HMGR protein in plants treated with myriocin. Intensities of the HMGR protein bands (upper panel) and the Coomassie blue stained Rubisco large subunit (lower panel) were quantified by densitometric scanning. HMGR protein levels were normalized to the levels of the Rubisco large subunit and are expressed relative to the amount of HMGR protein in untreated plants (set at 1) at the top of each lane. Relative HMGR protein levels from two independent experiments are shown in boxes. Images show the results from one of the experiments (quantitative data shown in the upper box).

gene in myriocin-treated plants with levels in untreated plants revealed that myriocin caused no relevant change in the expression levels of *AtHMG1* and *AtHMG2* (Fig. 2E). *AtHMG1* transcripts were ca. 17-fold more abundant than *AtHMG2* transcripts. The availability of polyclonal antibodies against the catalytic domain of *A. thaliana* HMGR1 isoforms allowed us to conduct immunoblot analysis in protein extracts from plants treated with the above-mentioned concentrations of myriocin. These antibodies are able to recognize isoform HMGR2 as well, given that the catalytic domains of isoforms HMGR1 and HMGR2 are highly conserved (78% amino acid identity) (Enjuto et al., 1994). As expected, a prominent 63-kD immuno-reactive band corresponding to HMGR1S was detected in all samples analyzed (Fig. 2F), whereas no bands corresponding to HMGR1L (69 kD) and HMGR2 (60 kD) were detected, which confirmed that these two isoforms exist at much lower levels than HMGR1S (Leivar et al., 2005). Thus, the bulk of HMGR protein and enzyme activity at this stage of development is attributable to HMGR1S. The intensities of the 63-kD band were normalized to those of the Coomassie-stained large subunit of Rubisco. The amount of immuno-reactive HMGR protein in extracts from myriocin-treated plants was nearly identical to the amount in untreated plants (Fig. 2F). This, together with the results of qRT-PCR analysis, indicated that down-regulation of HMGR activity in response to depletion of sphingolipid biosynthesis is mediated by mechanisms operating at the post-translational level.

2.3. Effects of MVA, squalestatin, and terbinafine on HMGR activity and expression levels

For greater insight into the regulation of *A. thaliana* HMGR in response to metabolic stimuli, we investigated the effects on HMGR activity of either enhancement or depletion of the flux through the MVA pathway. To enhance the flux of the pathway plants were grown on GM or GM supplemented with 5 mM MVA for 20 days and, in both cases, transferred to plates containing either GM or GM with 5 mM MVA and grown for another 9 days. Sterol measurements in plants grown with MVA from the beginning confirmed the stimulatory effect of MVA on the flux of the pathway, as the total amount of campesterol, stigmasterol and sitosterol in these plants was ca. 2.2-fold higher than in untreated plants (Fig. 3A). Nevertheless, plants grown in the presence of MVA showed no visible phenotypic alterations. Plants subjected to the experimental conditions described above were collected and extracts assayed for HMGR activity. Whether plants were grown with MVA from the beginning or only during the last 9 days, HMGR activity was drastically reduced to 25% of the activity in plants grown in the absence of MVA. Plants grown without MVA during the last 9 days still showed a severe, though less pronounced, reduction in HMGR activity, which decreased to 60% of the activity in control plants (Fig. 3B). However, in all cases the significant reduction of HMGR activity did not correlate with changes in both the expression of *AtHMG1* and *AtHMG2* genes (Fig. 3C) and the

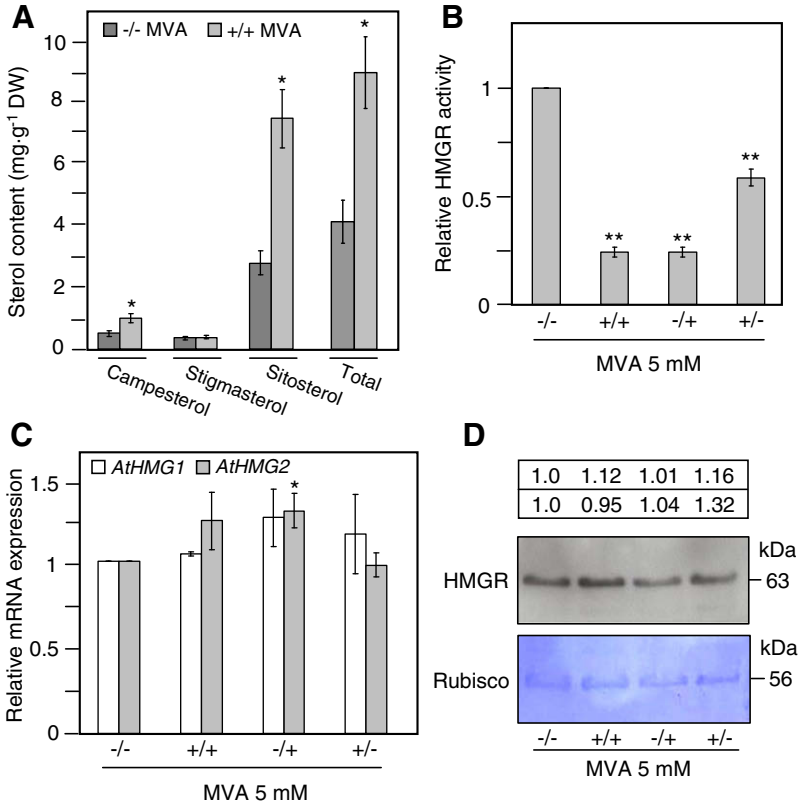

Fig. 3. Effects of MVA on sterol content, HMGR activity and protein, and *AtHMG* gene expression levels. (A) Content of the individual sterols, campesterol, stigmasterol and sitosterol, and total content of these three sterol species. Sterol amounts are expressed in milligrams per gram of DW in plants grown for 29 days on GM with 5 mM MVA (+/+). Sterol amounts are expressed in milligrams per gram of DW. (B) HMGR activity in *A. thaliana* grown for 29 days on GM with 5 mM MVA (+/+), 20 days on GM and 9 days on GM with 5 mM MVA (−/+), and 20 days on GM with 5 mM MVA and 9 days on GM (+/−). Enzyme activities are expressed relative to the activity in plants grown for 29 days on GM (−/−) (set at 1). (C) Steady state transcript levels of the *AtHMG* genes in plants treated as indicated above. Transcript levels were normalized as indicated in the legend of Fig. 2 and are expressed relative to their levels in plants grown for 29 days on GM (set at 1). The mean values and s.d. shown in panels A to C were calculated from at least three independent experiments. Asterisks show the values that are significantly different ($^{*}p < 0.05$; $^{**}p < 0.005$) compared to their corresponding controls. (D) Western blot analysis of HMGR protein in plants treated as indicated above. HMGR protein levels (upper panel) were quantified, normalized to Rubisco large subunit (lower panel), and expressed (at the top of each lane) as indicated in the legend of Fig. 2. Relative HMGR protein levels from two independent experiments are shown in boxes. Images show the results from one of the experiments (quantitative data shown in the upper box).

amount of HMGR protein (Fig. 3D). These results indicate that MVA triggers a negative feed-back regulatory response on HMGR activity, which is again exerted at the post-translational level.

The effect on HMGR activity of depletion of the sterol pathway was evaluated in *A. thaliana* treated with squalestatin and terbinafine. Squalestatin, also called zaragozic acid, is a highly specific competitive inhibitor of SQS that shows structural analogy with presqualene diphosphate (Bergstrom et al., 1993), the intermediate of the reaction catalyzed by SQS. Terbinafine belongs to the class of allylamines and is a specific non-competitive inhibitor of fungal SQE, whereas it is a less potent and competitive inhibitor of the mammalian enzyme (Ryder, 1992). HMGR activity was measured in extracts from plants grown for 9 days on GM plates supplemented with different concentrations of squalestatin (2–10 μM) or terbinafine (5–150 μM). Although no relevant phenotypic effects were observed under these growing conditions, both treatments led to a significant concentration-dependent activation of HMGR of ca. 3-fold the activity in untreated plants. Maximal HMGR activation was observed at 6 μM squalestatin (Fig. 4A) and 75 μM terbinafine (Fig. 4B). These concentrations of inhibitors also gave rise to a moderate decrease in the total content of campesterol, stigmasterol and sitosterol, which was reduced by ca. 17% at 6 μM squalestatin (Fig. 4C) and ca. 21% at 75 μM terbinafine (Fig. 4D). These results confirmed the inhibitory effect of both compounds on the sterol pathway. Although both inhibitors triggered an up-regulatory response of HMGR, none of them led to relevant changes either in the level of expression of the *AtHMG* genes (Figs. 5A and B) or in the amount of immuno-detectable HMGR protein (Figs. 5C and D). The conclusion from all these results is that both up- and down-regulation of HMGR activity in response to changes in the flux of the *A. thaliana* MVA pathway occur *via* post-translational control.

3. Discussion

A. thaliana is a suitable model to study the regulatory mechanisms controlling HMGR in intact plants since it contains only two HMGR genes coding for three HMGR isoforms (Enjuto et al., 1994; Lumbreras et al., 1995) and, moreover, neither of them seems to be stress-responsive. Here we report for the first time that treatment of plants with the sphingolipid biosynthesis inhibitor, myriocin, causes concomitant depletion of the MVA pathway, as attested by a marked reduction in the levels of the major sterols. This effect is most probably due to the observed reduction of HMGR activity, which cannot otherwise be attributed to a direct interaction of the inhibitor with HMGR, as myriocin did not inhibit enzyme activity *in vitro*. Despite the reduced levels of HMGR activity, which is mainly contributed by isoform HMGR1S, expression of *AtHMG1* and *AtHMG2* mRNAs and HMGR protein levels remained unchanged, indicating that down-regulation of HMGR activity in

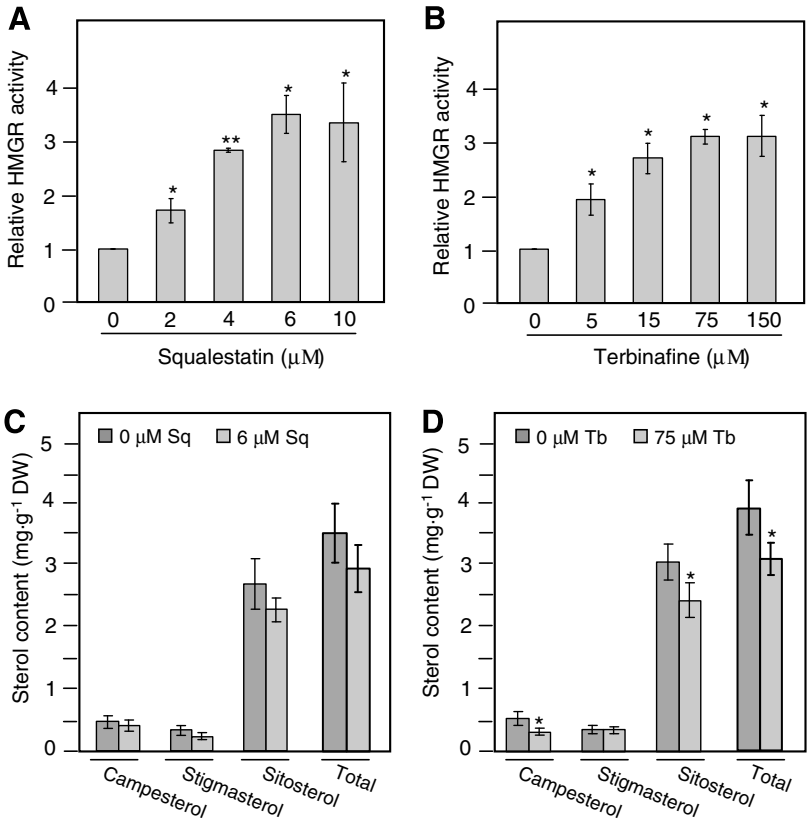

Fig. 4. Effects of squalestatin and terbinafine on HMGR activity and sterol content. HMGR activity in plants grown for 9 days on GM plates supplemented with the indicated concentrations of squalestatin (A) and terbinafine (B). For each concentration of the inhibitors, enzyme activities are expressed relative to the activity in the corresponding control samples without inhibitors (set at 1). Content of the individual sterols, campesterol, stigmasterol and sitosterol, and total content of these three sterol species in plants treated with 6 μM squalestatin (C) and 75 μM terbinafine (D). Sterol amounts are expressed in milligrams per gram of DW. The mean values and s.d. shown in panels A to D were calculated from at least three independent experiments. Asterisks show the values that are significantly different ($^{*}p < 0.05$; $^{**}p < 0.005$) compared to their corresponding controls.

response to inhibition of sphingolipid biosynthesis occurs at the post-translational level. These results indicate that plants adjust their rate of sterol biosynthesis to that of sphingolipids and lead us to suggest that HMGR is the primary target site in the MVA pathway mediating such a compensatory response, although it remains to be determined whether other enzymes of the pathway are also involved. Our finding in *A. thaliana*, along with the observation that inhibition of the sterol pathway in leek seedlings leads to a decrease in glucosylceramide synthesis (Hartmann et al., 2002), clearly argues in favour of bidirectional regulatory cross-talk between sterol and sphingolipid biosynthesis in plants, as reported in other eukaryotes (Ridgway, 2000; Veen and Lang, 2005). Sterols and sphingolipids are major components of endomembranes and both are enriched in plasma membrane lipid rafts (Riyaz and Panstruga, 2005). It is therefore possible that the regulatory relationship between both plant pathways might contribute to maintaining a proper relative ratio of these lipid species and hence ensuring proper lipid raft formation and functioning.

The finding that HMGR activity is post-translationally regulated in response to a perturbation of a metabolic pathway other than the MVA pathway, prompted us to investigate the regulatory response of *A. thaliana* HMGR to changes in the flux through the MVA pathway itself. Treatment of plants with MVA to increase the flux of the pathway triggered a negative feed-back regulatory response in HMGR activity, which again appears to be exerted at the post-translational level. A previous report showing that MVA restored control levels of HMGR activity and protein in tobacco BY-2 cells treated with mevinolin, a competitive inhibitor of HMGR, does not argue against our finding, because the experimental conditions were clearly different. The effect of MVA was tested in cultured cells that had, moreover, greater levels of HMGR than control cells because of mevinolin treatment (Hemmerlin et al., 2003). It is also conceivable that HMGR regulation in *A. thaliana* might be different from its regulation in tobacco. Structural differences between *A. thaliana* and tobacco HMGR isoforms possibly involved in differential regulation of HMGR in these species have been reported (Merret et al., 2007). Our studies using squalestatin and terbinafine also support this notion, since, although both inhibitors trigger ca. a 3-fold increase of HMGR activity in *A. thaliana* and BY-2 cells, up-regulation of HMGR activity in *A. thaliana* occurs *via* post-translational control whereas in tobacco cells squalestatin and terbinafine induce distinct regulatory responses, namely transcriptional in response to squalestatin and post-transcriptional in the case of terbinafine (Wentzinger et al., 2002).

The results presented here indicate that in the different metabolic settings investigated regulation of *A. thaliana* HMGR activity is exerted at the post-translational level, which raises the question of the nature of the mechanism(s) underlying post-translational control of *A. thaliana* HMGR. *In vivo* control of HMGR mediated by regulated proteolysis of the enzyme has been demonstrated in potato (Korth et al., 2000), although this does not seem to be the case in *A. thaliana*. It is conceivable that changes in the sterol/sphingolipid ratio in the ER membrane may perturb its structure, with subsequent effects on HMGR activity through the two amino-terminal trans-membrane sequences that anchor the enzyme to the ER. In fact removal of the HMGR membrane domain leads to deregulation of its activity, which suggests a key role of the endomembrane system in the control of plant HMGR activity

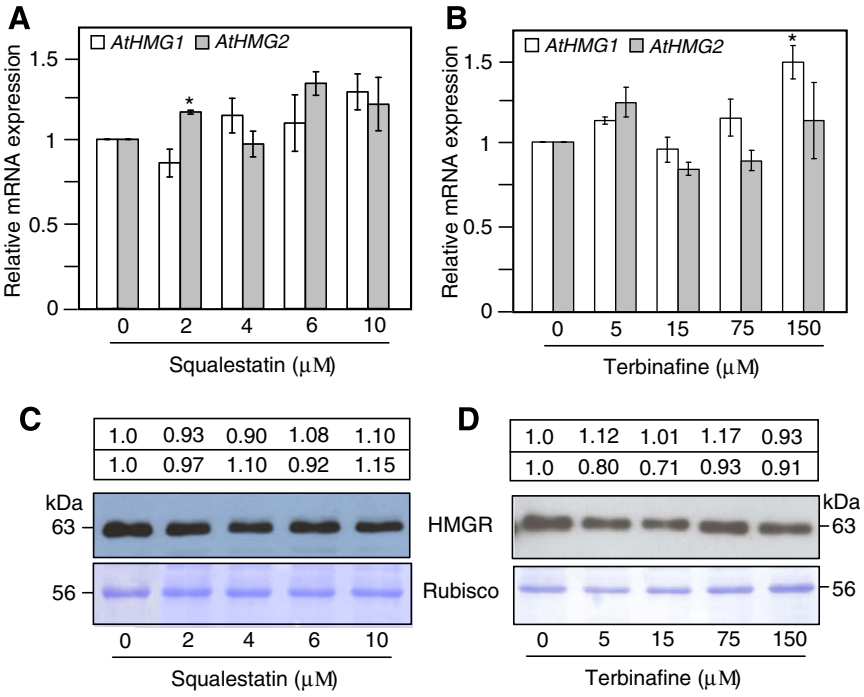

Fig. 5. Effects of squalestatin and terbinafine on *AtHMG* gene expression and HMGR protein levels. Steady state transcript levels of the *AtHMG* genes in plants grown for 9 days on GM plates supplemented with the indicated concentrations of squalestatin (A) and terbinafine (B). Transcript levels were normalized as indicated in the legend of Fig. 2, and are expressed relative to their levels in plants grown without the inhibitors (set at 1). The mean values and s.d. shown in panels A and B were calculated from at least three independent experiments. Asterisks show the values that are significantly different ($^{*}p < 0.05$) compared to their corresponding controls. Western blot analysis of HMGR protein in plants treated as indicated above with squalestatin (C) and terbinafine (D). HMGR protein levels (upper panel) were quantified, normalized to Rubisco large subunit (lower panel), and expressed (top of each lane) as indicated in the legend of Fig. 2. Relative HMGR protein levels from two independent experiments are shown in boxes. Images show the results from one of the experiments (quantitative data shown in the upper box).

(Leivar et al., 2005). Modulation of HMGR activity by reversible phosphorylation cannot be excluded either, as the enzyme can be phosphorylated and inactivated in vitro by Ca^{2+}-dependent and -independent protein kinases (Dale et al., 1995; Douglas et al., 1997), and evidence of the operation of this regulatory mechanism *in vivo* has been reported (Hey et al., 2006). The challenge is now to clarify the precise mechanism(s) by which HMGR activity is regulated in response to metabolic perturbations affecting not only the MVA pathway, but also other lipid pathways, which will be key for future understanding of the regulatory circuits controlling plant intracellular lipid homeostasis.

4. Experimental

4.1. Chemicals

MVA lactone, myriocin and squalestatin and other chemicals used in this study were purchased from Sigma, unless otherwise stated. Terbinafine was kindly supplied by Novartis Farmacéutica S.A. MVA was converted to the acid form (Hemmerlin et al., 2003) and stored as a 5 M stock solution. Myriocin, squalestatin, and terbinafine were dissolved in MeOH, water, and DMSO to prepare 2.5, 10, and 200 mM stock solutions, respectively.

4.2. Plant material

A. thaliana (Col-3) seeds were surface-sterilized, sowed on chemically inert polyester membranes (31 μm pore diameter; Büchkmann GmbH) placed on Petri dishes containing solid (0.8% w/v agar) germination medium (GM; Murashige and Skoog medium supplemented with 0.5 g/l MES, pH 5.7), and grown for 20 days under short-day conditions (8 h light/16 h dark) at 22 °C. Membranes were then transferred onto plates containing either GM or GM supplemented with MVA or the corresponding inhibitors at the indicated concentrations. Plants were grown under the same conditions for another 9 days.

4.3. HMGR activity assay, immunoblot analysis, and sterol measurements

For HMGR activity measurements, ca. 200 mg of plants was frozen in liquid nitrogen, ground to a fine powder, and mixed with 2 ml of pre-chilled extraction buffer (100 mM sucrose, 40 mM sodium phosphate, pH 7.5, 30 mM EDTA, 50 mM NaCl, 10 mM DTT, 10 μg/ml aprotinin, 1 μg/ml E64, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, and 0.25% (w/v) Triton X-100). The slurry was centrifuged at 200*g* for 10 min at 4 °C to remove cell debris and HMGR activity was immediately measured in the supernatant as previously described in Dale et al. (1995). Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Values of enzyme activity in extracts from plants subjected to the different treatments are expressed relative to the activity in the corresponding untreated control plants, which was assigned a value of 1. One unit of HMGR activity is defined as the amount of enzyme that converts one picomol of 3-hydroxy-3-methylglutaryl coenzyme A into MVA per min and mg of protein at 37 °C. Immunoblot analysis was performed in the same extracts (3 μg of protein) fractionated by 10% SDS–PAGE (Laemmli, 1970), using rabbit polyclonal antibodies (1:5000 dilution) raised against the catalytic domain of HMGR1 isoforms (Leivar et al., 2005). Protein loading was assessed by Coomassie blue staining of the membrane blots. Quantification of

HMGR and Rubisco bands was obtained using Scion Image Software. Sterols were measured as described (Masferrer et al., 2002) and are expressed on a dry weight (DW) basis.

4.4. Analysis of AtHMG gene expression by quantitative real-time PCR

One microgram of total RNA extracted using the RNeasy Mini plant kit (Qiagen) was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen) and 100 pmol of an oligo(dT) primer in a 20-μl reaction. After 50 min at 42 °C, reactions were diluted 1:10 with water and a 1.5-μl aliquot was analyzed in triplicate in 20 μl SYBR Green assays using an ABI PRISM 7000® Sequence Detection System (Applied Biosystems). Relative quantification of *AtHMG1* and *AtHMG2* expression at each experimental condition was calculated according to the comparative threshold (C_T) method ($\Delta\Delta C_T$) using the *AtGAPDH* gene (At3g26650) as a reference. Amplification efficiencies of target and reference genes were ca. equal (Livak and Schmittgen, 2001). Dissociation curves for each product were examined for non-specific amplification. Primers specific for mRNAs expressed from *AtHMG1*, HMG1-F (5′-GGAGATTGTGAAATCGGTTATCG-3′) and HMG1-R (5′-GCAACGCCTCACGACGAATCG-3′), *AtHMG2*, HMG2-F (5′-CTAA TCGGTTTCGTTGCTTCG-3′) and HMG2-R (5′-ACCCAAACATCAT CATCGGAA-3′), and *AtGAPDH*, GAPDH-F (5′-CTCCCTTGGAAGGAGCT AGG-3′) and GAPDH-R (5′-TTCTTGGCACCAGCTTCAAT-3′) were designed using the Primer Express software (Applied Biosystems).

4.5. Data analysis

Statistical significance of changes in HMGR activity, *AtHMG1* and *AtHMG2* transcript levels, and sterol content was calculated by using paired *t*-tests.

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