Transgenic Tobacco Overexpressing *Brassica juncea* HMG-CoA Synthase 1 Shows Increased Plant Growth, Pod Size and Seed Yield

Pan Liao1, Hui Wang1*, Mingfu Wang1, An-Shan Hsiao1, Thomas J. Bach2, Mee-Len Chye1¤

1 School of Biological Sciences, The University of Hong Kong, Hong Kong, China 2 Centre National de la Recherche Scientifique, UPR 2357, Institut de Biologie Moléculaire des Plantes, Strasbourg, France

**Abstract**

Seeds are very important not only in the life cycle of the plant but they represent food sources for man and animals. We report herein a mutant of 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGS), the second enzyme in the mevalonate (MVA) pathway that can improve seed yield when overexpressed in a phylogenetically distant species. In *Brassica juncea*, the characterisation of four isogenes encoding HMGS has been previously reported. Enzyme kinetics on recombinant wild-type (wt) and mutant BjHMGS1 had revealed that S359A displayed a 10-fold higher enzyme activity. The overexpression of wt and mutant (S359A) BjHMGS1 in *Arabidopsis* had up-regulated several genes in sterol biosynthesis, increasing sterol content. To quickly assess the effects of BjHMGS1 overexpression in a phylogenetically more distant species beyond the Brassicaceae, wt and mutant (S359A) BjHMGS1 were expressed in tobacco (*Nicotiana tabacum* L. cv. Xanthi) of the family Solanaceae. New observations on tobacco OEs not previously reported for *Arabidopsis* OEs included: (i) phenotypic changes in enhanced plant growth, pod size and seed yield (more significant in OE-S359A than OE-wtBjHMGS1) in comparison to vector-transformed tobacco, (ii) higher *NtSMT2-1* expression and sterol content in OE-S359A than OE-wtBjHMGS1 corresponding to greater increase in growth and seed yield, and (iii) induction of *NtIPPI2* and *NtGGPPS2* and downregulation of *NtIPPI1*, *NtGGPPS1*, *NtGGPPS3* and *NtGGPPS4*. Resembling *Arabidopsis* HMGS-OEs, tobacco HMGS-OEs displayed an enhanced expression of *NtHMGR1*, *NtSMT2-1*, *NtSMT2-2* and *NtCYP85A1*. Overall, increased growth, pod size and seed yield in tobacco HMGS-OEs were attributed to the up-regulation of native *NtHMGR1*, *NtIPPI2*, *NtSMT2-1*, *NtSMT2-2* and *NtCYP85A1*. Hence, S359A has potential in agriculture not only in improving phytosterol content but also seed yield, which may be desirable in food crops. This work further demonstrates HMGS function in plant reproduction that is reminiscent to reduced fertility of *hmgs* RNAi lines in *let-7* mutants of *Caenorhabditis elegans*.

**Introduction**

Isoprenoids form a large and diverse group of natural products, which have promising pharmacological applications including anti-cancer, antibacterial and anti-malarial properties [1–4]. Some isoprenoids including gibererlic acids, abscisic acid, cytokinins, sterols and brassinosteroids (BRs) play significant roles in plant growth and development [4–6]. Furthermore, carotenoids and chlorophylls are involved in photosynthesis [7]. Phytosterols are important in regulating growth and mediates stress tolerance in plants [4,8] and their nutritional value and health benefits in the human diet has been recognized [9–11].

In higher plants, two pathways generate isopentenyl diphosphate (IPP), which constitutes the universal precursor of all isoprenoids: the mevalonate (MVA) pathway in the cytosol, and the non-MVA, methylenylthriol phosphate (MEP) pathway in plastids [1,3,12] and references cited therein), with some crosstalk between them [13,14] (Figure 1). Sterols and BRs are synthesized in the cytoplasm and thereby derive from MVA, while giberellic acids and abscisic acid precursors, active cytokinins, carotenoids and chlorophylls are produced in plastids [1,15–21] and thus depend on the MEP pathway (Figure 1).

In agriculture, it is desirable to increase seed yield because grains represent significant sources of food, and the relevant key genes must be identified. Plant isoprenoids including sterols and BRs are essential in plant growth and reproduction [6,22–24] and genes from the BR-specific biosynthetic pathway, including *DWF4* and *DWF5*, affect seed production [22–24]. Transgenic *Arabidopsis* overexpressing *DWF4* showed better vegetative growth and seed yield [23], while the *Arabidopsis daf5* mutant demonstrated a dwarf phenotype accompanied by abnormal seeds [22]. The genes in the first and third steps of the MVA pathway also affect plant growth and development. RNAi lines of *Arabidopsis* downregulated for cytoplasmic *ACETOACETYL-COA THIOLASE* (*AACT2*) displayed reduction in apical dominance, seed yield and root length, accompanied by sterility and dwarfing [25]. Also, the *Arabidopsis*...
hmgr1 mutant is dwarf-like and male sterile, and has a lower sterol content [26].

3-Hydroxy-3-methylglutaryl-coenzyme A synthase (HMGS) is the second enzyme in the MVA pathway [27–31]. Besides 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), HMGS is a key enzyme in cholesterol biosynthesis in mammals and cytoplasmic isoprenoid biosynthesis in plants [3,4,32–36]. Four genes designated BjHMGS1 to BjHMGS4 encode HMGS in Brassica juncea [34] and investigations revealed that BjHMGS1 is cytosolic. The expression of recombinant BjHMGS1 led to the elucidation of its kinetic and physiological properties [37,38] and of its crystal structure [39]. Enzyme kinetics of recombinant wild-type (wt) and mutant BjHMGS1 had revealed that H188N showed 8-fold lower enzyme activity and loss of acetoacetyl-CoA inhibition, while S359A displayed a 10-fold higher enzyme activity [37]. Given these interesting results, mutant (H188N, S359A and H188N/S359A) and wt BjHMGS1 were overexpressed in Arabidopsis, which like Brassica, belongs to the family Brassicaceae [4]. BjHMGS1 overexpression in transgenic Arabidopsis up-regulated several genes in sterol biosynthesis (cf. Figure 1), for instance those encoding HMGR, SMT2 (sterol methyltransferase 2), DWF1 (sterol C-24 reductase), CYP710A1 (sterol C-22 desaturase) and BR6OX2 (brassinosteroid-6-oxidase 2), increasing sterol content and thereby enhancing stress tolerance [4]. Analysis of the Arabidopsis hmgs mutant demonstrated the role of HMGS in tapetal development and pollen fertility [35].

To quickly assess the effects of BjHMGS1 overexpression in a more distant species, the overexpression of BjHMGS1 was carried out on a plant outside the Brassicaceae family. Hence, tobacco (Nicotiana tabacum L. cv. Xanthi), another model plant from the family of Solanaceae was selected, also because of the easiness of its genetic transformation. Subsequently, the genes downstream of HMGS that were tested encode enzymes that produce intermediates in phytosterol and BR biosynthesis, for instance
N. tabacum 3-hydroxy-3-methylglutaryl-CoA reductase (NtHMGR1 and NtHMGR2), isopentenyl diphosphate isomerase (NtIPPI1 and NtIPPI2), farnesyl diphosphate synthase (NtFPPS), squalene synthase (NtSQS), and sterol methyltransferases (NtSMT1-2, NtSMT2-1 and NtSMT2-2) and cytochrome P450 monoxygenase (NtCYP83A1). In addition, we examined the expression of genes encoding geranylgeranyl diphosphate synthases (NtGGPPS1, NtGGPPS2, NtGGPPS3 and NtGGPPS4), enzymes that are not implied in the formation of an intermediate in the sterol pathway. Resultant transgenic tobacco (OE-wtBjHMGS1 and OE-S359A) not only showed an increased sterol content but also displayed enhanced plant growth, pod size and seed yield that were not previously observed in transgenic Arabidopsis HMGS-OEs. Furthermore, OE-S359A conferred better plant growth and seed production than OE-wtBjHMGS1, and this was attributed to higher NtSQS expression and total sterol content, realizing the potential application of BjHMGS1 in being quite active in phylogenetically distant species.

Materials and Methods

Plant materials and growth conditions

Wt tobacco (N. tabacum L cv. Xanthi) obtained from the Institute of Molecular and Cell Biology (Singapore) was used in this study. Tobacco plants were grown at 25°C (16 h light)/22°C (8 h dark). Tobacco seedlings were cultured in Murashige and Skoog (MS) medium [40].

Generation of transgenic plants overexpressing HMGS

Plasmids pBj134 (wtBJHMGS1) and pBj136 (S359A) were used in Agrobacterium-mediated leaf disc transformation of N. tabacum [4,41]. The binary vector pSa13 [42] was used as vector control in transformation. T1 transgenic tobacco seeds were selected on MS containing kanamycin (50 μg ml⁻¹) and verified using PCR and DNA sequence [4]. T2 homozygous plants with a single-copy transgene were compared in mRNA expression, metabolite composition, plant growth and seed yield.

Western blot analysis

Total protein was extracted [43] from 21-d-old tobacco leaves. Protein concentration was determined using the Bio-Rad Protein Assay Kit I (Bio-Rad). Protein (20 μg per well) separated on 12% SDS-PAGE was transferred onto Hybond-ECL membrane (Amersham) using a Trans-Blot® cell (Bio-Rad). Antibodies raised against the synthetic peptide (DESYQSRDLEKVSQQ) corresponding to BjHMGS1 amino acids 290 to 304 were used in western blot analyses [4,44]. Cross-reacting bands were detected using the ECL™ Western Blotting Detection Kit (Amersham).

Northern blot analysis

Tobacco total RNA was extracted from 21-d-old tobacco leaves using TRIzol reagent (Invitrogen). RNA (20 μg per well), separated on 1.3% agarose gels containing 6% formaldehyde, was transferred to Hybond-N membrane (Amersham) for northern blot analysis [45]. Digoxigenin-labelled probes were synthesized using the PCR Digoxigenin Probe Synthesis (Roche) with primer pair ML276 and ML860. Primers are listed in Table S1.

Southern blot analysis

Genomic DNA (40 μg) from 4-week-old tobacco leaves prepared by the CTAB method [46] was digested by EcoRI and separated on 0.7% agarose gel by electrophoresis, together with a 1-kb plus DNA standard ladder (Invitrogen). DNA was transferred from the agarose gel onto Hybond-N membrane (Amersham) by capillary transfer [47]. Southern blot analysis of tobacco using a 32P-labelled full-length of BjHMGS1 cDNA probe with primer pair ML264 and ML860 was performed [4]. Primers are listed in Table S1.

Extraction and quantitative analysis of sterols

For sterol profiling, freeze-dried materials from 20 mg of 60-d-old soil-grown tobacco leaves and 10 mg of 20-d-old MS plate-cultured tobacco seedlings were used. Extraction and quantitative analysis of sterols were carried out as described [4,48]. GC-MS analysis (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) was used to determine sterol content, with He as the carrier gas (1 ml/min). The column temperature program used included a fast rise from 60°C to 220°C (30°C/min) and a slow rise from 220°C to 300°C (5°C/min), then kept at 300°C for 10 min. The inlet temperature was 280°C. Compounds were identified using the National Institute of Standards and Technology (NIST) libraries of peptide tandem mass spectra (Agilent, USA). The sterol masses were determined by comparison of the peak area of each compound with that of the internal standard (lupenyl-3,28-diacetate). Two independent lines for each OE genotype were analysed. Five independent repeats (samples) for each independent line were used for sterol extraction. Each sample was injected twice in GC-MS analyses and an average of the sterol mass was taken. Sitosterol, campesterol and stigmastanol contents in transgenic tobacco HMGS-OEs were compared to those in vector (pSa13)-transformed plants following previous reports [4,17].

Seed germination assay

Tobacco seeds collected simultaneously from vector (pSa13)-transformed control and HMGS-OE lines were sterilized in 20% bleach, 70% ethanol and then spread on MS medium agar plates supplemented with kanamycin (50 mg/l). About 30 tobacco seeds were sown on one plate. Five duplicate plates were used for each independent line [4]. All the plates were incubated at 4°C for 4 days and transferred to a culture room for 2 days under a photoperiod of 22°C 8-h dark and 23°C 16-h light. Subsequently, the number of germinated seeds was counted every 12 h for 60 h using a dissecting microscope. The emergence of the radicle was defined as germination [4]. The germination rates were calculated and compared using the Student’s t-test. Two independent lines of OE-wtBjHMGS1 (“401” and “402”) and two independent lines of OE-S359A (“603” and “606”) were tested in seed germination assays. The experiment to measure seed germination was repeated twice.

Growth rate measurements

Growth rate was measured according to previous reports [49–53]. Four-d-old seedlings were transferred onto fresh MS plates placed vertically for a further 10-d growth. The dry weight of 4-d-old seedlings was then measured. Five seedlings were grouped for weight measurements and a total of 30 groups were analysed per individual line.

For greenhouse plants, 7-d-old tobacco seedlings of similar size were transferred from MS medium to soil for further growth rate measurements. The height of 80-, 98- and 210-d-old tobacco were measured. As 80-d-old plants did not have flowers, the height measurement did not include the inflorescence. However, 98- and 210-d-old plants were flowering and the height measurement included the inflorescence. For 98-d-old tobacco, measurements of leaf fresh weight, length and width of the four bottom-most leaves
were also analysed for the vector-transformed control, OE-wtBjHMGS1 and OE-S359A. Two independent lines from each OE construct were analysed for 80-d-old tobacco plants and three independent lines from each OE construct were analysed for 90- and 210-d-old tobacco plants. For each line, six plants were used.

Comparison in tobacco seed yield
Seed yield was measured [49,51,52,54] to test the differences between HMGS-OEs (OE-wtBjHMGS1 and OE-S359A) and the vector-transformed control. Ten plants each from two independent lines from each OE construct were examined and T2 homozygous seeds of each line were germinated on MS. Fourteen-d-old seedlings were transferred to soil in a greenhouse. Pods (30 per group) were harvested at maturity from each of 10 plants per line to determine total dry pod weight, average dry pod weight, total dry seed weight and total seed number. The experiment to measure seed yield was repeated twice (2–3 groups were analysed for each repeat).

To further determine if increase in seed size occurred, the dry weight of 100 seeds from each line was measured and 29 repeats were carried out per line. The average dry weight was calculated from 30 measurements of 100 seeds per line.

RNA analysis
Total RNA (5 μg) of 20-d-old tobacco seedlings and 14-d-old Arabidopsis were extracted using RNeasy Plant Mini Kit (Qiagen) and were reverse-transcribed into first-strand cDNA using the SuperScript First-Strand Synthesis System (Invitrogen). Quantitative Reverse-Transcription-PCR (qRT-PCR) was carried out with a StepOne Plus Real-time PCR System (Applied Biosystems) and FastStart Universal SYBR Green Mater (Roche). The conditions for qRT-PCR were as follows: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Three experimental replicates for each reaction were carried out using gene-specific primers and tobacco ACTIN and Arabidopsis ACTIN2 were used as internal controls. The relative changes in expression from three independent experiments were analysed [55]. Primers for qRT-PCR are listed in Table S1.

Accession numbers
Sequence data included herein can be found in the GenBank/EMBL data libraries under accession numbers AF148847 (BjHMGS1), AV140008 (AtHMGS), U60452 (NtHMGR1), AFO02432 (NtHMGR2), ABO49815 (NtHIP1), ABO49816 (NtHIP2), GQ410573 (NtFPPS), U60057 (NtSQS), GQ911583 (NtGGPPS1), GQ911584 (NtGGPPS2), AFO53766 (NtSMIT2-1), U71108 (NtSMIT2-2), U71107.1 (NtSMIT2-2), DQ649022 (NtIPI1), U60489 (NtACTIN) and AY096381 (AtACTIN2).

Statistical analysis
Analyses of data in this work was carried out using the Student’s t-test to determine any significant differences between means.

Results
Molecular analyses of transgenic tobacco HMGS-OEs
The presence of wt and mutant BjHMGS1 in transgenic tobacco was verified by PCR [Figure S1A-B] following by DNA sequence analysis of the PCR product. Putative tobacco HMGS-OEs were designated as OE-wtBjHMGS1 (lines “401”, “402” and “404”) and OE-S359A (lines “602”, “603” and “606”). PCR-positive HMGS-OE lines were confirmed by western blot analysis (Figure 2A). As the peptide used to generate anti-BjHMGS1 antibodies shows 100% homology to tobacco HMGS (GenBank accession number EF636813), a faint band was detected in the vector (pSa13)-transformed control (Figure 2A). Northern blot analyses revealed that transgenic lines verified by western blot analysis expressed BjHMGS1 mRNA (Figure 2B). Single-insertion-alias identified by Southern blot analyses (Figure S2) were selected for further experiments.

Tobacco HMGS-OEs accumulate sterols in both seedlings and leaves
The contents of the three major sterols (campesterol, stigmasterol and sitosterol) in 20-d-old tobacco HMGS-OE seedlings and leaves show rRNA (20 μg per well). Three independent lines per construct were analysed. (B) Northern blot analysis of BjHMGS1 in representative vector (pSa13)-transformed control and HMGS-OEs (OE-wtBjHMGS1 and OE-S359A). Putative tobacco HMGS-OEs were designated as OE-wtBjHMGS1 (lines “401”, “402” and “404”) and OE-S359A (lines “602”, “603” and “606”). Bottom, Coomassie Blue-stained gel of total protein loaded (20 μg per well). Three independent lines per construct were analysed. (B) Northern blot analysis of BjHMGS1 in representative vector (pSa13)-transformed control and HMGS-OEs. The expected 1.7-kb BjHMGS1 band is marked with an arrowhead. Bottom gels show rRNA (20 μg per lane). Two independent lines per construct are shown. The two independent lines of OE-wtBjHMGS1 plants labelled “401” and “402”, and two independent lines of OE-S359A plants labelled “603” and “606” used in further tests are underlined.

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60-d-old leaves were analysed. GC-MS results of changes represented in µg per mg dry weight showed that the average campesterol, stigmasterol, sitosterol and total sterol contents of the OE-S359A seedlings were significantly higher than the vector (pSa13)-transformed control and OE-wtBjHMGS1 (Table 1). In particular, the average elevations over the vector (pSa13)-transformed control in OE-S359A seedlings were noted for campesterol (31.7%), stigmasterol (24.0%), sitosterol (25%) and total sterol (25.7%) (Table 2) and average elevations over OE-wtBjHMGS1 for campesterol (25.4%), stigmasterol (19.0%), sitosterol (20%) and total sterol (20.4%) (Table 2). However, OE-wtBjHMGS1 seedlings did not show significant changes from the vector-transformed control and increases were merely ~4–5% for each sterol (Table 2).

In leaves, except for stigmasterol, the average amounts of campesterol, sitosterol and total sterol were significantly higher in OE-wtBjHMGS1 than the vector (pSa13)-transformed control (Table 1): campesterol (12.9%), sitosterol (42.9%) and total sterol (12.1%) (Table 2). Furthermore, the average amounts of stigmasterol and total sterol in OE-S359A leaves were significantly higher (31.8% and 19.0%, respectively) over the vector (pSa13)-transformed control (Table 2). The differences between OE-wtBjHMGS1 and OE-S359A leaves were not significant and OE-S359A average stigmasterol and total sterol contents were only slightly higher than OE-wtBjHMGS1 (Table 1).

The % increase of sterols between transgenic tobacco (observed herein) and transgenic Arabidopsis (OE-wtBjHMGS1 and OE-S359A) [4] were also compared (Table 2 and S2). A similar trend was observed in transgenic Arabidopsis and tobacco seedlings; OE-S359A transformants displayed higher increase than the OE-wtBjHMGS1 not only in each sterol (campesterol, stigmasterol and sitosterol) but also in total sterol (Table S2); OE-S359A transformants also showed similar increase over the OE-wtBjHMGS1 in both Arabidopsis and tobacco leaves for stigmasterol and total sterol (Table 2 and S2).

**Tobacco HMGS-OE seeds germinated earlier**

As seeds from Arabidopsis HMGS-OEs were observed to germinate earlier than the vector (pSa13)-transformed control [4], the germination of tobacco HMGS-OE seeds was investigated. Tobacco seeds of OE-wtBjHMGS1 and OE-S359A not only germinated earlier but also displayed significantly higher germination rates than the control at 60 to 120 h post-germination (Figure S3). Also, OE-S359A germinated faster than OE-wtBjHMGS1 (Figure S3).

**Tobacco HMGS-OE plants show increased growth**

As sterols or steroid plant hormones have been reported to regulate plant growth [8,56], phenotyping was carried out on 14-d-old seedlings and 60-d-old plants. In 14-d-old HMGS-OE (OE-wtBjHMGS1 and OE-S359A) seedlings, root length (Figure 3A–B) and dry weight (Figure 3C) were significantly greater than the vector (pSa13)-transformed controls. Although the root length of 14-d-old seedlings in OE-S359A was not significantly greater than the OE-wtBjHMGS1 (Figure 3B), their dry weight was significantly heavier than OE-wtBjHMGS1 (Figure 3C). Consistently, 80-d-old tobacco HMGS-OE greenhouse plants grew better than the vector-transformed control (Figure 3D). HMGS-OEs (OE-wtBjHMGS1 and OE-S359A) were taller at 80-d than the control (Figure 3E). More interestingly, 80-d-old OE-S359A displayed significantly greater height than the OE-wtBjHMGS1 (Figure 3E).

Growth differences in height (Figure 4A–B) and leaf size (Figure 4C–D) between 98-d-old HMGS-OEs (OE-wtBjHMGS1 and OE-S359A) and vector (pSa13)-transformed plants were also represented in Figure 4C–D.

### Table 1. Sterol profiles of tobacco HMGS-OE seedlings and leaves (µg/mg dry weight)

| Construct | Sterol content of 20-d-old seedlings | Sterol content of 60-d-old leaves |
|-----------|-------------------------------------|----------------------------------|
| pSa13     | Campesterol 0.60 ± 0.08            | Campesterol 0.64 ± 0.05          |
|           | Stigmasterol 0.64 ± 0.05            | Stigmasterol 0.63 ± 0.03          |
|           | Sitosterol 0.63 ± 0.03              | Sitosterol 0.64 ± 0.02ab          |
|           | Total sterol 0.79 ± 0.02ab          | Total sterol 0.79 ± 0.02ab        |
| pSi13     | Campesterol 0.66 ± 0.05             | Campesterol 0.66 ± 0.05           |
|           | Stigmasterol 0.63 ± 0.03             | Stigmasterol 0.64 ± 0.02ab        |
|           | Sitosterol 0.64 ± 0.02ab             | Sitosterol 0.65 ± 0.02ab          |
|           | Total sterol 0.79 ± 0.02ab           | Total sterol 0.79 ± 0.02ab        |
| OE-wtBjHMGS1 | Campesterol 1.21 ± 0.15             | Campesterol 1.25 ± 0.06           |
|           | Stigmasterol 1.26 ± 0.06             | Stigmasterol 1.26 ± 0.06           |
|           | Sitosterol 1.26 ± 0.06                | Sitosterol 1.26 ± 0.06             |
|           | Total sterol 1.50 ± 0.06ab           | Total sterol 1.50 ± 0.06ab         |
| OE-S359A | Campesterol 1.29 ± 0.06ab           | Campesterol 1.29 ± 0.06ab         |
|           | Stigmasterol 1.29 ± 0.06ab           | Stigmasterol 1.29 ± 0.06ab        |
|           | Sitosterol 1.29 ± 0.06ab             | Sitosterol 1.29 ± 0.06ab          |
|           | Total sterol 1.50 ± 0.06ab           | Total sterol 1.50 ± 0.06ab        |

Two independent lines for each OE genotype were analysed. For OE-wtBjHMGS1, transformants “a” and “b” were tested. For OE-S359A, transformants “a” and “b” were tested. Two independent lines for each vector (pSa13) controls were tested. Asterisks indicate significant difference between OE-wtBjHMGS1 and OE-S359A, Bold font indicates significant higher sterol content than vector (pSa13) transformed control and/or OE-wtBjHMGS1.
Table 2. Increase (%) of sterol composition in tobacco HMGS-OE seedlings and leaves in comparison to vector (pSa13)-transformed control and elevation of OE-S359A over OE-wtBjHMGS1.

| Construct Compared to Elevation (%) in 20-d-old seedlings Elevation (%) in 60-d-old leaves | Campesterol | Stigmasterol | Sitosterol | Total sterol | Campesterol | Stigmasterol | Sitosterol | Total sterol |
|---|---|---|---|---|---|---|---|---|
| OE-S359A | 401 | 6.7 | 3.3 | 6.3 | 4.0 | 15.3 | 3.0 | 50.0 | 14.4 |
| OE-S359A | 402 | 5.0 | 4.1 | 2.1 | 4.8 | 9.4 | 10.6 | 28.6 | 9.8 |
| OE-S359A | 603 | 31.7 | 24.0 | 22.9 | 26.1 | 2.4 | 4.7 | 24.2 | 14.3 |
| OE-wtBjHMGS1 | 606 | 31.7 | 23.1 | 27.1 | 25.3 | 4.7 | 24.2 | 14.3 | 15.5 |

Two independent lines for each OE genotype were analysed. For tobacco OE-wtBjHMGS1, transformants “401” and “402” were tested. For tobacco OE-S359A, transformants “603” and “606” were tested. Values = [(meanOE - meanpSa13)/meanpSa13]*100. The data presented for OE-S359A in comparison to OE-wtBjHMGS1 was calculated from an average of two transformants (average of “603” and “606” for OE-S359A in comparison to average of “401” and “402”) by the Student’s t-test (P<0.05) (Figure 6D–F). No significant difference in dry seed weight of 100 seeds was noted between the vector-transformed control and HMGS-OEs (Figure 6G), suggesting that seed size was not affected. Hence, HMGS-OE increase in seed yield was attributed to increase in pod size and seed number rather than seed size (Figure 6).

Tobacco HMGS-OEs produce an enhanced seed yield

Comparison in seed yield by seed weight measurement between HMGS-OEs (OE-wtBjHMGS1 and OE-S359A) and the vector (pSa13)-transformed control indicated that both OE-wtBjHMGS1 and OE-S359A were higher than the control (Figure 6A–D); seed yield of OE-wtBjHMGS1 increased by 21 to 32% (P< 0.05) (Figure 6D–F), while OE-S359A showed a 55 to 80% rise (P< 0.01) (Figure 6D–F). OE-S359A lines “603” and “606” showed an average of 32% increase over OE-wtBjHMGS1 lines “401” and “402” by the Student’s t-test (P<0.05) (Figure 6D–F). No significant difference in dry seed weight of 100 seeds was noted between the vector-transformed control and HMGS-OEs (Figure 6G), suggesting that seed size was not affected. Hence, HMGS-OE increase in seed yield was attributed to increase in pod size and seed number rather than seed size (Figure 6).

Change in expression of isoprenoid biosynthesis genes in tobacco HMGS-OEs

qRT-PCR was performed to check the effect of BjHMGS1 overexpression on the expression of genes downstream of HMGS in tobacco HMGS-OE seedlings and to explore possible molecular mechanism of HMGS function in plant growth and seed production. The results from qRT-PCR revealed that the expression of NtHMGR1, NtIPPI2, NtSQS, NtSMT1-2, NtSMT2-1, NtSMT2-2 and NtCYP85A1 was significantly higher than in the vector (pSa13)-transformed control for both OE-wtBjHMGS1 and OE-S359A tobacco seedlings with the exception of NtSQS, NtSMT1-2, NtSMT2-2 and NtCYP85A1 in one OE-wtBjHMGS1 line (401) (P<0.01) (Figure 7). However, there was no difference in the expression of NtHMGR2 between all the HMGS-OE lines and the vector-transformed control (Figure 7). For the expression of NtFPPS, there was no disparity amongst the two lines of OE-wtBjHMGS1 (401 and 402) and the vector-transformed control, while the expression of NtFPPS in another OE-wtBjHMGS1 line (404) and in two OE-S359A lines (602 and 606) was slightly higher than the control (P<0.05) (Figure 7). Conversely, the expression of NtIPPI1, NtGGPPS1, NtGGPPS3 and NtGGPPS4 were downregulated in tobacco HMGS-OE seedlings (P<0.01) (Figures 7–8) while the expression of NtGGPPS2 was higher than the control (P<0.05) in two OE-wtBjHMGS1 lines (402 and 404) and two OE-S359A lines (602 and 606) (Figure 8). Observations that (i) NtSQS expression in all three OE-S359A lines was higher than all three OE-wtBjHMGS1 lines, (ii) NtHMGR1 and NtCYP85A1 expression in all three OE-S359A lines was higher than two (“401” and “402”) of three OE-wtBjHMGS1 lines, and (iii) NtSMT2-1 expression in two (“602” and “603”) of three OE-S359A lines was higher than two (“401” and “402”) of three OE-wtBjHMGS1 lines suggest that the differences in expression levels of NtSQS, NtHMGR1, NtSMT2-1 and NtCYP85A1 in OE-wtBjHMGS1 and OE-S359A do correspond to the expected...
differences in enzyme activities between recombinant wtBjHMGS1 and S359A [37].

Discussion

New observations from tobacco HMGS-OEs

Our investigations on the overexpression of HMGS in transgenic tobacco revealed new observations not previously evident in Arabidopsis HMGS-OEs including the upregulation of NtIPPI2, NtSQS and NtGGPPS2 and downregulation of NtIPPI1, NtGGPPS1, NtGGPPS3 and NtGGPPS4 (Figures 7–8). However, similar to findings from Arabidopsis HMGS-OEs, enhanced NtHMGR1, NtSMT1-2, NtSMT2-1, NtSMT2-2 and NtCYP85A1 expression in tobacco HMGS-OEs was seen (Figure 7). Other new findings from tobacco HMGS-OEs included growth stimulation in the tobacco HMGS-OE lines, confirming the positive role of HMGS overexpression in plant growth. Furthermore, tobacco HMGS-OEs show increased pod size and seed yield (Figure 6), indicative of a specific HMGS function in seed production. Improved growth, pod size and seed yield of OE-S359A in comparison to OE-wtBjHMGS1 may be attributed to the higher NtSQS expression (Figure 7) and sterol content in OE-S359A transformants (Table 1).

Function of HMGS in reproduction and development

In plants, the floral organs are involved in reproduction. HMGS has been shown to play a crucial role in floral development [4, 34, 35, 37]. In Arabidopsis, higher AhtHMGS expression had been observed in flowers than seedlings or leaves from RT-PCR analysis [4]. Using mutants in Arabidopsis, AhtHMGS was demonstrated essential for pollen fertility and proper development of tapetum-specific organelles in Arabidopsis [35]. In B. juncea, northern blot analysis had previously revealed that BjHMGS1 mRNA was highly expressed in flowers and seedling hypocotyls [34] and in situ hybridization analysis had shown that HMGS mRNA was predominantly localized in the stigmata and ovules of flower buds and in the piths of seedling hypocotyls [37]. BjHMGS1 and BjHMGS2, but not BjHMGS3 and BjHMGS4 expression was
**Figure 4. Comparison in plant growth between 98-d-old greenhouse-grown HMGS-OEs and vector-transformed tobacco.** (A) Representative plants photographed 98-d after germination show differences in growth between HMGS-OE tobacco plants and vector-transformed control. Bar = 10 cm. (B) Analysis on height of 98-d-old transgenic plants. (C) Representative tobacco leaves photographed 98-d after germination with growth differences between HMGS-OE and vector-transformed tobacco. Bar = 10 cm. (D) Analysis on fresh weight, length and width of bottom-most four leaves from a 98-d-old tobacco plant. Values are mean ± SD (n = 6); Bars are SD; **, P<0.01; *, P<0.05; ** and *, significantly higher than control, by the Student’s t-test. The vector-transformed control is labelled “pSa13”, three independent lines of OE-wtBjHMGS1 plants are labelled “401”, “402” and “404”, and three independent lines of OE-S359A plants are labelled “602”, “603” and “606”. doi:10.1371/journal.pone.0098264.g004
detection of HMGS in tobacco HMGS-OEs

In transgenic Arabidopsis, the overexpression of wt and mutant (H188N, S359A and H188N/S359A) BjHMGS1 caused a feed-forward effect in the upregulation of several genes in sterol biosynthesis including HMG1, SMT2, DWF1, CYP710A1 and BBG0X2 [4]. This study using tobacco HMGS-OEs demonstrated that some differences exist between tobacco and Arabidopsis HMGS-OEs in the expression of genes encoding HMG1 and SMT (cf. Figure 1). Although HMG1 is considered to be the rate-limiting enzyme in the MVA pathway in plants [62], only NiHMG1 but not NiHMG2 was upregulated in tobacco HMGS-OEs (Figure 7). This can perhaps be attributed to some differences in the localization and function of NiHMG1 and NiHMG2 [63,64]. NiHMG1 is a house-keeping gene that likely participates in sterol biosynthesis, plant growth and development, while NiHMG2 is stress-inducible [63,64]. Also elicitor-inducible HMG1 activity is known to be associated with defence-related sesquiterpenoid accumulation in tobacco cell suspension cultures [65]. Thus it was not surprising that rather than NiHMG2, NiHMG1 was upregulated in seedlings undergoing rapid growth and development.

Isopentenyl diphosphate isomerase (IPPI) catalyses the interconversion of IPP and its ally isomer dimethylallyl diphosphate (DMAPP) and provides the first key intermediate for the biosynthesis of all kinds of isoprenoids including sterols in the MVA pathway and carotenoids in the MEP pathway [1,3,12,66] (and references cited therein) (cf. Figure 1). IPP is most likely involved in cross-talk between the cytosolic MVA pathway and the plastidial MEP pathway [13,14]. AtIPPI1 and AtIPPI2 have been reported to be critical to sterol biosynthesis in the MVA pathway and Arabidopsis development [67]. Analysis of the expression of the two AtIPPI genes in tobacco HMGS-5 OE seedlings revealed that AtIPPI1 was downregulated, while AtIPPI2 was upregulated (Figure 7). Their corresponding proteins are apparently differentially localized in tobacco [68]. NtIPPI1 is targeted to the chloroplast, while NtIPPI2 is cytosolic, similar to BjHMGS1 [37,68]. Possibly, upregulation of BjHMGS1 and NtIPPI2 in the cytosol of tobacco HMGS-OE seedlings promoted cross-talk between the MVA and MEP pathways. The MEP pathway
Figure 6. Tobacco HMGS-OEs show increased seed yield. (A) Phenotype of tobacco pods. pSa13, vector-transformed control; “401” and “402”, two independent lines of OE-wtBjHMGS1 and “603” and “606”, two independent lines of OE-S359A. Scale bar = 1 cm. (B) Total dry weight of 30 tobacco pods. (C) Average dry weight per pod. (D) Total dry weight of seeds from 30 pods. (E) Total seed number per 30 pods. (F) Average seed number per pod. (G) Average dry weight of 100 seeds in control and HMGS-OEs. Thirty independent readings were taken for each line. Values are means ± SD, n = 30. a indicates significant difference between HMGS-OE and the vector (pSa13)-transformed control; b indicates significant difference between OE-wtBjHMGS1 and OE-S359A. H, value higher than the control (P<0.05 or 0.01 by the Student’s t-test).

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produces simultaneously IPP and DMAPP, and plastidial NtIPPI1 is possibly needed to adjust the ratio of starter DMAPP to elongation units IPP for longer prenyl chains. If IPP is imported from the cytosol because of “overproduction”, then plastidial NtIPPI1 would be downregulated.

FPPS catalyses the condensation of two molecules of IPP with DMAPP to form farnesyl diphosphate (FPP) (C15) (cf. Figure 1), which provides the key precursor for the biosynthesis of essential isoprenoids such as sesquiterpenes, ubiquinones, polyterpenes, dolichols and sterols [69,70]. In plants, FPPS isozymes that are encoded by a small gene family, exert differential roles, based on their subcellular localisation [69,71].

NtFPPS expression was slightly elevated in seedlings of only one OE-wtBjHMGS1 line (Figure 7). Given that NtFPPS functions as the key provider of the universal product FPP in the biosynthesis of many C-15 related products, a moderate change in NtFPPS mRNA in the HMGS-OE lines may not be significant enough to affect sterol accumulation. Also, other NtFPPS isogenes or post-translational regulation may be involved [72–74].

SQS catalyses the biosynthesis of squalene by the reductive dimerization of two FPP molecules (cf. Figure 1), and represents the first committed step in the biosynthesis of sterols, BRs and triterpenes [75–79]. The change in NtSQS expression in seedlings was the most dramatic, with a 2.1-fold increase in two lines of OE-wtBjHMGS1 and 36.5-fold in OE-S359A, in comparison to the vector-transformed control (Figure 7). The increase of NtSQS mRNA in OE-S359A seedlings was also much higher (11.1-fold) than OE-wtBjHMGS1 (Figure 7). Interestingly, NtSQS expression and NtSQS activity have been detected predominantly at the shoot apical meristem (SAM) rather than leaves or roots, implying that sterol biosynthesis occurs especially in the SAM [77]. Furthermore, the SAM is critical in plant growth and development, and stem cells from the SAM continuously generate all the aerial organs and tissues of a plant [80]. Results from qRT-PCR (Figure 7) herein support a role for NtSQS in HMGS-associated sterol accumulation related to growth and seed yield. Also, enhanced sterol accumulation, growth and seed yield in OE-S359A, over OE-wtBjHMGS1 (Figure 7), corresponded to higher NtSQS expression (Figure 7). Consistently, Arabidopsis SQS (AtSQS) displayed higher expression in HMGS-OEs than the vector-transformed control; and AtSQS expression in OE-S359A was higher than OE-wtBjHMGS1 (Figure S4). However the elevation of AtSQS in tobacco OE-S359A over OE-wtBjHMGS1 (Figure 7)
was greater in comparison to AtSQS in Arabidopsis OE-S359A (Figure S4). Furthermore, our results correspond well to a recent study on the overexpression of G. max SQS1 (GmSQS1) in Arabidopsis that yielded a 50% increase of seed sterol content [91]. An enhanced flux of MVA to FPP might present some risk as phosphatases always being present might liberate farnesol, which can be quite toxic to cells [92]. Thus SQS could remove a potentially dangerous intermediate and get it channelled into the synthesis and accumulation of chemically inert sterols and their derivatives.

In the MEP pathway, GGPPS catalyses the consecutive condensation of three molecules of IPP and one DMAPP to generate the 20-carbon geranylgeranyl diphosphate (GGPP) (cf. Figure 1), which is the universal key intermediate for the biosynthesis of carotenoids and of abscisic acid as derivative, of cellulose-induced sesquiterpenoid phytoalexin in tobacco by SMT2 and of sesquiterpenoids in cypridina green shrimp [12,83]. Although biosynthesis of carotenoids and of abscisic acid (derivatives) is upregulated in Arabidopsis that yielded a 50% increase of seed sterol content [91–93], the expression of all these genes was maintained at a level similar to the control (Figures 7–8). Furthermore, AtHMGR1, AtPP2 and NtSMT2 displayed significantly higher expression in this line than the control (Figure 7), implying that they positively affected plant growth and seed yield. Taken together with observations on a general up-regulation of AtCYP73A2 (BR60X2) in 21-d-old rosette leaves of transgenic Arabidopsis overexpressing BjHMGS1 [4], our studies reinforce that HMGS overexpression likely leads to up-regulation of BR synthesis, and thereby promotes growth and seed production.

Supporting Information

Figure S1 The BjHMGS1 constructs used in tobacco transformation and resultant PCR analysis on transgenic tobacco lines. (A) Schematic map of transformation vector indicating primer location. BjHMGS1 wild-type and mutant inserts were derived from plasmids, pBj134 (WT BjHMGS1) and pBj136 (S359A) [4]. CaMV353: Cauliflower Mosaic Virus 353 promoter; NOSsp: nopaline synthase (NOS) promoter; NOSter: NOS terminator; NPTII: gene encoding neomycin phosphotransferase II conferring resistance to kanamycin; RB: right border of T-DNA; LB: left border of T-DNA. 353: 35S promoter 3′-end forward primer; ML264: BjHMGS1-specific 3′-end reverse primer. (B) Agarose gel showing the expected 1.65-kb BjHMGS1 cDNA band (arrowed) from transgenic tobacco following PCR using primer pair 35S/ML264; representative lines are shown here. OE-wtBjHMGS1 (lanes 1–3); OE-S359A (lanes 4–6); positive control (lane 7, PCR template plasmid pBj134); blank control (BC) (lane 8, no DNA band after PCR). Putative tobacco HMGS-OEs were designated as OE-wtBjHMGS1 (lines “401”, “402” and “404”) and OE-S359A (lines “602”, “603” and “606”). (TIF)

Figure S2 Southern blot analysis on transgenic tobacco plants. (A) Schematic map of transformation vector indicating EzO1 (E) sites. BjHMGS1 wild-type and mutant inserts were derived from plasmids pBj134 (wt BjHMGS1) and pBj136 (S359A). CaMV353: Cauliflower Mosaic Virus 353 promoter; NOSsp: nopaline synthase (NOS) promoter; NOSter: NOS terminator; NPTII: gene encoding neomycin phosphotransferase II conferring resistance to kanamycin; RB: right border of T-DNA; LB: left border of T-DNA. Dotted lines denote position of nucleotide on vector. (B) Southern blot analysis of genomic DNA digested by restrictive endonuclease EzO1 and probed with 32P-labelled BjHMGS1 full-length cDNA in representative blots. Arrowheads indicate hybridizing bands. OE-wtBjHMGS1 transformants (lanes 1–2), OE-S359A transformants (lanes 3–5). Representative single insertion lines (transformants “401” and “402”) were OE-wtBjHMGS1 and “603” and “606” for OE-S359A were underlined. Transformant “601” likely has a more than one inserts and was not included in further analysis. (TIF)

Figure S3 Comparison in seed germination of tobacco HMGS-OEs. Statistical data on seed germination rates recorded at 60, 72, 84, 96, 108 and 120 h after incubation at 23°C indicates (a) significant difference (P<0.01 by the Student’s t-test) between HMGS-OE and the vector (pSa13)-transformed control; (b) indicates significant difference (P<0.01 by the Student’s t-test).
between OE-wtBHMGSl and OE-S359A. Values are mean ± SD (n = 5); bars represent SD. pSas13, vector-transformed control; the two independent lines of OE-wtBHMGSl (“401” and “402”) and two independent lines of OE-S359A (“603” and “606”) were tested in seed germination assays. The data represents the average from two transformants.

**Table S4 Expression of Arabidopsis SQS by qRT-PCR in 14-d-old HMGS-OE seedlings.** Total RNA was extracted from 14-d-old Arabidopsis seedlings of vector (pSas13)-transformed control, two independent lines of OE-wtBHMGSl (lines “134-L1” and “134-L2”) and two independent lines of OE-S359A (lines “136-L1” and “136-L2”) previously generated [4]. H, value higher than the control (P < 0.01, Student’s t-test). Values are means ± SD (n = 3), a indicates significant difference between HMGS-OE and the vector (pSas13)-transformed control.

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**Author Contributions**

Conceived and designed the experiments: PL, HW, TJB, MLC. Performed the experiments: PL, HW, TJB, ASH, TJB. Analyzed the data: PL, HW, TJB, MLC. Contributed reagents/materials/analysis tools: MLC, TJB. Wrote the paper: PL, HW, TJB, MLC. Coordinated the project: TJB, MLC.

**Table S2 Increase (%) of sterol composition in Arabidopsis HMGS-OE seedlings and leaves in comparison to vector (pSas13)-transformed control.** (DOCX)
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