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Research article

Overexpression of *Brassica juncea* wild-type and mutant HMG-CoA synthase 1 in Arabidopsis up-regulates genes in sterol biosynthesis and enhances sterol production and stress tolerance

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Summary

Brassica juncea 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) is encoded by four isogenes (*BjHMGS1-BjHMGS4*). *In vitro* enzyme assays had indicated that the recombinant BjHMGS1 H188N mutant lacked substrate inhibition by acetoacetyl-CoA (AcAc-CoA) and showed 8-fold decreased enzyme activity. The S359A mutant demonstrated 10-fold higher activity, while the H188N/S359A double mutant displayed a 10-fold increased enzyme activity and lacked inhibition by AcAc-CoA. Here, wild-type and mutant BjHMGS1 were overexpressed in Arabidopsis to examine their effects *in planta*. The expression of selected genes in isoprenoid biosynthesis, isoprenoid content, seed germination and stress tolerance was analysed in HMGS overexpressors (OEs). Those mRNAs encoding enzymes 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), sterol methyltransferase 2 (SMT2), delta-24 sterol reductase (DWF1), C-22 sterol desaturase (CYP710A1) and brassinosteroid-6-oxidase 2 (BR6OX2) were up-regulated in HMGS-OEs. The total sterol content in leaves and seedlings of OE-wtBjHMGS1, OE-S359A and OE-H188N/S359A was significantly higher than OE-H188N. HMGS-OE seeds germinated earlier than wild-type and vector-transformed controls. HMGS-OEs further displayed reduced hydrogen peroxide (H_2O_2)–induced cell death and constitutive expression of salicylic acid (SA)-dependent pathogenesis-related genes (*PR1*, *PR2* and *PR5*), resulting in an increased resistance to *Botrytis cinerea,* with OE-S359A showing the highest and OE-H188N the lowest tolerance. These results suggest that overexpression of *HMGS* up-regulates *HMGR*, *SMT2, DWF1*, *CYP710A1* and *BR6OX2*, leading to enhanced sterol content and stress tolerance in Arabidopsis.

Introduction

Isoprenoids (terpenoids or terpenes) from eukaryotes and prokaryotes represent a large class of over 50 000 natural products, of which most are derived from plants (Bach, 1995; Roberts, 2007; Kirby and Keasling, 2008). In higher plants, isoprenoids originate from a common 5-carbon unit, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which are biosynthesized *via* either the cytosolic mevalonate (MVA) pathway (cf. Bach, 1995) or the plastidial methylerythritol phosphate (MEP) pathway (cf. Rohmer, 1999) (Figure 1). The MVA pathway is responsible for supplying the precursors for sterols, ubiquinone and some sesquiterpenes (Edwards and Ericsson, 1999). 3-Hydroxy-3-methylglutaryl-CoA synthase (HMGS) is the second enzyme in the MVA pathway and catalyses the condensation of AcAc-CoA with acetyl-CoA (Ac-CoA) to produce 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (Lynen *et al.*, 1958; Ferguson and Rudney, 1959; Rudney and Ferguson, 1959; Stewart and Rudney, 1966; Lynen, 1967; Balasubramanlam *et al.*, 1977). 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR) then converts HMG-CoA to MVA, which further undergoes two phosphorylation and a decarboxylation reactions to form IPP, the universal precursor of all isoprenoids (cf. Nagegowda, 2010). IPP units for the formation of carotenoids, the side chains of chlorophylls, abscisic acid (ABA), gibberellins (GA), plastoquinones, monoterpenes and tocopherols are synthesized *via* the MEP pathway (cf. Eisenreich *et al.*, 2001). Although the MVA and MEP pathways can operate independently, metabolic flow has been reported to occur between these pathways, suggesting some cross-talk between them (Hemmerlin *et al.*, 2003; Laule *et al.*, 2003; Chow *et al.*, 2007).

Isoprenoids have been proven to play significant roles in plant–environment interactions including plant–plant, plant–insect, plant–microbe, plant–stresses and plant–animal commu-

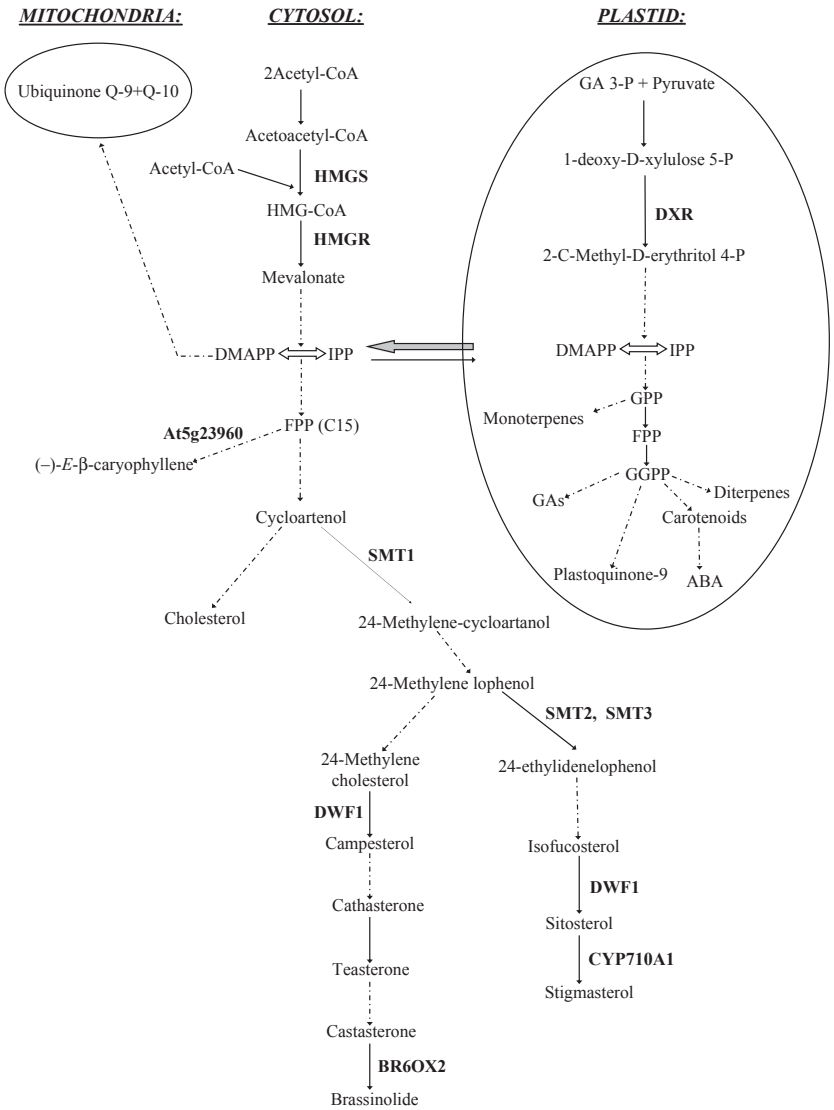

Figure 1 Simplified isoprenoid biosynthesis pathways in Arabidopsis. Subcellular compartments are indicated in uppercase italics. Solid arrows indicate single-step reactions; dashed arrows denote several steps. Enzymes are shown in bold. Arrows between cytosolic and plastid compartments represent metabolic flow between them (thicker arrow for greater flux). At5g23960, *Arabidopsis thaliana* sesquiterpene synthase. Abbreviations: ABA, abscisic acid; BR6OX2, brassinosteroid-6-oxidase 2; CYP710A1, C-22 sterol desaturase; DMAPP, dimethylallyl diphosphate; DWF1, delta-24 sterol reductase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; FPP, farnesyl diphosphate; GA-3P, glycerol-3 phosphate; GAs, gibberellins; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; IPP, isopentenyl diphosphate. SMT1, sterol methyltransferase 1; SMT2, sterol methyltransferase 2; SMT3, sterol methyltransferase 3.

nications (Pichersky and Gershenzon, 2002; Violeta *et al.*, 2005; Keeling and Bohlmann, 2006; Floß *et al.*, 2008; Dudareva and Pichersky, 2008). For example, accumulated sesquiterpenoid phytoalexins (rishitin and lubimin) in potato enhanced disease resistance to *P. infestans* (Choi *et al.*, 1994). Furthermore, isoprenoids have essential physiological functions in membrane biogenesis (sterols), photosynthesis (carotenoids and chlorophyll), respiration (quinones) and regulation of growth and development (ABA, GA and brassinosteroids) (Bush and Grunwald, 1972; Bach and Lichtenthaler, 1983; Bach, 1995; Hedden and Kamiya, 1997; Clouse and Sasse, 1998; Montoya *et al.*, 2005). Sterols are also important in embryogenesis (Clouse, 2000) and in mediating stress tolerance (Posé *et al.*, 2009).

Given the importance of isoprenoids, attempts have been made to overaccumulate them in transgenic plants. Overexpression of *Hevea brasiliensis* HMGR in transgenic tobacco resulted in 2- to 6-fold increase in sterol production, with most detected as steryl esters (Schaller *et al.*, 1995). Transgenic tobacco overexpressing a hamster HMGR also accumulated sterol intermediates such as cycloartenol, while end-product sterols (campesterol, sitosterol and stigmasterol) merely doubled (Chappell *et al.*, 1995). Zook *et al.* (1996) expressed a fungal-derived trichodiene synthase in tobacco, resulting in overproduction of sesquiterpene in elicited cells. Furthermore, traits to combat plant pests have been added through isoprenoid engineering (cf. Dudareva and Pichersky, 2008).

In *B. juncea* (Indian mustard), four isogenes (*BjHMGS1–BjHMGS4*) encode HMGS (Alex *et al.*, 2000). *BjHMGS* was down-regulated by ABA, osmotic stress and dehydration and up-regulated by salicylic acid (SA), methyl jasmonate (MeJA) and wounding (Alex *et al.*, 2000). *BjHMGS1* and *BjHMGS2* were confirmed to be expressed in young floral buds and leaves, while *BjHMGS3* and *BjHMGS4* were mainly expressed in leaves (Nagegowda *et al.*, 2005). BjHMGS1 has been demonstrated to be a cytosolic enzyme using transient expression of *N*- and *C*-terminal green fluorescent protein fusions in onion epidermal cells and tobacco BY-2 cells (Nagegowda *et al.*, 2005). His-tagged BjHMGS1 is inhibited *in vitro* by one of its substrates (AcAc-CoA) and by both products (HMG-CoA and CoA-HS) (Nagegowda *et al.*, 2004). Replacement of conserved amino acid residues in BjHMGS1 by site-directed mutagenesis and the expression of such mutants in *Escherichia coli* resulted in alterations in BjHMGS1 activity *in vitro*. Enzyme kinetic analyses revealed that the H188N (His → Asn) mutant did not display substrate inhibition by AcAc-CoA and showed 8-fold lower enzyme activity; substitution S359A (Ser → Ala) resulted in a 10-fold increased specific activity, while double mutant H188N/S359A demonstrated 10-fold increased specific activity and lacked inhibition by AcAc-CoA (Nagegowda *et al.*, 2004).

The aim of this study was to investigate the effects of overexpression of wild-type (wt) and mutant (H188N, S359A and H188N/S359A) BjHMGS1 in *Arabidopsis thaliana* Col-0. *HMGS* overexpression in Arabidopsis HMGS-OEs not only elevated the expression of *HMGR*, given that both are co-ordinately regulated in *B. juncea* (Alex *et al.*, 2000), but *SMT2, DWF1*, *CYP710A1* and *BR6OX2*, further down in the MVA pathway related to sterol biosynthesis, were also induced. Subsequently, changes in end products and phenotype of HMGS-OEs were investigated.

Results

Generation and molecular analyses of transgenic Arabidopsis HMGS-OEs

The wild-type and mutant *BjHMGS1* cDNAs were cloned into the plant binary vector pSa13 (Xiao *et al.*, 2008) in which the cauliflower mosaic virus *35S* promoter (CaMV *35S*) directs the expression of the cloned cDNA. Plasmid constructs pBj132 (encoding BjHMGS1 H188N/S359A), pBj134 (wtBjHMGS1), pBj136 (BjHMGS1 S359A) and pBj137 (BjHMGS1 H188N) were generated, and resultant *Agrobacterium* transformants were used to transform Arabidopsis yielding 30–60 kanamycin-resistant transgenic lines per construct. Putative Arabidopsis HMGS-OEs were designated as OE-wtBjHMGS1 (wild-type BjHMGS1), OE-H188N (BjHMGS1 H188N), OE-S359A (BjHMGS1 S359A) and OE-H188N/S359A (BjHMGS1 H188N/S359A). The presence of each transgene in Arabidopsis was preliminarily verified by PCR (Figure S1a,b). Mutations on the cDNA were further validated by DNA sequence analysis of PCR products amplified from genomic DNA of Arabidopsis HMGS-OEs. PCR-positive lines were then tested by Western blot analysis for overproduction of HMGS (Figure 2a). Given that the peptide used to generate anti-BjHMGS antibodies in rabbit showed 100% homology to Arabidopsis HMGS (At4g11820), the faint band in protein of the vector (pSa13)-transformant is attributed to cross-reaction with endogenous HMGS (Figure 2a).

Northern blot analysis indicated that all HMGS-OEs accumulated higher levels of *BjHMGS1* mRNA (Figure 2b) than the

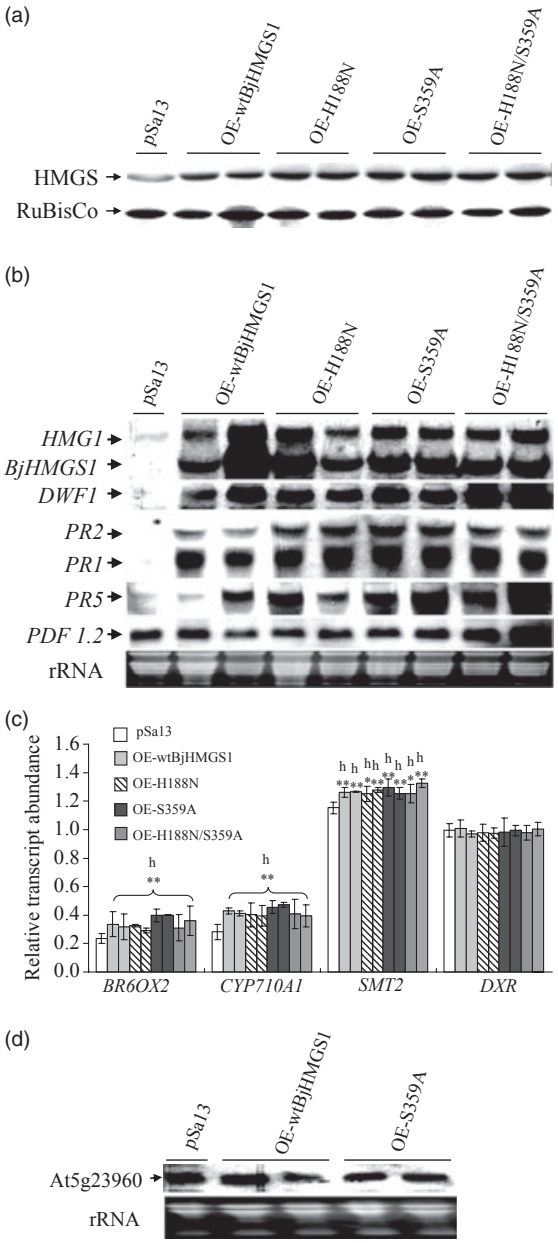

Figure 2 Molecular analyses on rosette leaves of 3-week-old Arabidopsis HMGS-OEs. (a) Western blot analysis using antibodies against BjHMGS indicates overproduction of HMGS (52.4-kDa) in representative Arabidopsis vector-transformed control (pSa13) and HMGS-OEs (OE-wtBjHMGS1, OE-H188N, OE-S359A and OE-H188N/S359A). (b) Northern blot analyses showing the mRNA levels of *HMG1* (2.2-kb), *BjHMGS1* (1.7-kb), *DWF1* (2.0-kb), *PR2* (1.2-kb), *PR1* (0.7-kb), *PR5* (0.5-kb) and *PDF 1.2* (0.8-kb) in representative pSa13 and OE lines. (c) RT-PCR demonstrates the relative transcript abundance (as compared to *ACTIN*) of endogenous *BR6OX2*, *CYP710A1, SMT2* and *DXR* mRNAs in representative pSa13 and OE lines. *BR6OX2*, *CYP710A1* and *SMT2* mRNAs in HMGS-OE lines were significantly higher than in the vector (pSa13)-transformed line. h, Higher than the vector (pSa13)-transformed line. Values are means ± SD ($n = 3$); $^{**}P < 0.01$ and $^{*}P < 0.05$ by *U* test. Bars represent SD. (d) Northern blot analysis of mRNA encoding floral sesquiterpene synthase (At5g23960, 1.8-kb), which was not induced in OE-wtBjHMGS1 and OE-S359A, representative of OE lines.

pSa13-transformant, which lacks the *AtHMGS* hybridizing band following stringent washing. OEs were confirmed to be independent lines by Southern blot analysis that also revealed the number of copies of *BjHMGS1* inserted in the Arabidopsis genome (Figure S1c). Two independent OE lines from each construct with single *BjHMGS1* inserts and of similar HMGS expression levels were subsequently chosen for further analyses on isoprenoid content, germination rate, resistance to fungal infection and sensitivity to H_2O_2 treatment.

Arabidopsis HMGS-OEs show induced expression of *SMT2, DWF1, CYP710A1* and *BR6OX2* besides *HMGR*

The co-ordinated regulation of *HMGS* and *HMGR* encoding two adjacent enzymes in the MVA pathway has been previously reported (Alex *et al.*, 2000). However, it is not known whether the expression of mRNAs encoding these enzymes in early MVA biosynthesis is co-regulated with those further downstream in the pathway. In Arabidopsis, HMGR, DWF1, CYP710A1, BR6OX2 (or CYP85A2) and sesquiterpene synthase (At5g23960) catalyse the production of MVA (Suzuki and Muranaka, 2007), campesterol/sitosterol (Choe *et al.*, 1999), stigmasterol (Arnqvist *et al.*, 2008), brassinolide (BL; Nomura *et al.*, 2005) and floral sesquiterpenes [(–)-α-copaene, (–)-*E*-β-caryophyllene and α-humulene] (Tholl *et al.*, 2005) biosyntheses, respectively. All five enzymes occur after HMGS in the MVA pathway of isoprenoid biosynthesis, in contrast to 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), a key enzyme of the MEP pathway (Figure 1). SMT1, SMT2 and SMT3 have been reported to control the ratio of sterol content in Arabidopsis (Schaller *et al.*, 1998; Schaeffer *et al.*, 2001; Carland *et al.*, 2010). To address whether their mRNA expression is affected in Arabidopsis HMGS-OEs, Northern blot (Figure 2b,d) and RT-PCR (Figures 2c and S2a,d) analyses were carried out. *HMG1*, *HMG2*, *SMT2, DWF1*, *CYP710A1* and *BR6OX2* mRNAs were induced in all OEs (Figures 2b–c and S2a), whereas the mRNAs encoding sesquiterpene synthase (At5g23960; Figure 2d), DXR (Figure 2c), SMT1 (Figure S2d) and SMT3 (Figure S2d) remained unaffected.

Arabidopsis HMGS-OEs accumulate sterols

The overexpression of HMGR has been studied in eukaryotes and results in sterol accumulation in plants (Chappell *et al.*, 1995; Schaller *et al.*, 1995). However, the overexpression of HMGS in eukaryotes has not been investigated; hence, Arabidopsis HMGS-OEs were analysed for sterol contents using gas chromatography–mass spectrometry (GC-MS) analysis. The results indicated that the sterol contents of OEs generally increased in both 14-day-old seedlings cultured in petri dishes (Table 1) and 3-week-old soil-grown rosettes leaves (Table 2). In comparison with total sterol content of wild-type seedlings (Tables 1 and S1) and leaves (Tables 2 and S2), there was a significant increase in all OE lines except for OE-H188N. OE-S359A showed the highest increment in seedlings (24.5%–29.0%) (Table S1) and leaves (18.7%–25.9%) (Table S2), followed by OE-H188N/S359A, while OE-H188N had the least increment. Among individual sterols, the sitosterol content also displayed a similar trend as that of total sterol with an increase of 19.4%–24.0% in seedlings and 12.0%–22.7% in leaves of OE-S359A (Tables S1 and S2). Campesterol content was significantly increased in OE-S359A (20.3%–26.2%) and OE-H188N/S359A (8.7%–12.6%) seedlings (Table S1), whereas the leaf campesterol content was significantly increased in all OE-lines except

Table 1 Sterol composition (μg/mg dry weight) in HMGS-OE, wild-type and vector (pSa13)-transformed Arabidopsis seedlings

	Control		OE-wtBjHMGS1		OE-H188N		OE-S359A		OE-H188N/S359A	
Sterols	Wt	pSa13	Line 1	Line 2	Line 1	Line 2	Line 1	Line 2	Line 1	Line 2
Campesterol	1.02 ± 0.02	1.03 ± 0.05	1.04 ± 0.03	1.05 ± 0.03	0.99 ± 0.05	1.00 ± 0.04	**1.24 ± 0.04****	**1.30 ± 0.13****	**1.16 ± 0.07****	**1.12 ± 0.05****
Stigmasterol	0.15 ± 0.05	0.16 ± 0.01	**0.34 ± 0.05****	**0.36 ± 0.05****	**0.34 ± 0.06****	**0.35 ± 0.04****	**0.38 ± 0.03****	**0.40 ± 0.04****	**0.37 ± 0.04****	**0.35 ± 0.03****
Sitosterol	3.86 ± 0.06	3.87 ± 0.22	**4.21 ± 0.17****	**4.21 ± 0.13****	3.78 ± 0.21	3.82 ± 0.18	**4.62 ± 0.09****	**4.85 ± 0.40****	**4.38 ± 0.22****	**4.28 ± 0.18****
Total sterol	5.11 ± 0.19	5.19 ± 0.13	**5.73 ± 0.15****	**5.81 ± 0.19****	5.26 ± 0.26	5.30 ± 0.21	**6.46 ± 0.17****	**6.70 ± 0.56****	**6.04 ± 0.27****	**5.94 ± 0.21****

Wt, wild-type.
Values are means ± SD, $n = 5$. *$P < 0.05$; **$P < 0.01$ by *U* test.
Bold font indicates value that is significantly higher than the vector-transformed control (pSa13).

Table 2 Sterol composition (μg/mg dry weight) in HMGS-OE, wild-type and vector (pSa13)-transformed Arabidopsis rosette leaves

	Control		OE-wtBjHMGS1		OE-H188N		OE-S359A		OE-H188N/S359A	
Sterols	Wt	pSa13	Line 1	Line 2	Line 1	Line 2	Line 1	Line 2	Line 1	Line 2
Campesterol	0.43 ± 0.04	0.43 ± 0.02	**0.46 ± 0.03***	**0.47 ± 0.05***	0.44 ± 0.03	0.46 ± 0.05	**0.54 ± 0.07****	**0.54 ± 0.05****	**0.51 ± 0.06****	**0.50 ± 0.06****
Stigmasterol	0.042 ± 0.007	0.042 ± 0.007	0.036 ± 0.004	0.043 ± 0.010	0.046 ± 0.005	0.044 ± 0.005	**0.052 ± 0.009****	**0.065 ± 0.012****	0.046 ± 0.008	0.044 ± 0.005
Sitosterol	3.11 ± 0.20	2.99 ± 0.21	**3.37 ± 0.14****	**3.58 ± 0.19****	3.06 ± 0.10	3.10 ± 0.18	**3.35 ± 0.31****	**3.67 ± 0.19****	**3.62 ± 0.35****	**3.28 ± 0.19****
Total sterol	3.84 ± 0.16	3.79 ± 0.23	**4.22 ± 0.27****	**4.39 ± 0.32****	3.76 ± 0.16	3.84 ± 0.27	**4.50 ± 0.38****	**4.77 ± 0.47****	**4.75 ± 0.46***	**4.34 ± 0.31****

Wt, wild-type.
Values are means ± SD, $n = 5$. *$P < 0.05$; **$P < 0.01$ by *U* test.
Bold font indicates value that is significantly higher than the vector-transformed control (pSa13).

OE-H188N (Table S2). Stigmasterol content of seedlings showed the highest percentage increment (112.5%–150%) among the individual sterols in all OE lines (Table S1), whereas its leaf content was significantly increased only in OE-S359A (23.8%–54.8%) (Table S2). Although OE-H188N indicated no significant difference in total sterol, campesterol and sitosterol, its seedlings accumulated stigmasterol as other OE lines. Overall, OE-S359A displayed the highest increase in total as well as individual sterol contents both in seedlings and in leaves. The ratio of campesterol to sitosterol was calculated in wild-type, vector-transformed control and HMGS-OEs, but no significant differences were observed (Figure S2c) in contrast to results from analysis on sterol methyltransferase (SMT)-OE plants (Schaller *et al.*, 1998; Schaeffer *et al.*, 2001). Trace amounts of cholesterol, isofucosterol, cycloartenol, delta-7-sitosterol, delta-7-avenasterol, 24-methylene cycloartanol and 24-ethylidene lophenol were also detected in all lines tested, but no significant differences between BjHMGS-OEs and vector (pSa13)-transformed controls (data not shown) were observed. Also, no significant differences in floral (–)-*E*-β-caryophyllene (Figure S4a), plastid chlorophyll (Figure S4b) and carotenoid contents (Figure S4c) were observed between HMGS-OEs and pSa13-transformants. In all experiments, wild-type Arabidopsis was also tested, but it did not demonstrate any significant differences from the vector-transformed control (Tables 1 and 2).

Arabidopsis HMGS-OE seeds germinate earlier

Arabidopsis HMGS-OE seedlings showed induced *DWF1 and CYP710A1* mRNA expression and consequently overaccumulated stigmasterol. In tobacco, stigmasterol is known to significantly increase during seed germination (Bush and Grunwald, 1972). Hence, the germination rates of HMGS-OE seeds were examined next. The results from germination assays indicated that OE seeds germinated approximately 1 day earlier than wild-type and pSa13-transformed seeds (Figure 3). The germination rates of wild-type and pSa13-transformed vector control seeds were significantly lower ($P < 0.01$) than OE seeds at 36, 48 and 60 h following transfer from 4 °C to normal growth conditions (Figure 3). Furthermore, 3-day-old OE seedlings show

Figure 3 Seed germination in Arabidopsis HMGS-OEs. Statistical data on germination rates recorded at 36, 48, 60 and 72 post-germination, showing differences at 36, 48 and 60, but not 72 h. Values are means ± SD ($n = 5$); H, Higher than pSa13-transformants; **$P < 0.01$ by *U* test. Bars represent SD.

faster growth than wild-type and vector (pSa13)-transformed seedlings (data not shown). Similar results were obtained in this test using two generations of seeds.

Arabidopsis HMGS-OEs show induced PR gene expression and enhanced resistance to *Botrytis cinerea*

As both *BjHMGS* and *BjHMGR* mRNAs are SA-inducible (Alex *et al.*, 2000), we were interested to test whether the elevation of *HMGR* mRNA in HMGS-OEs would confer SA-associated fungal resistance. It has also been reported very recently that brassinosteroids (BR) which appears elevated in HMGS-OEs up-regulates *PR-1* (Divi *et al.*, 2010). Hence, the expression of SA-dependent *PR* genes was investigated, and HMGS-OEs were subject to fungal infection by *B. cinerea*. Northern blot analysis using PCR-generated cDNA probes demonstrated that the expression of SA-dependent PR genes, *PR1* (At2g14610, unknown protein), *PR2* (At3g57260, β-1,3-glucanase) and *PR5* (At1g75040, thaumatin-like protein), was induced in OE lines similar to the induction of *HMGR* and *DWF1* (Figure 2b). The results from infection assays with *B. cinerea* 3 days after treatment revealed that detached leaves of OE lines were more resistant and showed reduction (17.9%–22.8%) in diameter size of lesions (Figure 4a,b).

Detached leaves of Arabidopsis HMGS-OEs are delayed in H_2O_2-induced cell death

Paniagua-Pérez *et al.* (2008) have verified that β-sitosterol functions as an antioxidant by removing free radicals from diphenylpicrylhydrazyl. As sitosterol content was elevated in OE-wtBjHMGS1, OE-S359A and OE-H188N/S359A lines, their 3-week-old rosette leaves were subjected to H_2O_2 (3 or 5 mM) treatment. All OE leaves exhibited higher tolerance to H_2O_2 at both concentrations than vector-transformed leaves, following 72-h treatment. Figure 5a indicates the phenotype of H_2O_2-induced cell death photographed 72 h after treatment. The results of trypan blue staining confirmed that more cells of the vector-transformed leaves had undergone cell death in comparison with OE leaves treated with either 3 or 5 mM H_2O_2 (Figure 5b). According to the electrolyte conductivity data, OE-S359A leaves showed highest tolerance to 3 and 5 mM H_2O_2, while OE-H188N leaves were the least tolerant ones among OE lines (Figure 5c). Interestingly, the OE-H188N leaves, which had not exhibited a significant increase in sitosterol content in comparison with the other OEs, demonstrated highest electrolyte leakage (15.2% or 18.1% in Figure 5c) when treated with H_2O_2 (3 or 5 mM, respectively).

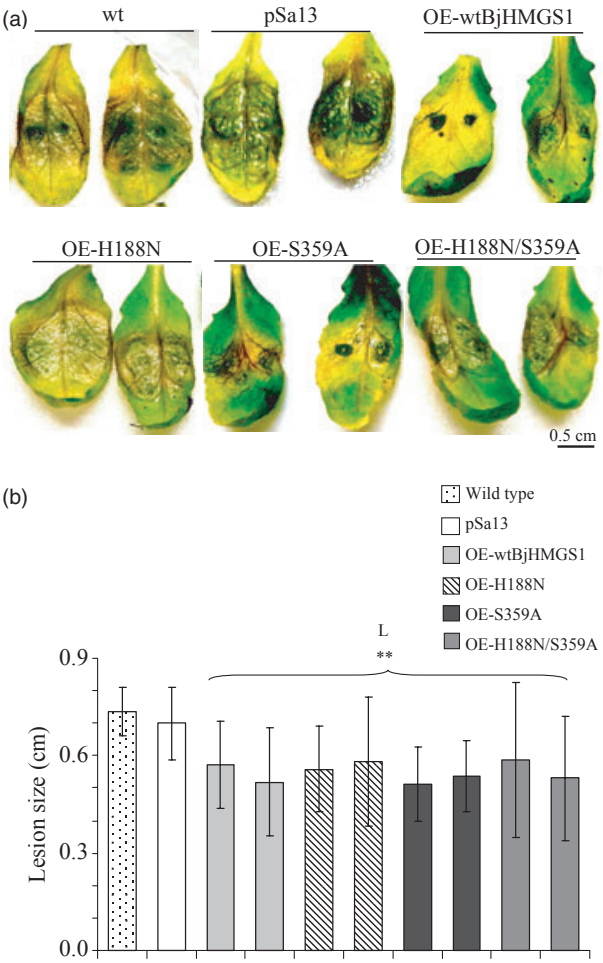

Figure 4 *Botrytis cinerea* infection assay on rosette leaves of Arabidopsis HMGS-OEs. (a) Phenotype of *B. cinerea* infection on detached rosette leaves, 3 days after treatment. Bar, 50 mm. (b) Statistical data of lesion size shown in (a), demonstrating all OE leaves showed reduction in lesion sized in comparison with pSa13-transformed leaves (100%); reductions being OE-wtBjHMGS1 by 21.7%, OE-H188N 17.9%, OE-S359A 22.8% and OE-H188N/S359A 20.7%. Values are means ± SD (n = 30); L, lower than pSa13; $^{**}P < 0.01$ by U test. Bars represent SD.

Discussion

The aims of this study were to phenotypically characterize Arabidopsis plants transformed with wild-type and mutant *BjHMGS1*, including effects on expression of downstream isoprenoid genes and products, seed germination, as well as consequent stress responses. Northern blot analyses revealed that the expression of ectopic *BjHMGS1* mRNA and endogenous Arabidopsis *HMG1* (Figure 2b) *and HMG2* (Figure S2a) is induced in all HMGS-OE lines. Co-ordinated expression between endogenous *HMGR* and *HMGS* has been previously reported in *B. juncea* (Alex *et al.*, 2000) and in animals (Gil *et al.*, 1986; cf. Goldstein and Brown, 1990). In this study, we present new evidence that the mRNAs encoding DWF1, CYP710A1, SMT2 and BR6OX2, located further downstream of HMGS in the MVA-dependent steroid pathway, are also up-regulated in Arabidopsis HMGS-OEs. The coordination between *HMGS*, *HMG2* and *BR6OX2* was also observed in wild-type Arabidopsis inflorescence (Figure S2b). Thus, in addition to HMGR (Bach, 1986), it is evident that HMGS plays a crucial role in sterol biosynthesis, as it is required for MVA synthesis. The importance of HMGS in plant pollen development has been recently verified in Arabidopsis (Ishiguro *et al.*, 2010), indicating that HMGS has significant functions in the life cycle of the plant.

No significant changes were evident in plastidial *DXR* mRNA expression in all OEs. Correspondingly, chlorophyll and carotenoid contents remained unaltered in the sterol-accumulating Arabidopsis OEs (Figure S4), indicating that HMGS overexpression has little bearing on the MEP pathway, consistent with reports (Hemmerlin *et al.*, 2003; Laule *et al.*, 2003) that metabolic flow is mainly from the plastid located MEP pathway to the cytosolic MVA pathway. The expression of sesquiterpene synthase At5g23960 and the formation of its major product floral (–)-*E*-β-caryophyllene did not show significant changes in

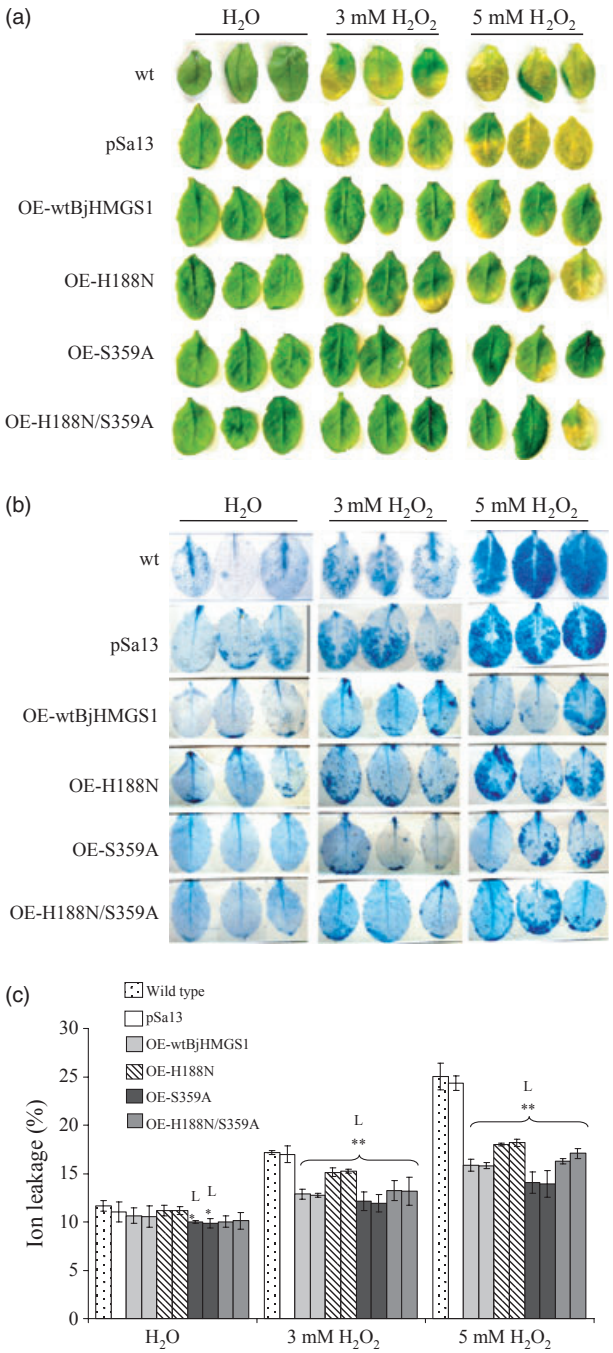

Figure 5 Treatment with H_2O_2 on rosette leaves of Arabidopsis HMGS-OEs. (a) Phenotype of H_2O_2-induced cell death photographed 72 h after treatment. Representative leaves are shown here. (b) Trypan blue-stained leaves after treatment for 72 h. Dark blue indicates cell death. Representative leaves were shown here. (c) Ion leakage indicates cell death of leaves treated by H_2O_2. L, lower than vector (pSa13)-transformed control; DW, dry weight; values are means ± SD (n = 3); $^{**}P < 0.01$ by U test. Bars represent SD.

OE-S359A, further confirming a specific link between HMGS and sterol isoprenoid biosynthesis. SMT2 has been reported to control the ratio of campesterol to sitosterol content in Arabidopsis SMT2-OEs (Schaeffer *et al.*, 2001). However, the ratio of campesterol to sitosterol in HMGS-OEs did not differ from wild-type or vector-transformed control (Figure S2c), although *SMT2* mRNA increased in HMGS-OEs. Perhaps the slightly induced *SMT2* mRNA was not sufficient to influence sterol composition in comparison with SMT2-OE Arabidopsis which was driven by the *35S* promoter. Furthermore, HMGS is located in the second step of MVA isoprenoid biosynthesis pathway and its overproduction in HMGS-OEs most probably had resulted in a greater effect on the metabolic flux of the whole pathway. Nevertheless, *SMTs* located further downstream would need to depend on the steroid intermediates to influence sterol content. Hence, when *SMT2* is overexpressed, sitosterol is overproduced at the expense of campesterol which shares a common precursor (24-methylene lophenol) with sitosterol biosynthesis and then the ratio of campesterol to sitosterol is significantly changed (Schaeffer *et al.*, 2001).

Gas chromatography–mass spectrometry analyses revealed that the OE-S359A lines accumulated the highest amounts of total sterol, campesterol and sitosterol in both seedlings and rosette leaves, consistent with the highest activity of recombinant S359A mutant of HMGS in *in vitro* assays (Nagegowda *et al.*, 2004). Correspondingly, OE-H188N leaves and seedlings accumulated the lowest amounts of total and individual sterols (with the exception of stigmasterol) among all OE lines. Loss of inhibition by superfluous AcAc-CoA was not apparent in the OE-H188N lines from analysis of sterol content, perhaps attributed to relatively limited level of endogenous AcAc-CoA, which is not inhibitory. Although the bacterial-expressed BjMHGS1-H1188N/S359A enzyme displayed both characteristics of the two single mutants, H188N and S359A, its corresponding Arabidopsis OE lines (OE-H188N/S359A) did not accumulate the highest contents of sterols and was even somewhat lower than the OE-S359A lines. It is likely that the H188N mutation masked the S359A phenotype of increased activity (10-fold) when expressed *in vivo* because of the limited affinity to AcAc-CoA, which is only present in submicromolar concentrations in the cytosol. Moreover, the enzyme preceding HMGS, AcAc-CoA thiolase favours the thiolytic cleavage of AcAc-CoA to Ac-CoA in the presence of HS-CoA (see Ahumada *et al.*, 2008).

Interestingly, in the OE-H188N seedlings, stigmasterol significantly increased, whereas campesterol and sitosterol levels remained relatively unchanged. It is likely that more acetyl-CoA and AcAc-CoA units are available for MVA biosynthesis in seedlings than in leaves. During seed germination, AcAc-CoA is released from lipid mobilization and catabolism, while acetyl-CoA is further derived from seed protein and carbohydrate catabolism. Therefore, H188N may function more efficiently in seedlings than in leaves. Given that the enzyme activity of H188N is 8-fold lower than wild-type BjHMGS1 and 80-fold lower than the S359A mutant (Nagegowda *et al.*, 2004), H188N-OEs may not have as much HMG-CoA for subsequent sterol biosynthesis in comparison with other HMGS mutants. Although H188N-OEs could not overaccumulate total sterols, sitosterol and campesterol, the stigmasterol level in H188N-OE seedlings was significantly greater than the controls (Tables 1 and S1), suggesting that stigmasterol (rather than sitosterol or campesterol) plays a very important role in the rapid early development of HMGS-OEs. This was further supported by observations in higher *CYP710A1* expression in wild-type developing seedlings than in leaves (Figure S3). It should be noted that stigmasterol is an end product in the pathway. In comparison, sitosterol and campesterol are intermediates in stigmasterol and BL biosynthesis (Grove *et al.*, 1979). When either

CYP710A1 or CYP710A4 was overexpressed in *Arabidopsis*, Arnqvist *et al.* (2008) observed that stigmasterol increased at the expense of sitosterol. Given the induction of *CYP710A1* in HMGS-OEs, OE-H188N seedlings may accumulate stigmasterol at the cost of sitosterol (Table S1). Although stigmasterol increased >2-fold in HMGS-OEs, it did not drastically alter total sterol content in Arabidopsis seedlings because only 3% of total seedling sterols consist of stigmasterol, while in leaves stigmasterol levels are even lower (1%). A previous report (Schaeffer *et al.*, 2001) showed that stigmasterol constitutes only 0.2% of total sterol in Arabidopsis ground materials.

Several studies have demonstrated that phytosterols particularly β-sitosterol possess high antioxidant activity (Weng and Wang, 2000; Wang *et al.*, 2002; Vivancos and Moreno, 2005; Li *et al.*, 2007; Posé *et al.*, 2009). Wegener *et al.* (1997) have demonstrated that *Pinus sylvestris* L. *HMGS* mRNA is induced by ozone. Such evidence suggests that HMGS and sterols are essential in protection against oxidative damage. Sitosterol was observed to account for about 80% of total sterols in Arabidopsis OE lines (Table S1). The phenotypes of H_2O_2-treated Arabidopsis OE leaves (Figure 5a) are consistent with the sitosterol content of leaves determined in this study (Tables 2 and S2). Both OE-H188N and vector-transformed leaves accumulated less sitosterol, in comparison with the other OE leaves, and were correspondingly more sensitive to H_2O_2. Thus, having an ability to accumulate sitosterol would be applicable for crops to combat stresses caused by H_2O_2 and to protect against ultraviolet-B (UVB) light, as the ozone layer declines as a consequence of atmospheric pollution.

Besides contribution to growth and development (Suzuki *et al.*, 2004, 2009; Okada *et al.*, 2008), the MVA pathway also plays a major role in plant–pathogen interaction. Arabidopsis *HMG2*, one of two genes encoding HMGR, is pathogen inducible because its root mRNA was co-activated with *PR*3 after *Orobanche ramosa* infection (Santos *et al.*, 2003). Alex *et al.* (2000) showed that both *BjHMGS* and *BjHMGR* were highly induced following SA treatment in *B. juncea* leaves. In this study, we detected constitutive activation of *HMG2* together with SA-dependent pathogenesis-related genes *PR1, PR2* and *PR5* mRNA in Arabidopsis HMGS-OE lines. The function of PR1 is yet unclear, but *PR2* encoding β-1,3-glucanase can degrade the fungal cell wall and *PR5* encoding a thaumatin-like protein permeabilizes fungal membranes (van Loon *et al.*, 2006). Fungal resistance of HMGS-OEs could be attributed to the SA pathway as apparent BL induction would have had up-regulated *PR-1* (Divi *et al.*, 2010). In comparison, this study revealed that expression of the jasmonate (JA)-dependent pathogenesis-related gene *PDF1.2* mRNA (van Loon *et al.*, 2006) was not significantly different between vector-transformed control and OE lines (Figure 2b), implying that JA does not seem to play a role in the HMGS-mediated defence response in Arabidopsis. This is consistent with our previous report that *BjHMGS1* mRNA is only slightly induced by MeJA in *B. juncea*, in comparison with greater induction by SA (Alex *et al.*, 2000).

In conclusion, the overexpression of wild-type and mutant BjHMGS1 in Arabidopsis up-regulated the expression of *HMGR, SMT2, DWF1, CYP710A1* and *BR6OX2* involved in MVA-dependent steroid biosynthesis and enhanced seed germination, *B. cinerea* resistance and H_2O_2 tolerance. Further, the up-regulation of *HMGR, SMT2, DWF1, CYP710A1* and *BR6OX2* in HMGS-OEs tempts us to suggest that all these genes could be possibly co-regulated, which would be the subject of future investigations.

Materials and methods

Construction of plasmids overexpressing wild-type and mutant BjHMGS1 and generation of transgenic Arabidopsis

A 1.4-kb wild-type or mutant *BjHMGS1* cDNA *Bam*HI fragment was excised from plasmids pBj110 (H188N/S359A), pBj75 (wtBjHMGS1), pBj89 (S359A) or pBj76 (H188N) (Nagegowda *et al.*, 2004) and cloned into binary vector pSa13 (Xiao *et al.*, 2008) to generate plasmids pBj132, pBj134, pBj136 and pBj137, respectively. The resultant plasmids and pSa13 vector control were mobilized into *Agrobacterium tumefaciens* LBA4404 by triparental mating, after which *A. thaliana* Col-0 was transformed using the floral dip transformation method (Clough and Bent, 1998). T_1 seeds from each transformant were selected on MS medium (Murashige and Skoog, 1962) for resistance to 50 mg/L kanamycin, and the resistant seedlings were transferred to soil. To preliminarily confirm Arabidopsis transformants, the *35S* promoter forward primer (5′-CAATCCCACTATCCTTCGCAAGACC-3′) and a *BjHMGS1* 3′-end cDNA reverse primer ML264 (5′-GGATCCATAACCAATGGACACTGAGGATCC-3′) were used to amplify inserts (Figure S1a,b). Mutations on the *BjHMGS1* cDNA were validated by DNA sequencing of PCR products amplified from total DNA of transgenic Arabidopsis using primer ML915 (5′-CATTGCTATGTTGATAGGAC-3′). Wild-type and transgenic Arabidopsis were grown under cycles of 8 h dark at 22 °C and 16 h light at 23 °C. T_2–T_6 transgenic plants were used for subsequent analyses of isoprenoid content, germination rates and stress tolerance.

Protein extraction and Western blot analysis

Total protein was extracted from 3-week-old Arabidopsis rosette leaves following Xu *et al.* (2002). Protein (20 μg per well) was separated on 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) gel and transferred to Hybond-ECL membrane (Amersham, Piscataway, NJ). Western blot analysis was carried out according to ECL Western blotting instructions (Amersham) and Chen *et al.* (2010). Antibodies against HMGS were used in Western blot analysis. A synthetic peptide (DESYQSRDLEKVSQQ) corresponding to BjHMGS1 amino acids 290–304 was used for the immunization of rabbits.

Northern blot analysis

Total RNA was extracted from 3-week-old Arabidopsis rosette leaves using TRIzol reagent (Invitrogen, Carlsbad, CA) for Northern blot analysis. RNA (20 μg per well) was separated on 1.3% agarose gels containing 6% formaldehyde and transferred to a Hybond-N membrane. Northern blot analysis was carried out according to Chen *et al.* (2008) using cDNA probes encoding Arabidopsis HMGR (HMG1, At1g76490), DWF1(At3g19820), PR1 (At2g14610), PR2 (At3g57260) and PR5 (At1g75040), PDF1.2 (At5g44420) and sesquiterpene synthase (At2g37620). The gene-specific primers used in labelling probes were ML276 (5′-GGATCCATGGCGAAGAACGTAGGGATATTG-3′) and ML860 (5′-GGAGACTGGTTCTCGCAGAGAC-3′) for *BjHMGS1*; ML1038 (5′-TCTCTCCTTCACACTTATCACGC-3′) and ML1039 (5′-GTTGTCGTTGTCGTTGCTCCAG-3′) for *HMG1*; ML1084 (5′-ATGTCGG

ATCTTCAGACACCGC-3′) and ML1085 (5′-CCCAGTACAAGCA CCTTGTGTG-3′) for *DWF1*; ML739 (5′-CTCTTTGTAGCTCTTGT AGGTGC-3′) and ML740 (5′-GGCTTCTCGTTCACATAATTCCC-3′) for *PR1*; ML755 (5′-TCTGAATCAAGGAGCTTAGC-3′) and ML756 (5′-CATACTTAGGCTGTCGATCT-3′) for *PR2*; ML757 (5′-ATCTCCAGTATTCACATTCT-3′) and ML758 (5′-CTTCGGTTTT TAAGGGCAGA-3′) for *PR5*; ML741 (5′-TAAGTTTGCTTCCAT CATCACCC-3′) and ML742 (5′-TTAACATGGGACGTAACAGAT AC-3′) for *PDF1.2*; and ML1100 (5′-AAGGACACTTGGTGACAG ATGC-3′) and ML1101 (5′-TGCGACATCTGTCATTCTCACG-3′) for sesquiterpene synthase.

RT-PCR

Those genes with low mRNA levels were detected using RT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen) from 14-day-old seedlings (except for Figure S2b) for RT-PCR analyses. RNA (2.5 μg) was treated by DNase (Promega, Madison, WI) and used for first-strand synthesis following the instructions of the SuperScript ™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The RT product (2 μL) was used as a PCR template to amplify the 1.4-kb *DXR* (At5g62790), 1.0-kb *BR6OX2* (At3g30180), 0.5-kb *CYP710A1* (At2g34500), 234-bp *ACTIN1* (At2g37620), 0.45-kb *SMT1* (At5g13170), 0.5-kb *SMT2* (At1g20330), 0.4-kb *SMT3* (At1g76090) and 0.5-kb *ACTIN8* (At1g49240) cDNAs in Arabidopsis pSa13-transformed and HMGS-OE lines under a temperature programme of 94 °C, 30 s; 54 °C, 30 s and 72 °C, 90 s; running for 30 cycles. Equal volumes of PCR products were loaded on 0.8% agarose in gel electrophoresis. Gels were scanned using PharosFX Plus Molecular Imager, and the expected bands were quantified using Quantity One software (Bio-Rad, Hercules, CA). The adjusted volume of each band for each gene was divided by that of the corresponding *ACTIN1* (Figures 2c and S2d) or *ACTIN8* (Figures S2a–b and S3) band to obtain the relative quantity. Primer pairs used were ML1042 (5′-AACTCACTATCTCCAGCTGAATC-3′) and ML1043 (5′-GAACTG GCCTAGCACCAGAAG-3′) for *DXR*; ML1086 (5′-TCTTCTCCG ATGGAACCAGATGC-3′) and ML1087 (5′-ACCGTTGAGTTC TAAGTCGTGAG-3′) for *BR6OX2*; primer pairs 5′-CACCGCTT AACCCGAA-3′ and 5′-GTGAGGTCACGACCAG-3′ for *ACTIN1*; ML1175 (5′-ATGGCCGATGCTGATGACATTC-3′) and ML1176 (5′-GTAGATAGGCACAGTGTGAGAC-3′) for *ACTIN8*; ML1181 (5′-TGCAAGAAGAGCTGCTCTTGTC-3′) and ML1182 (5′-AGAT GTGAAGGTCGTCACCATC-3′) for *HMG2* and ML1258 (5′-GAC AAGCAATCCTCCACGGC-3′) and ML1259 (5′-CTATGGGGAG AGCCATGGATC-3′) for *CYP710A1*; ML1564 (5′-CGTCGAATCT TGGTGGGAAGATC-3′) and ML1565 (5′-CACCTGCAAGTCGGTT TAGTTCC-3′) for *SMT1*; ML1566 (5′-ACACTCTTCTTCACCG GTGCAC-3′) and ML1567 (5′-CCTCGCAAAGCGCGTCGAGAC-3′) for *SMT2*; ML1568 (5′-CTCTCTACTGCACCGCTGGTC-3′) and ML1569 (5′-GGCTCGTTGCACTTGGTACTC-3′) for *SMT3*.

Quantitative analysis of sterols

Sterols were extracted and analysed according to Babiychuk *et al.* (2008) with some modifications. Fourteen-day-old freeze-dried Arabidopsis seedlings grown on MS medium or 3-week-old soil-grown rosette leaves (50 mg) were homogenized in 15 mL methanol containing 6% potassium hydroxide (KOH) and incubated in a 65 °C water bath for 2 h. Water (5 mL) was added, and then 20 mL hexane (HPLC grade, Fluka) was used twice to extract the neutral lipids (including sterols). The top hexane phases were pooled and evaporated to dryness. Acetylation was performed by adding toluene (100 μL), acetic anhydride (100 μL) and pyridine (50 μL). The mixture was incubated overnight at room temperature. The acetylation reactants were evaporated under hot drought, and lupenyl-3,28-diacetate (20 μg) was added as internal standard to the acetylated sterols. The mixture was then dissolved in hexane (500 μL), and 1 μL was used for GC-MS analyses (GC: Hewlett Packard 6890 with an HP-5MS capillary column: 30 m long, 0.25 mm i.d., film thickness 0.25 μm; MS: Hewlett Packard 5973 mass selective detector, 70 eV) to determine the sterol content, with He as the carrier gas (1 mL/min). The column temperature programme used included a fast rise from 60 to 220 °C (30 °C/min) and a slow rise from 220 to 300 °C (5 °C/min), then kept at 300 °C for 10 min. The inlet temperature was 280 °C. Compounds were identified using mass spectral libraries NIST (Agilent, Santa Clara, CA). The sterol masses were determined by comparing the peak area of each compound with that of the internal standard. Each sample was injected twice in GC-MS analyses, and an average of its sterol mass was taken. Sitosterol, campesterol and stigmasterol contents in Arabidopsis OE were compared with those of vector (pSa13)-transformed plants (Tables 1 and 2).

Quantitative analysis of floral sesquiterpene (–)-*E*-β-caryophyllene in HMGS-OEs

Volatiles were collected using solid phase micro extraction (SPME) (Tholl *et al.*, 2005; Abel *et al.*, 2009) from flowers of 5-week-old Arabidopsis transformed with vector pSa13 and representative constructs OE-wtBjHMGS1 and OE-S359A. The fibre coating was PDMS-30 for the manual holder (30 μm polydimethylsiloxane) (57308; Supelco, Bellefonte, PA). Twenty detached flowers were placed in a 1.5-mL glass vial sealed with a septum cap containing 100 μL water mixed with 0.01 μL (8.67 μg) anhydrous toluene (internal standard). Water with toluene minus flowers was set as a blank control. Samples were kept at 25 °C, and the fibres were exposed for 10 min. GC-MS system and temperature programme used in this assay were similar to those described by Abel *et al.* (2009). (–)-*E*-β-caryophyllene was identified according to the Agilent mass library and commercial standard β-caryophyllene (Aldrich, Hercules, CA). The retention time and peak pattern were similar to those obtained by Abel *et al.* (2009). The (–)-*E*-β-caryophyllene level (Figure S4a) was determined by comparing the peak area of the volatile compound to that of the internal standard.

Spectrometry quantification of chlorophyll and carotenoid contents

Fresh rosette leaves of 3-week-old Arabidopsis plants were weighed and transferred to eppendorf tubes. The material was ground on ice using a Teflon grinding bar, and chlorophyll and carotenoids were extracted in dim light using 90% methanol, following Laule *et al.* (2003). After centrifugation at 4 °C, absorbance was measured at 470, 653 and 666 nm. The amount of chlorophyll a, chlorophyll b and total carotenoids was calculated according to Lichtenthaler (1987).

Germination assay

Seeds collected simultaneously from wild-type, vector (pSa13)-transformed and HMGS-OE Arabidopsis lines were dried at 37 °C for 2 days and subsequently sterilized in 20% bleach and

70% ethanol. After five rinses in sterile water, seeds were spread onto MS medium agar plates. Plates were incubated at 4 °C for 4 days (Uchida and Yamamoto, 2002) and transferred to a growth room (22 °C 8-h dark/23 °C 16-h light). The emergence of the radicle was defined as germination. The number of germinated seeds was counted every 12 h within 3 days.

Fungal infection assays

Botrytis cinerea was incubated on Potato Dextrose Agar medium (BD, Lakewood, CO) for 3 weeks at 23 °C. Spores were washed off from the medium using 1% glucose, and the number of spores was calculated using a haemocytometer. Spores (3.5×10^5 mL^{-1}, 5 μL per drop) were used for the droplet infection (Dhawan *et al.*, 2009). Fungal infection assays were carried out at 23 °C in 100% humidity. The diameters (width) of fungal lesions were measured in the droplet infection assay after 3 days (Ferrari *et al.*, 2003; Dhawan *et al.*, 2009).

Hydrogen peroxide treatment assay

The detached rosettes leaves from 3-week-old plants (pSa13-transformed control and HMGS-OE lines) were soaked in water, 3 or 5 mM H_2O_2 for 72 h at room temperature under dim light. Ion leakage (IL) was determined and expressed as increased electrolyte conductivity according to Gechev *et al.* (2005). To estimate cell death induced by H_2O_2, H_2O_2-treated leaves were soaked in Milli Q water and shaken for 1 h before electrolyte conductivity (C) was measured using a conductivity meter (YSI model 55) (Chen *et al.*, 2008). Total conductivity (Tc) was obtained after boiling the treated leaves. Ion leakage was calculated by the equation: IL = (C/Tc)%. Treated leaves were also stained with trypan blue (Heese *et al.*, 2007) to directly indicate the dead cells.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 PCR and Southern blot analyses on transgenic Arabidopsis plants.
Figure S2 RT-PCR analysis on the expression of *HMG2* and *SMTs* (1 and 3) in HMGS-OEs and the expression of *HMGS*, *HMG2* and *BR6OX2* at different developmental stages in Arabidopsis Col-0.
Figure S3 RT-PCR on *CYP710A1* expression in developing seedlings and leaves of wild-type Arabidopsis.
Figure S4 Quantification of floral (–)-*E*-β-caryophyllene, total chlorophyll and carotenoids in HMGS OEs.
Table S1 Increase (%) of sterol composition in *Arabidopsis* HMGS-OE seedlings in comparison to pSa13-vector transformed control as calculated from Table 1.
Table S2 Increase (%) of sterol composition in *Arabidopsis* HMGS-OE rosette leaves in comparison to pSa13-vector transformed control as calculated from Table 2.

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