Plant Mol Biol (2010) 74:503–518
DOI 10.1007/s11103-010-9692-6

Molecular characterization and functional analysis of *Glycine max sterol methyl transferase 2* genes involved in plant membrane sterol biosynthesis

Anjanasree K. Neelakandan · Hanh T. M. Nguyen · Rajesh Kumar · Lam-Son Phan Tran · Satish K. Guttikonda · Truyen Ngoc Quach · Donovan L. Aldrich · W. David Nes · Henry T. Nguyen

Received: 5 June 2010 / Accepted: 11 September 2010 / Published online: 24 September 2010

Abstract Sterol C24 methyltransferase (*SMT2*) genes governing the pattern of phytosterols synthesized in higher plants have been studied in *Glycine* seedlings and wild-type and engineered *Arabidopsis thaliana* plants. The *SMT2* genes of soybean (*SMT2-1* and *SMT2-2*) previously cloned and characterized (Neelakandan et al. 2009) were shown to complement the SMT deficient *cvp1* mutant Arabidopsis plants, consistent with their role in regulation of 24-alkyl sterol-controlled plant physiology. Further analysis of these genes showed that environmental cues, including dehydration, cold, and abscisic acid induced differential changes in transcript levels of the *SMT2* during soybean seedling growth. Sterol analyses of transgenic Arabidopsis seeds originating in variant constructs of *AtHMGR1*, *GmSMT1*, and *GmSMT2* engineered in seeds showed relevant modifications in the ratio of 24-methyl to 24-ethyl sterol in the direction of sitosterol formation. To provide insight into the structural features of the sterol gene that affects transcript regulation, the upstream promoter sequences of soybean *SMT2* genes were cloned and characterized. Sequence analysis revealed several important *cis*-elements and transcription factor binding sites. The analysis of promoter-GUS fusions in transgenic Arabidopsis plants revealed shared and distinct expression features in different developmental stages and tissues. The data are interpreted to imply that SMT2 is an important contributor to normal plant growth and development.

Electronic supplementary material The online version of this article (doi:10.1007/s11103-010-9692-6) contains supplementary material, which is available to authorized users.

A. K. Neelakandan · H. T. M. Nguyen · R. Kumar · L.-S. P. Tran · S. K. Guttikonda · T. N. Quach · D. L. Aldrich · H. T. Nguyen (✉)
Division of Plant Sciences and National Center for Soybean Biotechnology, University of Missouri, Columbia, MO 65211, USA
e-mail: nguyenhenry@missouri.edu

W. D. Nes
Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX 79409, USA

Present Address:
H. T. M. Nguyen
Center for Plant Science Innovation, Department of Agronomy & Horticulture, University of Nebraska, Lincoln, NE 68588-0660, USA

Present Address:
R. Kumar
National Research Center on DNA Fingerprinting, National Bureau of Plant Genetic Resources, New Delhi 110012, India

Present Address:
L.-S. P. Tran
Signaling Pathway Unit, Gene Discovery Group, RIKEN Plant Science Center, 1-7-22 Suehiro, Tsurumi, Yokohama 230-0045, Japan

Present Address:
S. K. Guttikonda
Dow AgroSciences LLC, Indianapolis, IN 46268, USA

Present Address:
D. L. Aldrich
School of Management, University of Texas at Dallas, Richardson, TX 75080-3021, USA

Keywords Sterol 24-C-methyltransferase · Sitosterol · Campesterol · Promoter · *Cis*-elements · Gene expression · Development · Mutant complementation · Regulation

Abbreviations

SMT	Sterol 24-C-methyltransferase
PCR	Polymerase chain reaction
GC-FID	Gas-chromatography-flame ionization detector
BR	Brassinosteroids
SAM	*S*-Adenosyl methionine
cvp	Cotyledon vascular pattern
HMGR	3-Hydroxy, 3-methylglutaryl coenzyme A reductase
GUS	Beta glucuronidase
2,4-D	2,4-Dichlorophenoxy acetic acid
ABA	Abscisic acid
START	Steroidogenic acute regulatory protein (StAR)-related lipid transfer domain

Introduction

Phytosterols are a family of natural products characterized by their unique side chain construction of a 24-alkyl group that in nature can differ in size and stereochemistry. In crop plants, 24α/β-methyl and 24α-ethyl sterols generally account for more than 85% of the total sterols (Jayasimha et al. 2006). These 24-alkyl sterols possess multiple roles in tracheophytes, including that of an architectural component of membranes and as a precursor to steroid hormones (Beck et al. 2007; Clouse 2000; Hartmann 1998). The biosynthesis of phytosterols originates in the isoprenoid pathway, in which the starting C5-unit can be derived either from the acetate-mevalonate or mevalonate-independent pathways (Fig. 1; Benveniste 2004; Rohmer 2008). Recent studies have shown that compartmentation of isoprenoid enzymes and co-ordinate regulation of enzyme activities involved in sterol biosynthesis can affect the sterol fluxome across kingdoms and in plant development (Benveniste 2004; Jayasimha et al. 2006).

Enzyme activities crucial to carbon flux in the direction of 24-alkyl sterol synthesis are 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), and sterol C-24 methyltransferase (SMT), which act as a "course control" and "fine control" of metabolism, respectively (Chappell et al. 1995; Hemmerlin et al. 2004; Schaller et al. 1995). Transgenic upregulation of SMT1 (Holmberg et al. 2002) or pharmacological inhibition of squalene synthase (Wentzinger et al. 2002) feed-back to modulate HMGR activity in such a way to affect phytosterol production. Alternatively, sitosterol can feed-back and down-regulate SMT activity, presumably as membrane biogenesis plateaus at growth arrest (Nes 2000). HMGR activity is also regulated by alterations of sphingolipid levels, thus presenting an important junction for regulatory crosstalk between lipid biosynthetic pathways (Nieto et al. 2009). In transgenic studies targeted at HMGR, over-expression of the enzyme led to the accumulation of intermediate sterols thereby facilitating the identification of additional slow steps in the pathway, mediated by SMT1, which catalyses the first committed step of sterol biosynthesis (Bach 1986; Chappell et al. 1995; Nes 2000). Further engineering efforts of different combinations of HMGR and SMT1 in crop plants resulted in improved total sterol levels (Holmberg et al. 2003), suggesting that appropriate modification of the isoprenoid-phytosterol genes might produce beneficial increases in phytosterol levels of seed oils.

Another distinct class of SMT enzymes termed SMT2 (EC 2.1.1.143), consisting of two isoforms, SMT2-1 and SMT2-2 (Neelakandan et al. 2009), catalyzing a second methyl transfer further downstream the pathway, was identified (Bouvier-Navé et al. 1997, 1998; Zhou and Nes 2003). The plant SMT2 is believed to be an ER localized enzyme, which catalyses *S*-adenosylmethionine-dependent transfer of methyl groups to the C-24(28)-methylene lophenol substrate. This step represents a branch point reaction directing carbon flux towards synthesis of C-24 ethyl sterols and away from brassinosteroid biosynthesis. The transcriptional regulation of plant *SMT1* and *SMT2* genes of the sterol pathway have been investigated in different plant species (Carland et al. 2002; Diener et al. 2000; Luo et al. 2008; Neelakandan et al. 2009; Shi et al. 1996). The mutants of the *SMT2-1* gene in Arabidopsis, cotyledon vascular pattern 1 (*cvp1*), has pleiotropic phenotypes including a defect in vascular patterning and development, serrated petal and sepal margins and reduced intermodal and silique elongation (Carland et al. 2002). Another Arabidopsis mutant, *frl1*, was reported with serrated sepal and petal tips, which were attributed to ectopic endoreduplication (Hase et al. 2000, 2005).

The reaction catalyzed by the SMT2 enzyme diverts carbon flux towards the C-24 ethyl sterols, which represents the final segment of phytosterol biosynthesis. These molecules, especially sitosterol, have tremendous applications in the functional food industry mainly due to the cholesterol-lowering effect in humans (Westrate and Meijer 1998). Constitutive over-expression of *SMT2* in model species resulted in the reduction of C24 methyl sterol (campesterol) levels and a concomitant increase in C24 ethyl sterol (sitosterol) species (Schaeffer et al. 2000, 2001; Schaller et al. 1998; Sitbon and Jonsson 2001). However, the plants had morphological alterations including reduction in plant height, apparently due to a decrease in cell number and reduced growth. These defects could be rescued by exogenous brassinosteroid treatment, indicating that the observed abnormal morphology was due to BR deficiency.

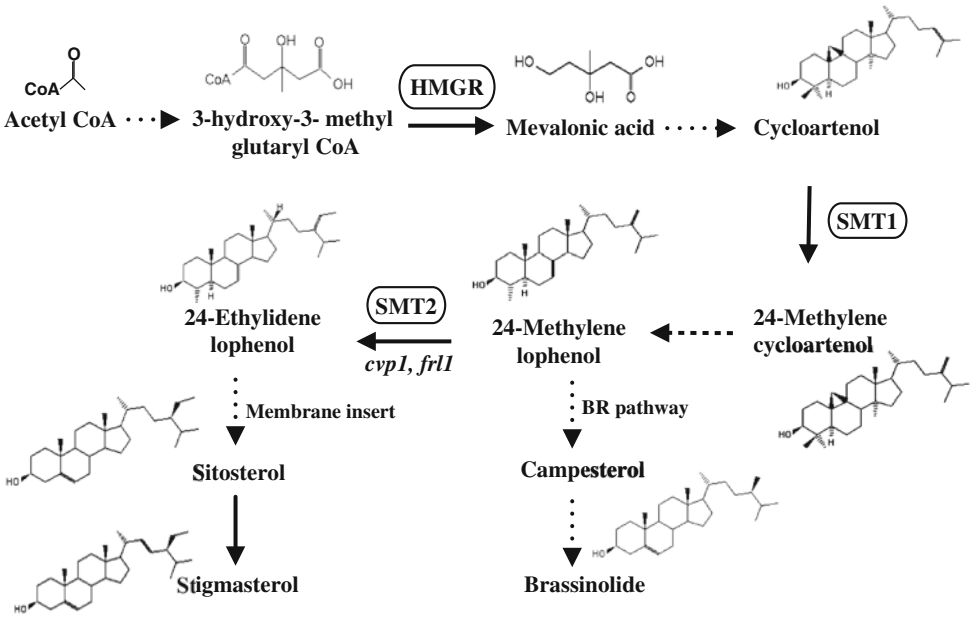

Fig. 1 Biosynthetic scheme of the major sterol biosynthesis pathway in plants. The enzymes involved in the main reactions involving committed steps in the isoprenoid, phytosterol and C24 ethyl sterols, examined in this study, are shown inside *rounded rectangles*. The mutants for *SMT2* gene are indicated in *italics*. *Dotted arrow* marks represent multiple enzymatic steps

Recently, we reported on soybean *SMT2* genes, *GmSMT2-1* and *GmSMT2-2*, with 96% identity at the amino acid level (Fig. 2) and they share 80% similarity to Arabidopsis (orthologues of *AtSMT2-1* and *AtSMT2-2*, respectively), and tobacco genes. These SMT2 proteins were cloned and characterized in *E. coli* for details of substrate specificity and inhibition (Neelakandan et al. 2009). They reside in chromosomes 4 (Glyma04g02270) and 6 (Glyma06g02330) respectively as per the soybean sequence assembly Glyma1.0 (http://www.phytozome.net/soybean.php). The enzyme encoded by the *GmSMT2-2* gene was found to have a better catalytic efficiency among the two and exhibited regulated transcript abundance in developing soybean seeds. To shed further light on the transcriptome—metabolome congruence affecting the function of SMT2 in soybean sterol biosynthesis, we prepared a set of transgenic plants harboring seed-targeted over-expression of *HMGR1*, *SMT1* and *SMT2* genes.

Materials and methods

Plant materials and treatments

Soybean seedlings of cv. Maverick were grown in 3 gallon pots under green house conditions of 28/20°C day/night temperature, photoperiod of 16/8 h, and the dehydration, ABA, salinity and cold treatments were performed on seedlings at different time points as per Tran et al. (2009).

Arabidopsis thaliana ecotype Columbia (Col 0-*gl*) seeds were used as the Arabidopsis wild-type genetic background. The SMT2 mutant, *cvp1-3* seeds used in this study were obtained from Dr. Timothy Nelson (Yale University). The Arabidopsis seeds were surface sterilized and sown in MS plates (with or without antibiotic), subjected to cold treatment (4°C) in dark for 2–3 days, and allowed to grow for 2 weeks. The seedlings were transplanted to small pots containing Promix media (Hummert International, Earth City, MO, USA) in growth chambers maintained at 22°C, 70% relative humidity, with 16/8 h photoperiod and fertilized with Scotts Peters 20–20–20 water soluble fertilizer as per requirement. The flowering stage plants were selected for transformation conducted as described by Clough and Bent (1998). The T1 transgenic seedlings were initially selected on MS plates in the presence of the selection agent, glufosinate ammonium (Crescent chemical company, Islandia, NY, USA) at a concentration of 20–25 mg/l and subsequently analyzed by gene-specific PCR. The T2 seeds were screened on antibiotic plates to determine segregation ratios, and only those events with single or two transgene insertion loci were selected for further analysis.

Generation of recombinant gene constructs for Arabidopsis transformation

The cloning strategy and details of the organization of gene cassettes generated in the binary vectors, pZY101Asc and

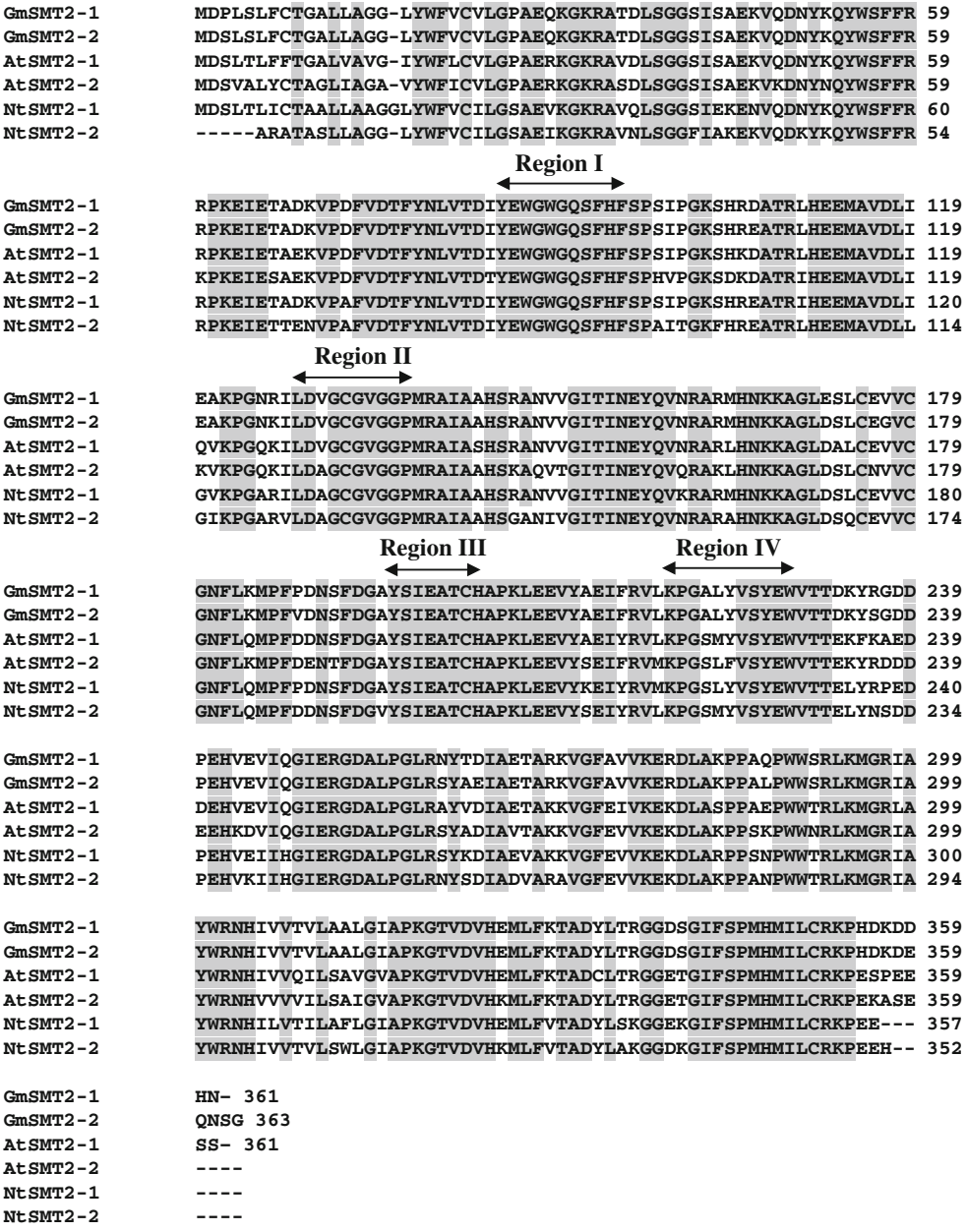

Fig. 2 Amino acid alignment of representative SMT2 proteins cloned and characterized from plants. The conserved sterol and Adomet binding sites are indicated with *arrow marks*

pCambia3301, for Arabidopsis transformation, are summarized below. The primer sequences used for amplification and cloning are detailed in Supplementary Table 1. The plant selection was conferred by the *bar* gene driven by the *CaMV35S* promoter sequence and *NOS* polyadenylation signal. For generating *Glycinin-GmSMT1* (SM1), Soybean *SMT1* (GeneBank accession number U43683) gene was amplified from the subcloning vector (GmSMTpET23a) with gene specific primers containing NotI sites and cloned into the intermediate vector pKMS3, downstream to the seed-specific glycinin-1 promoter, at the NotI site. The orientation was verified with respect to the glycinin promoter and the whole gene expression cassette (*Glycinin-1* promoter-*SMT1* gene-*Glycinin-1* terminator) was subsequently cloned into the binary vector, pZY101-Asc (SM1) at the AscI. The *β-Phaseolin-GmSMT2-2* (SM2) construct was developed by first amplifying the *phaseolin* promoter from the pPhas vector, which was subcloned into the pKMS3 vector replacing the *Glycinin-1* promoter (at the KpnI and NotI sites) creating an additional EcoRI site by incorporating EcoRI recognition sequence into the primer sequence. Subsequently, the *GmSMT2-2* cDNA sequences were cloned downstream of *Phaseolin* promoter at the EcoRI-NotI site and the whole gene cassette, together with *phaseolin* terminator was introduced into the destination vector at the SmaI site. To develop *β-Conglycinin-AtHMGR1: Glycinin-GmSMT1* (SH) construct, the C terminal catalytic domain along with a part of the linker region (amino acids 164–592)

of Arabidopsis *HMGR-1* gene (GeneBank accession number, P14891), was amplified using primers with NotI restriction site from leaf cDNA and subcloned into pBetaConSoyHyg, which harbors the seed-specific *Beta-conglycinin* promoter and *phaseolin* terminator (abbreviated as HM) and checked for the right orientation. The whole gene cassette was subsequently cloned into the binary vector SM1 at the Hind III site (abbreviated as SH). Subsequently a three gene casette vector *β-Conglycinin-AtHMGR1: Glycinin-GmSMT1: β-Phaseolin-GmSMT2-2* (SHS) was developed by inserting the *β-Phaseolin-GmSMT2-2* gene cassette into the SH vector at the SmaI site. For developing *35S-GmSMT2-1* (SM21C) and *35S-GmSMT2-2* (SM22C), the *GmSMT2* coding sequence is sub-cloned downstream to the *CaMV35S* promoter in pRTL2 vector (Carrington and Freed 1990) at EcoRI-SacI sites, and the whole gene cassette was subsequently cloned into the binary vector pZY101-Asc at the Hind III site. The vectors *GmSMT2-1-GUS* (G1) and *GmSMT2-2-GUS* (G2) were generated by amplifying the 5′ upstream sequence of soybean *SMT2-1* gene (591 bp) and *SMT2-2* gene (1,877 bp) using primers with incorporated restriction sites followed by cloning in pCambia3301 cut with XbaI and NcoI, replacing the *CaMV35S* promoter.

PCR analysis

The putative transgenic plants were screened by PCR for the presence of the transgene. Genomic DNA was isolated by from young leaf tissues using CTAB method (Saghai-Maroof et al. 1984) and electrophoresed on 0.8% agarose gels to verify the integrity and quantified by nanodrop model ND-1000 UV–Vis spectrophotometer (NanoDrop-Technologies, Wilmington, DE, USA). The PCR primers were designed for the transgene coding sequences, as listed in the Supplementary Table 1. Amplifications were performed with 50 ng genomic DNA using Taq 2X mastermix (New England Biolabs, Ipswich, MA, USA), along with the respective primers at the appropriate annealing temperatures for 30 cycles of amplification followed by electrophoresis on 1% agarose gels, visualized by ethidium bromide staining and photographed.

RNA isolation, cDNA synthesis and qRT–PCR analysis

The procedure for total RNA isolation from soybean tissues and transcript quantification are as described previously (Tran et al. 2009; Neelakandan et al. 2009). Gene-specific primers were designed using Primer Express 2.0 software (ABI); their sequences and the respective amplicon sizes are reported in the Supplementary Table 2. The relative quantifications of *SMT* gene expression in different tissues were performed using the comparative threshold cycle (C_t) method, after normalization to the expression levels of *CYP2* gene (Tran et al. 2009) selected as the internal reference.

RNA was isolated from wild-type and transgenic Arabidopsis plant tissues (seedlings and siliques) using RNEasy Plant Mini Kit (Qiagen Inc. Valencia, CA, USA) One microgram of total RNA was subjected to DNase I treatment and RT–PCR as described in Neelakandan et al. (2009). The *ubiquitin* gene (*UBQ5*:AT3G62250) was selected as the house keeping control. The amplified products were run on 1% agarose gels and visualized by ethidium bromide staining and photographed.

Sterol analysis

Sterol extraction and analysis was performed based on Neelakandan et al. (2009) and the Iowa State University's plant metabolomics analytical platform protocol, with slight modifications. 5-alpha cholestane (Sigma–Aldrich, St. Louis, MO, USA) was added as the internal standard. Lipids were extracted from 10 mg of Arabidopsis seeds in chloroform:methanol mixture, followed by saponification, solvent extraction with hexane and subsequent derivatisation by silylation to form TMS ether derivatives of sterols, which were used for quantification. GC analysis of sterols was performed with a Finnigan TraceGC Ultra gas chromatograph equipped with a Flame Ionization Detector. The column used was TRACE TR-5MS, which is a 5% Phenyl (equiv) polysilphenylene-siloxane capillary column of 30 m length, 0.25 mm ID and 0.25 μm film thickness. Helium was used as the carrier gas with a flow rate of 1.0 ml/min and a split injection ratio of 1:10 was employed. Standard sterols and mixtures were run under identical conditions to identify the analytes on the basis of their retention times.

Genome walking for identification of upstream sequences

The upstream sequences of the *Glycine max SMT2* genes were identified and cloned using Genome Walker Universal kit (Clontech Laboratories, Mountain View, CA, USA), as per the manufacturer's instructions. The gene-specific oligo sequences used in PCR amplification are as follows: SM21GR, 5′-GCCCAGAACGCACACGAACCAGTAGA 3′; SM21GNR (nested reverse primer), 5′-GGGGTCCATT GTGACTTTGTGAGAA 3′; SM22GR, 5′-CGGCGGAAG AAGGACCAGTACTGCTT 3′; SM22GNR, 5′-AGAAGG GCTCCGGTGCAGAAGAGAGA 3′. The PCR products were purified, cloned in Copy Control™ pCC1 vector (Epicentre Biotechnologies, Madison, WI, USA) and sequenced.

Promoter analysis in silico

The upstream sequences were analyzed computationally for the presence of potential *cis*-acting elements important for regulation of expression, using PLACE (Higo et al. 1999), PLANTCARE (Lescot et al. 2002) databases and putative transcription factor binding sites with the PlantPAN plant promoter analysis navigator tool (Chang et al. 2008).

GUS histochemical staining and microscopy

GUS histochemical staining was performed as per Jefferson et al. (1987). The tissues at the developmental stages of at least three independent plants of two independent events in T2 and T3 generations were sampled and stained in the GUS staining buffer at 37°C overnight. For dehydration treatments, 2-week-old seedlings were removed from the plate and subjected to bench drying in filter papers at room temperature for 1, 3 and 5 h time points, followed by GUS staining. The controls were also placed in filter papers saturated with water. For auxin treatment, 3-day-old seedlings were placed in a solution of 2, 4-Dichlorophenoxyacetic acid (2, 4-D), a synthetic auxin, at a concentration of 10 μM for a duration of 12 h, followed by staining in the GUS buffer. Arabidopsis tissues (treated and non treated) were immersed in GUS staining buffer [50 mM sodium phosphate, pH 7.0, 1 mM EDTA, 0.1 mM potassium ferrocyanide, 0.1 mM potassium ferricyanide, 2 mM of 5-Bromo-4-chloro-3-indolyl *β*-D-glucuronide cyclohexylammonium salt (X-gluc, Sigma–Aldrich, St. Louis, MO, USA) as substrate, which was pre-dissolved in *N*-dimethyl sulfoxide] and later photographed using a Leica EZ4 D stereo-microscope [Leica Microsystems (Switzerland) Ltd] equipped with a Leica EC3 camera digital camera and LAS EZ (Leica Application Suite Educational Zoom) software for image acquisition and analysis. For higher magnifications, Olympus Vanox AHBT3 upright microscope equipped with a 3-megapixel color CMOS digital camera was used with DIC optics.

Results

Expression of *GmSMT2* genes under stress and hormone treatments

Our earlier studies in soybean revealed that the expression of *SMT2* genes are more or less ubiquitous in different tissues (Neelakandan et al. 2009), and that *SMT2-2* shows higher transcriptional abundance in seed developmental stages. In the present study, we monitored the expression of soybean *SMT2* genes under a variety of treatments including dehydration, water (mock), ABA (100 μM), salt (NaCl, 250 mM), and cold, at the indicated time points in soybean seedlings (Fig. 3). Analysis of transcript abundance by qRT–PCR revealed that both the genes are down-regulated at 5 and 10 h after dehydration and markedly and consistently induced under cold conditions. ABA treatment elicited a slight increase in transcript levels, at 1 h time point for both the genes and consistently at the 5 and 10 h treatments for the *SMT2-2* gene, as compared to the water controls. A slight reduction in expression was associated with the salinity treatment for both the genes. Our findings suggest that the transcriptional regulation of soybean *SMT2* genes is influenced by environmental conditions to some extent. The data also suggests a co-ordinate regulation of the two genes to combat the environmental stresses and thereby point towards parallel functions, in tissue-dependent regulation of the carbon flux downstream in the pathway.

GmSMT2 genes functionally complement *cvp1* mutants

In order to ascertain the in planta functionality of soybean *SMT2* genes for potential applications in future, the respective cDNAs were cloned downstream of the constitutive CaMV35S promoter and used to transform homozygous Arabidopsis *cvp1-3* mutant plants (Carland et al.

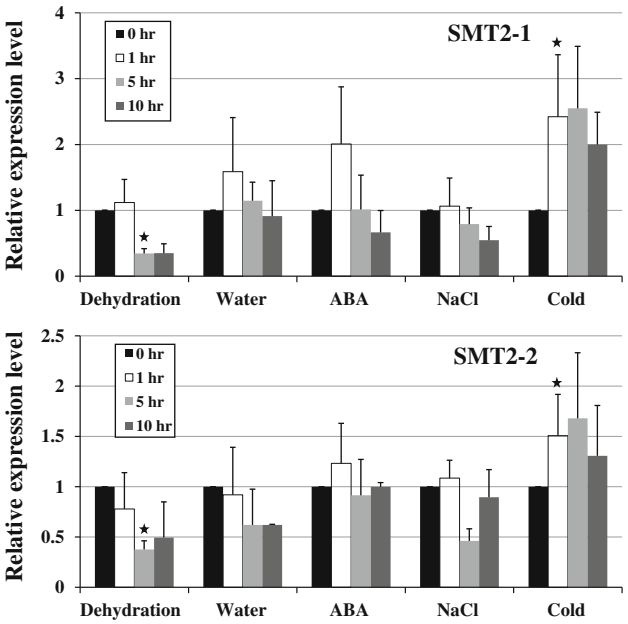

Fig. 3 ABA and abiotic stress-specific regulation of *SMT2* genes. Soybean tissues were subjected to treatments as described in the "Materials and methods" section and relative quantification of *SMT2* mRNA was performed. The control treatment values are normalized to 1 and the fold change in expression is depicted. The *error bars* denote standard error of three technical replicates. Statistically significant differences based on paired Students's *t* test at $P < 0.1$ is denoted by *asteriks*

2002). The mutant plants had pleiotropic phenotypes, including discontinuous cotyledon venation, serrated petal and sepal margins, delayed flowering and senescence and reduced plant height at maturity. The transgene expression by RT-PCR and morphological characteristics of the transgenic *cvp1-3* plants were evaluated in at least three independent events. The vascular patterning defect in the cotyledons of the mutant was completely rectified by both the soybean *SMT2* isoforms. Moreover, the dwarf mutant phenotype (Fig. 4) and the silique elongation defects (data not shown) were complemented in correlation with the transgene expression level. Other than a slight delay in flowering and senescence, the wild-type phenotype was almost completely restored. Our data thus demonstrates that the soybean *SMT2* genes are capable of producing a catalytically active enzyme, which could function in vivo in heterologous plant systems. In order to gain a deeper understanding towards the regulation of this gene family in soybean, we identified and cloned the respective promoter sequences and performed detailed in planta characterization.

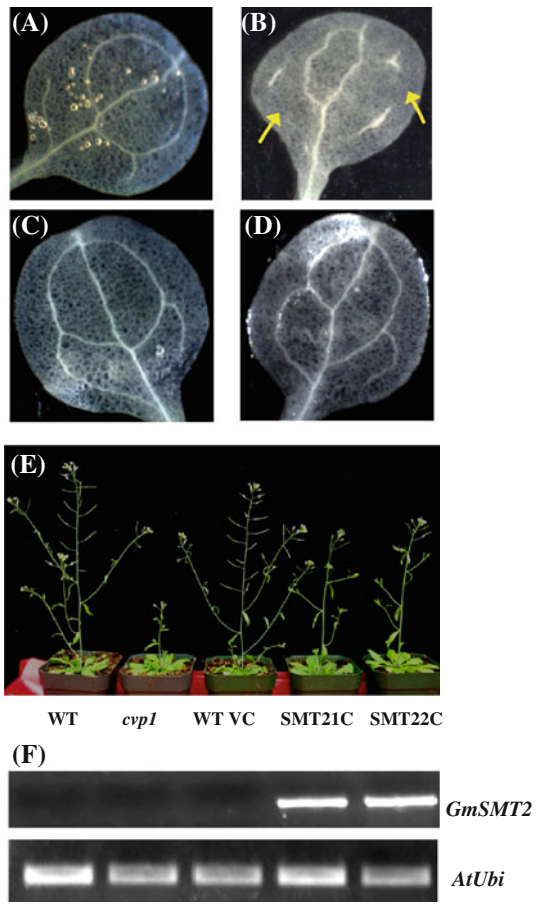

Fig. 4 Complementation of *cvp1* mutant Arabidopsis plants. The cotyledons are cleared of chlorophyll and the veination pattern is visualized in **a**–**d**. **a** Wild-type, **b** *cvp1-3* mutant, **c** SM21C and **d** SM22C, representing *GmSMT2* genes driven by the CaMV35S promoters in the mutant background. *Yellow arrows* mark discontinuities in the veination pattern in the *cvp1-3* mutant. **e** presents the morphology of the plants at the reproductive stage. WT stands for wild-type Arabidopsis plants and wild-type transformed the empty pZY101Asc vector (WTVC) is also included as a transgenic control. **f** represents the transgene expression by RT-PCR using *GmSMT2* gene-specific and *AtUBQ5* primers as the housekeeping control

Role of SMT2 in Arabidopsis seed sterol accumulation

The plant specific *SMT* genes code for enzymes that are involved in the transfer of methyl groups to sterol intermediates and it has been demonstrated earlier that SMT1 is a rate-limiting enzyme in channeling the carbon flux downstream towards C-24 alkyl sterol synthesis (Nes and Venkatramesh 1999; Holmberg et al. 2002), and that it is capable of acting in concert to enhance sterol levels (Holmberg et al. 2003). To investigate the role of plant *SMT2* genes in regulating the metabolic flux, we over-expressed the *GmSMT2-2* gene, selected based on higher transcriptional abundance among the two and better catalytic activity of the corresponding enzyme, in Arabidopsis seeds with the seed-specific *Beta Phaseolin* promoter (abbreviated as SM2). For comparison, transgenic plants were also developed with *GmSMT1* under the control of seed-specific *Glycinin-1* promoter alone (SM1) and in combination with *AtHMGR1* downstream to the seed-specific *Beta Conglycinin promoter* (SH). A fourth construct with seed-specific over-expression of all the three genes, *HMGR1*, *SMT1*, and *SMT2* (SHS) was also developed to ascertain the ability of *SMT2* gene to act in conjunction with known upstream rate-limiting enzymes in order to channel carbon flux towards the synthesis of C24-ethyl sterols.

The transgene expression was checked in the mid-mature siliques derived from transgenic T2 plants (see Supplementary Fig. 1 for details). In general, SHS seeds had a higher level of *SMT1* and *SMT2* expression, as compared to the rest of the constructs. The sterol content and composition from homozygous T4 seeds of three independent plants of three independent events, in two technical replicates were analyzed by GC-FID as shown in Fig. 5. The sterol content of the wild-type seeds comprising of sitosterol, stigmasterol, campesterol and cycloartenol was on an average, 2.82 μg/mg seeds. The alteration in sterol content was the least in SM2 seeds, whereas, the genetically modified SHS seeds, showed maximum increase in total content increased, i.e., up to 4.16 μg/mg seeds for the SHS2-2 event. In general, sitosterol and stigmasterol levels increased in all the transgenic seed samples, cycloartenol levels declined and campesterol levels were more or less static, except in the SHS seeds, where there was a reduction. The ratio R, representing campesterol: sitosterol was 0.25 for wild-type seeds, ranged from 0.22 to 0.24 for SM1 and SH seeds, 0.19 for SM2-2 and 0.13 in SHS-3 seeds. The proportion of sitosterol increased

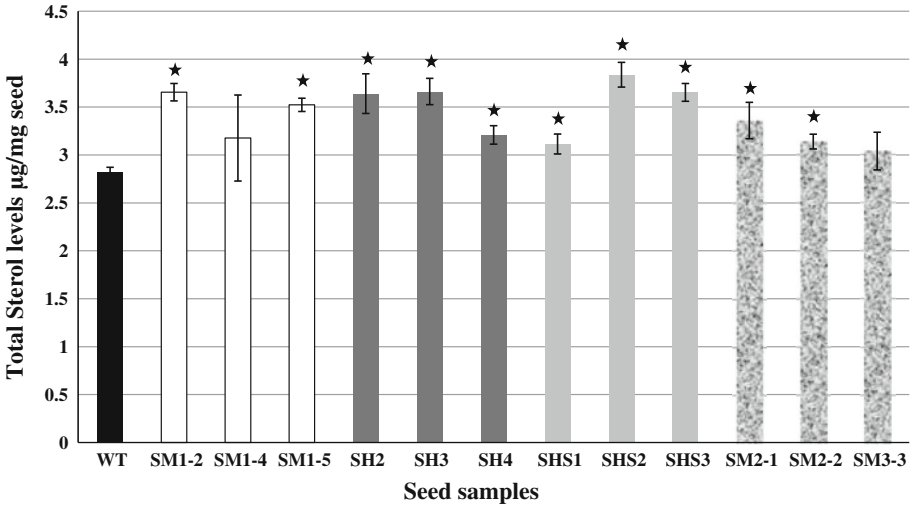

Fig. 5 Changes in the total sterol content in transgenic seeds. *Arabidopsis* seeds were analyzed for the total sterol content (sitosterol, campesterol, stigmasterol and cycloartenol) by GC-FID and the values for three independent events are depicted in the *graph*. The construct abbreviations are as follows: SM1-Seed-specific *GmSMT1* over-expression; SH-Seed-specific *GmSMT1* and *AtHMGR1* over-expression; SHS-Seed-specific *GmSMT*, *AtHMGR1* and *GmSMT2-2* over-expression; SM2: Seed-specific *GmSMT2-2* over-expression. The *error bars* represent standard error for three biological replicates. Statistically significant differences based on unpaired Students's *t* test at $P < 0.05$ are denoted by *asteriks*

to 85% in SHS as against 78% of the sterol pool in wild-type, at the expense of campesterol, which declined up to 14% in SHS as compared to 20% in wild-type (Fig. 6). The proportion of cycloartenol was around 1.8% of the total sterols in wild-type, 1.3% in SH, 0.8% in SM1, 1% in SM2 and 0.6% in SHS seeds. On an average, the levels of beneficial C-24 ethyl sterols increased by 49% in SHS-2 as against SM2-1 seeds, where the increase is only 22%, as compared to the wild-type. Our data indicates that *SMT2* genes do seem to have an effect on fine tuning the end-product C-24 ethyl sterol levels and engineering multiple rate-limiting steps leading to more efficient steering of metabolic flux downstream towards the sterol pathway.

Cis-acting elements in *SMT2* upstream sequences

The 5′ flanking sequences of the *SMT2* genes were cloned by adaptor mediated PCR based genome walking strategy and the sequences were analyzed for the presence of putative *cis*-acting motifs responsible for basal and regulated expression. The sequence lengths were 591 and 1,877 bp for the *GmSMT2-1* and *GmSMT2-2* respectively, with reference to the start codon, ATG. A number of *cis*-acting elements were identified based on homology to previously characterized regulatory motifs in plants as documented in the PLACE and PLANTCARE databases and are summarized in the Table 1. Many of these potential functional motifs were conserved in sequence and position for the two soybean *SMT2* genes, which include the ABRE elements (CACGTG) for ABA responsiveness, ARE elements (TGGTTT) for anaerobic induction, MBS motifs

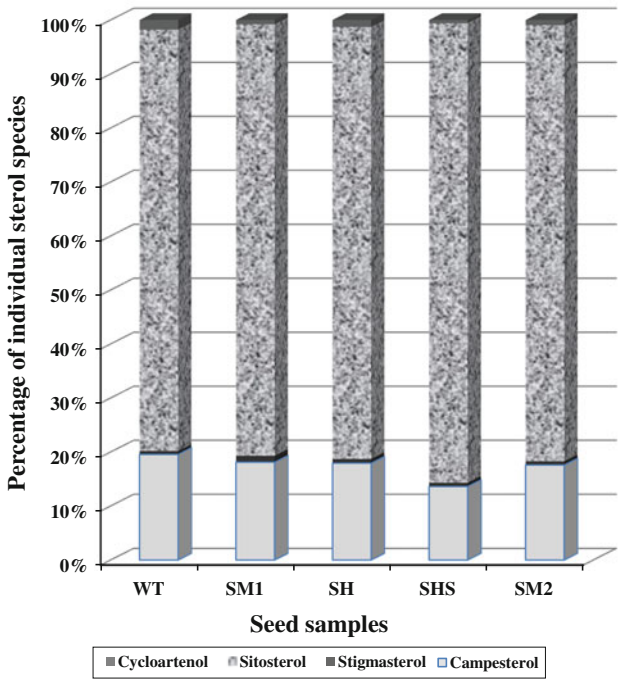

Fig. 6 Sterol composition in the genetically modified seeds. The relative proportion of individual sterol species in the total pool is represented *graphically* for the Arabidopsis seeds of different transgenic constructs, SM (Seed-specific *GmSMT1* over-expression), SH (Seed-specific *GmSMT1* and *AtHMGR1* over-expression), SHS (Seed-specific *GmSMT*, *AtHMGR1* and *GmSMT2-2* over-expression), and SM2 (Seed-specific *GmSMT2-2* over-expression)

(C/TAACTG) for drought responsiveness, LTRECORE (CCGAC) for low temperature responsive expression, and Skn-1 motif (GTCAT) for endosperm expression (Table 1;

Fig. 7). Furthermore, multiple regulatory elements for light responsive expression like GT1-motif, GA-motif, GAG-motif, I box, G box, ATCT-motif, etc. were identified (data not shown), along with several E box/MYC consensus sequences (CANNTG), which are characteristic sequence motifs for ABA and cold responsiveness. The cloned *SMT2-2* gene promoter has putative *cis*-elements like TGA-element (AACGAC) for auxin responsiveness, TATC-box (TATCCCA) for gibberellin responsiveness, T/G box (AACGTG) for induction by Jasmonic acid, and RY repeat or legumin box (GTGCATG) commonly found in legume storage protein gene promoters that are responsible for high levels of expression in seeds (Table 1; Supplementary Fig. 2). The analysis of these putative regulatory motifs suggests a potentially complex transcriptional regulation for the two *SMT-2* genes, which involves both shared and unique elements for modulating the gene expression.

Analysis of predicted transcription factor binding sites in the *SMT2-2* promoter sequence was also performed as summarized in Table 2. Binding sites for regulatory proteins belonging to HD-ZIP, bHLH, MADS box, Myb, Dof, AP2 domain, etc., were predicted by in silico analysis as summarized in Table 2. The presence of such diverse potential transcription factor binding sequence motifs based on homology to functional elements in heterologous systems indicate the significance of complex transcriptional regulation of this gene family in soybean.

Developmental and inducible regulation of *SMT2* promoter in transgenic Arabidopsis

In an effort to characterize the spatial, temporal and inducible transcriptional regulation, the *SMT2* promoter sequences were cloned upstream to the *β-glucuronidase* reporter gene to generate transcriptional fusions. These constructs were used to transform Arabidopsis wild-type plants by the floral dip method. The transgenic plants harboring the *SMT2-1* promoter showed GUS expression predominantly in the cotyledons and the base of the hypocotyls in seedlings, and the tips of young leaves. Transgenic plants with the *SMT2-2* promoter had a similar expression pattern, but with strong GUS expression throughout the leaves and stem, specifically in the vascular tissue, in both etiolated and light-grown plants (Fig. 8a-A–F). Figure 8a-G represents control plants with constitutive GUS expression driven by the *CaMV35S* promoter. Furthermore, *SMT2-2* expression was found prominently in the membrane adjacent to the inner walls of the guard cells of the stomata in leaves (Fig. 8a-H). This is also in agreement with similar studies on the squalene epoxygenase gene (Posé et al. 2009), acting upstream in the pathway and reportedly expressing in guard cells and affecting stomatal response features. In 7-day and 14-day-old seedlings, GUS expression was found in the older leaves and tips of younger leaves for *SMT2-1:GUS* (G1) plants whereas strong uniform expression was observed throughout the shoot in the case of G2 (*SMT2-2:GUS*) seedlings (Fig. 8b-I, J, M, N). At the reproductive stage plants, *SMT2-1* promoter controlled GUS was found to express strongly in the anthers, with a very low expression in petals, whereas GUS expression governed by the *SMT2-2* promoter was observed throughout the floral tissues, including stamen, petals, sepals, and stigma and in the developing pods at the receptacle and the apical region (Fig. 8b-K, L, O, P).

Although no detectable levels of GUS were found in roots of 3-day-old seedlings, the expression could be induced in the elongation zone of the root by exogenous supply of auxin in *SMT2-2* transgenic plants (Fig. 8c-Q–T). Our finding is consistent with studies of *SMT2* orthologues and related sterol genes in model systems (Carland et al. 2002; Posé et al. 2009). In order to study the treatment effects, the 14-day-old seedlings of *SMT2-2: GUS* transgenic plants were subjected to dehydration for different time periods to monitor the changes in expression patterns. A down-regulation of expression was observed as expected based on the transcript assay, spatially as visualized histochemically. In the water controls, the shoot had uniform distribution of GUS at different time points. After 3 h of dehydration, GUS expression could be detectable only in portions of veins towards the leaf apex, meristematic region and the base of the stem (Fig. 8c-U, V) and it progressed at 5 h time point as well (Fig. 8c-W).

Discussion

The ability to synthesize C-24 alkyl sterols and more specifically C-24α ethyl sterols is unique to the plant kingdom (Neelakandan et al. 2009). The soybean genome has a pair of *SMT2* genes encoding active enzymes as described in our earlier study (Neelakandan et al. 2009) and also based on the recently released soybean genome annotation (Schmutz et al. 2010). In the present study, we have characterized the soybean *SMT2* genes responsible for encoding enzymes catalyzing the first committed step in the final segment of the pathway leading to sitosterol biosynthesis. Furthermore, transgenic tissue-specific overexpression of *SMT2-2* gene in Arabidopsis provided novel insights on the regulation of partitioning of brassinosteroid precursors and membrane sterols in seeds.

In this study, our first objective was to analyze the transcriptional regulation of *SMT2* genes, in soybean seedlings with respect to environmental cues and signal molecules. It has been proposed that the ability of plants to synthesize sterols with branched ethyl groups (as in sitosterol and stigmasterol) might be part of an evolutionary adaptation

Table 1 Predicted *cis* elements in the soybean SMT2 promoters

Cis-element	Motif	Position	Attributed function	Reference	Database
SMT2-1					
TATA box	TAATA	-196, -129 (+), -132 (−)	Basal transcription	Deckert and Gresshoff (unpublished)	PLANTCARE
CAAT box	CCAAT	-153, -375 (+)	Basal transcription	Yamaguchi-Shinozaki and Shinozaki (1993)	PLANTCARE
ABRE	CACGTG	-432 (+)	Abscisic acid (ABA) and drought responsiveness	Baker et al. (1994)	PLANTCARE
ARE	TGGTTT	-149 (+), -378 (−)	Essential for anaerobic induction	Manjunath and Sachs (1997)	PLANTCARE
GT-1 motif	GGTTAA	-116 (−)	Light responsiveness	Vauterin et al. (1999)	PLANTCARE
Skn-1 motif	GTCAT	-549 (−)	Endopserm expression	Takaiwa et al. (1991)	PLANTCARE
MBS	CAACTG	-459 (+)	MYB binding site for drought inducibility	Yamaguchi-Shinozaki and Shinozaki (1993)	PLANTCARE
E box/MYC CONSENSUS	CANNTG	-340, -349, -432, -459, -552 (+) and (−)	ABA and Cold inducibility	Stalberg et al. (1996), Chinnusamy et al. (2003), and Abe et al. (2003)	PLACE
Anaero1consensus	AAACAAA	-58 (+), -307 (−)	Anaerobic inducibility	Mohanty et al. (2005)	PLACE
LTRECORE	CCGAC	-160 (−)	Core of low temperature responsive element	Baker et al. (1994)	PLACE
SMT2-2					
TATA box	TAATA	-124, -200, -269 (+)	Basal transcription	Deckert and Gresshoff (unpublished)	PLANTCARE
CAAT box	CAATT	-293, -219 (+)	Basal transcription	Deckert and Gresshoff (unpublished)	PLANTCARE
ABRE	CACGTG	-481, -1453 (+)	Abscisic acid (ABA) and drought responsiveness	Baker et al. (1994)	PLANTCARE
ARE	TGGTTT	-398 (+)	Essential for anaerobic induction	Manjunath and Sachs (1997)	PLANTCARE
GT-1 motif	GGTTAA	-1620 (−)	Light responsiveness	Vauterin et al. (1999)	PLANTCARE
GCN4 motif	TGTGTCA	-1567 (−)	Endosperm expression	Kim and Wu (1990)	PLANTCARE
Skn-1 motif	GTCAT	-614, -1568 (−)	Endosperm expression	Takaiwa et al. (1991)	PLANTCARE
HSE	AAAAAATTTC	-686, -739, -855, -1001 (+)	Heat stress responsiveness	Pastuglia et al. (1997)	PLANTCARE
MBS	T/CAACTG	-509, 1391 (+)	MYB binding site for drought inducibility	Yamaguchi-Shinozaki and Shinozaki (1993)	PLANTCARE
TGA-element	AACGAC	-101 (−)	Auxin responsiveness	Pastuglia et al. (1997)	PLANTCARE
SURECORE	GAGAC	-1194 (+)	Auxin Response Factor (ARF) binding sequence of Sulfur responsive element (SURE) core	Maruyama-Nakashita et al. (2005)	PLACE
Circadian	CAANNNNATC	-1686 (+)	Circadian control	Pichersky et al. (1985)	PLANTCARE
Evening element	AAAATATCT	-853, -737 (+)	Circadian control	Harmer et al. (2000)	PLACE
T/GBOX	AACGTG	-451, -1116 (−)	JA inducibility	Boter et al. (2004)	PLACE
E box/MYC CONSENSUS	CANNTG	-481, -508, -617, -1233, -1453, -1575 (+) and (−)	ABA and Cold inducibility	Stalberg et al. (1996), Chinnusamy et al. (2003), and Abe et al. (2003)	PLACE
Anaero2consensus	AGCAGC	-465, -468 (+)	Anaerobic inducibility	Mohanty et al. (2005)	PLACE
RY repeat/Legumin box	GTGCATG	-1572 (+)	Seed-specific regulation	Fujiwara and Beachy (1994)	PLACE
LTRECORE	CCGAC	-409 (−)	Core of low temperature responsive element	Baker et al. (1994)	PLACE

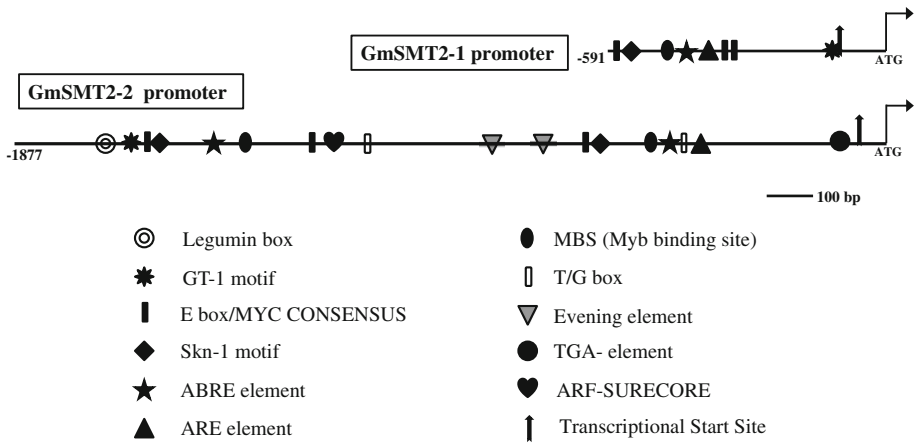

Fig. 7 *Cis*-acting elements predicted in the soybean *SMT2* sequences. The relative position and organization of putative *cis* acting functional motifs predicted computationally are indicated

process to cope up with wider temperature fluctuations, and to maintain the essential membrane associated metabolic processes, as compared to animals (Dufourc 2008). The *SMT2* genes catalyze the first committed step towards the synthesis of C-24 ethyl sterols. The cold induction of *SMT2* genes, therefore, seems likely to have a function in re-organizing sterol biosynthesis, to promote cold acclimation of membranes. The cold and dehydration regulated *cis*-acting elements (e.g. E box, LTRECORE, MBS) found conserved in the upstream sequences of the two *SMT2* genes could be implicated to have a role in this response. The ABRE sequences are also present in the *SMT2* gene promoters and they are possibly involved in the observed weak ABA responsiveness of the *SMT2* genes.

The first committed step in the isoprenoid biosynthesis reactions catalyzed by HMGR and the C-24 methyl transfer reaction catalyzed by SMT1 have been clearly elucidated to be two crucial control points in regulating sterol accumulation in plant systems (Chappell et al. 1995; Harker et al. 2003; Holmberg et al. 2002, 2003; Schaeffer et al. 2000). The potential role of *GmSMT2-2* gene in sterol synthesis and allocation of the campesterol: sitosterol ratio in seeds was investigated for the first time in transgenic Arabidopsis. BR-deficient mutants are not known to have embryogenic defects (Clouse 2000, 2002) and hence seed-specific over-expression of *SMT2* gene was undertaken as a logical approach to test the effect of this gene in elevating the levels of C-24 ethyl sterols. The transgenic seeds with seed-specific over-expression of *SMT2* gene and combinations displayed higher sitosterol and reduced cycloartenol levels. As observed, campesterol levels seem to be tightly regulated in seed tissues, since the R ratios were not drastically altered as compared to observations in vegetative tissues as a result of constitutive over-expression in earlier studies (Schaeffer et al. 2001).

In order to shed additional light on the transcriptional regulation of plant sterol genes, we have explored the nature of *cis* elements and transcription factor binding sites in the upstream sequences of *SMT2* genes. In humans and yeast, sterol response elements (SRE) have been identified as conserved *cis*-acting motifs in the upstream sequences of cholesterol and ergosterol biosynthesis genes respectively, to which the SRE binding protein (SREBP) binds and regulates sterol biosynthesis (Goldstein et al. 2002; Vik and Rine 2001). SREBPs comprise of a family of helix-loop-helix-leucine zipper membrane bound transcription factors, and they act as an oxygen sensor in *S pombe* (Hughes et al. 2005). Similarly, in the budding yeast, two activators of ergosterol pathway, Upc2p and Ecm22p, activate many of the ergosterol biosynthetic genes mediated by SRE elements. Furthermore, the ergosterol biosynthetic genes are also reportedly induced by hypoxia (Davies and Rine 2006). Plants appear to be devoid of protein homologues of mammalian and yeast sterol regulators. However, the presence of anaerobic response elements in *SMT2* promoters is interesting and points towards possible conserved modules in the regulation of phytosterol biosynthesis. Recently, a START domain family protein, PYL1, was identified as a receptor for ABA, a member of the terpenoid family, mediating ABA signaling (Miyazono et al. 2009). Our in silico analysis of the *SMT2-2* promoter revealed the presence of several putative transcription factor binding sites including the HD-START protein (ATHB-9), and this would be worth investigating in the future.

Subsequently, the in planta functionality and regulation of soybean *SMT2* genes were investigated in detail in transgenic Arabidopsis. Our data on mutant complementation and promoter analysis clearly shows that the two *SMT2* genes are functional, encoding catalytically active enzymes, and the promoters are capable of activating the transcription of reporter gene in heterologous plant systems, with shared and unique expression features. The RY repeat or legumin box found in legume storage protein gene promoters (Fujiwara and Beachy 1994) and GCN4 motif (Kim and Wu 1990) are present in the upstream sequence of *GmSMT2-2* and they are believed to be

Table 2 Predicted transcription factor binding sites in the GmSMT2-2 promoter sequence

Transcription factor	Family	Consensus sequence	Predicted binding site	Location	Species	Database	Function	Reference
Agamous	MADS	CCWWWWNNRGH	CCAAAATAAGT	-81 (+)	Arabidopsis	JASPER	Floral homeotic gene	Huang et al. (1993)
ABI4	AP2 domain	SYGCYYYY	GGAAGCAG	-471 (+)	Maize	JASPER	Binds coupling element1 in abscisic acid and sugar response genes	Niu et al. (2002)
LIM1	LIM	CCACCANMNNCN	tgcttgtGGTGG	-1229 (+)	Tobacco	JASPER	Pal box binding protein involved in the regulation of phenylpropanoid biosynthesis genes	Kawaoka et al. (2000)
ATMYC2	bHLH	CACATG	CACATG	-1233 (+)	Arabidopsis	AGRIS	Drought and ABA regulated gene expression	Abe et al. (1997)
ATHB-2	HD-ZIP	TAATMATTA	TAATTATTA	-832 (+)	Arabidopsis	PLACE	HD-ZIPII family proteins, mediate light signals in plant morphogenesis during shade avoidance responses	Ohgishi et al. (2001)
ATHB-9	HD-ZIP	NNNNGTAATGATTRCNYBS	atattttATGATggctttt	-567 (+)	Arabidopsis	TRANSFAC	Homeobox transcription factor belonging to the small family of HD-ZIPIII proteins, containing a START domain	Sessa et al. (1998)
			taatgctATCATtacttat	-1860 (+)				
			aaaaaatATCATttaagat	-686 (+)				
BIHD1OS	HD	TGTCA	TGACA	-1567 (+)	Rice	PLACE	Binding site of OsBIHD1, a rice BELL homeodomain transcription factor;	Luo et al. (2005)
MYB2AT	MYB	TAACTG	TAACTG	-1394 (+)	Arabidopsis	PLACE	Regulation of genes that are responsive to water stress	Urao et al. (1993)
PIF3	bHLH	GKRGGMCACGTGRMSWCK	acaattCACGTgaagaga	-1459 (+)	Arabidopsis	TRANSFAC	Act in phytochrome signalling pathways	Martínez-García et al. (2000)
			aaaagaCACGTtttttag	-1122 (+)				
			gcggagCACGTgaaccgg	-487 (+)				
			tcgatcCACGTtccgcaa	-459 (+)				
SEF4		RTTTTTR	GTTTTTA	-745, -1254 (+)	Soybean	PLACE	Positive transcriptional regulator of seed storage proteins	Allen et al. (1989)
			ATTTTTA	-291, -311, -666, -1782 (+), -1202 (−)				
SEF3		AACCCA	AACCCA	-1006 (+)	Soybean	PLACE	Positive transcriptional regulator of seed storage proteins	Allen et al. (1989)
Dof3	Dof	AAAGYV	GACTTT	-612, -1071, -1516 (+)	Maize	JASPER	Associated with diverse plant-specific phenomena including light, phytohormone and defense responses, seed development and germination	Yanagisawa and Schmidt (1999)

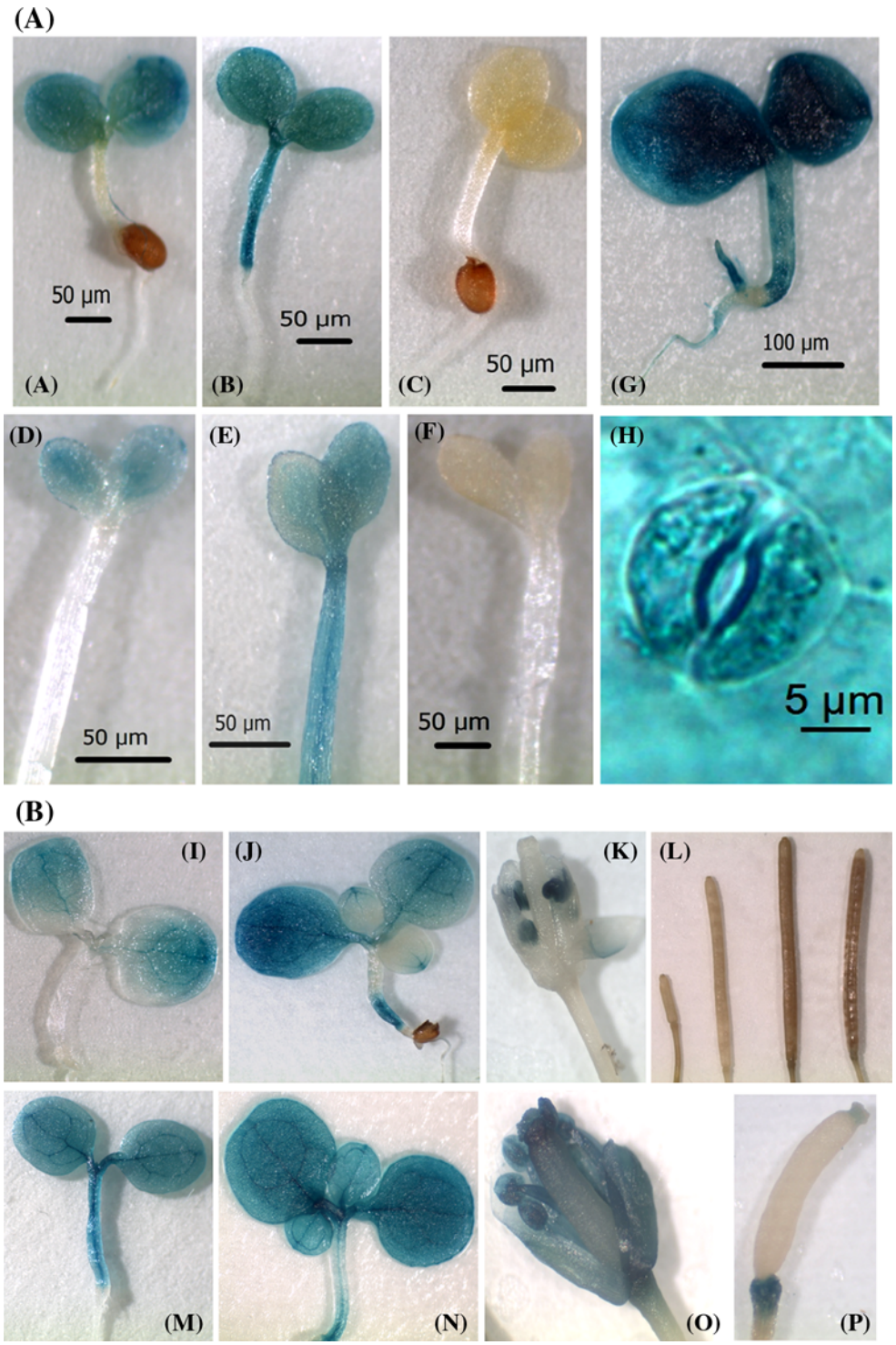

Fig. 8 Visualization of expression patterns of *SMT2 promoter: GUS* fusions. **a** has panels *A–C* that represent 3-day-old light-grown, and *D–F* which represent the corresponding etiolated seedlings, of G1 (*SMT2-1p:GUS*), G2 (*SMT2-2p:GUS*) and wild-type plants. *G* denotes seedlings with constitutive *35S* promoter driven GUS expression. *H* shows the close up of the cotyledon GUS expression showing prominent expression in the guard cells of the stomata. **b** contains panels *I–P*, which depicts GUS expression analysis of older seedlings and reproductive stage plants. *I–L* represents G1 and *M–P* shows G2 transgenic Arabidopsis plants. *I* and *M*: 7-day-old seedling; *J* and *N*: 14-day-old seedlings; *K* and *O*: flower; *L* and *P*; pods. **c** with the panels *Q–W* shows the analysis of auxin response and dehydration of G2 seedlings. *Q* to *T* demonstrates the results of auxin treatment on GUS expression in the roots of G2 plants. *Q* and *S* denote the seedling and root regions of water control plants and *R* and *T* represents the corresponding auxin treated samples. *U* represents the control and *V* and *W* denote plants subjected to dehydration stress or 3 and 5 h, respectively

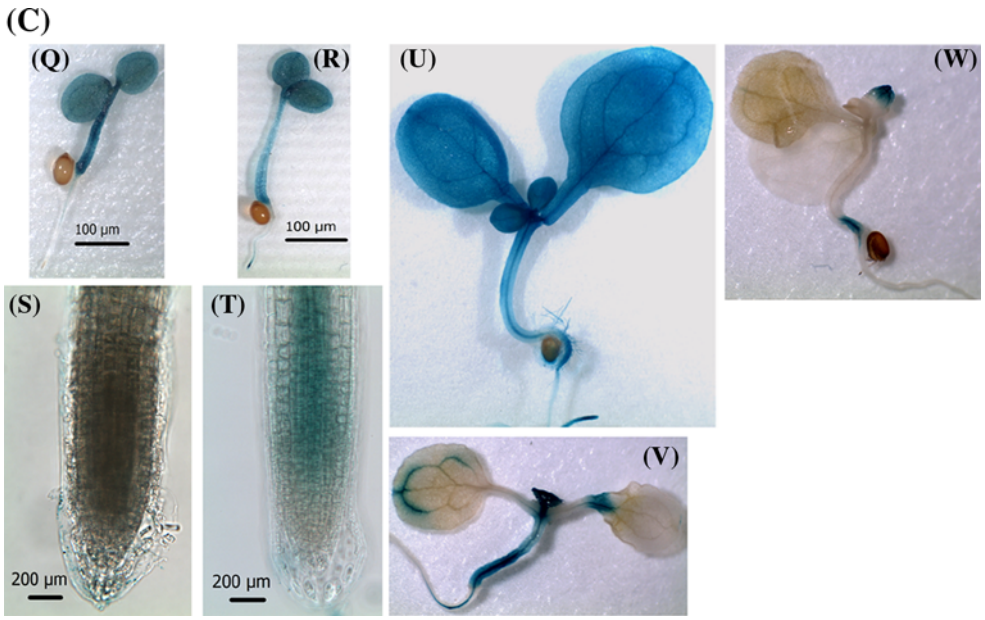

Fig. 8 continued

responsible for the high level of expression of this gene in seed tissues, as observed in our previous studies (Table 1; Supplementary Fig. 2). The observed auxin inducibility in *SMT2-2: GUS* plants indicate a functional auxin response element as predicted in silico. The fact that *SMT2-2* directed GUS expression was detected in guard cells is also significant, since recent studies on the drought hypersensitive/squalene epoxidase mutant *dry2/sqe1-5*, indicate a crucial role of sterols in the proper localization of NADPH oxidase protein in membranes (that are required for the efficient production of reactive oxygen species), in ABA mediated stomatal response characteristics, root growth, and in drought tolerance (Posé et al. 2009). The histochemical analysis of reporter expression in *SMT2-2: GUS* plants under dehydration also indicates spatial and differential transcriptional regulation, consistent with our data on soybean transcript studies.

In conclusion, our study has demonstrated the in planta functionality of soybean *SMT2* genes, the expression features with respect to environmental stimuli, and the utility of combinatorial engineering of sterol biosynthetic pathway for improved beneficial C24-ethyl sterol end-products. We surmise that the upstream sequences of soybean *SMT2* genes offer an opportunity towards a more precise study of sterol homeostasis in different environmental conditions and developmental stages. Future research on analysis of functional *cis*-elements and corresponding *trans* acting factors are bound to shed more light on the regulation of pathway towards membrane sterols and co-ordination of the sterol biosynthesis in plants.

Acknowledgments The support of the Missouri Soybean Merchandising Council (Project # 04-259) to H.T.N. and National Science Foundation (MCB-0920212) to W.D.N. is gratefully acknowledged. We would like to thank Drs. Tim Nelson and Francine Carland (Yale University, New Haven, CT) for the *cvp1-3* mutant seeds of Arabidopsis, Dr. Edgar Cahoon (University of Nebraska, Lincoln) for kindly providing the sub-cloning vectors, BetaConsoyHyg, pKMS3, the C58 strain of *Agrobacterium* and the binary vector, pZY101Asc, Dr. Zhanyuan Zhang for the pCAMBIA3301 vector and Dr. Bing Stacey and Prof. Gary Stacey (University of Missouri, Columbia) for the p35S: GUS Arabidopsis seeds and for the pPhas-Gly vector used in the study. We are grateful to Dr. Esteban Fernandez (Molecular Cytology Core, University of Missouri, Columbia) for help with imaging, Drs. Richard Jeannotte (Kansas Lipidomics Research Center), Babu Valliyodan and Lakshmi Praba Manavalan (University of Missouri, Columbia) for helpful suggestions. The nucleotide sequences of the upstream promoter sequence of soybean *SMT2-1* and *SMT2-2* genes have been submitted in Genbank and the accession numbers are HM185389 and HM185390, respectively.

References

Abe H, Yamaguchi-Shinozaki K, Urao T, Iwasaki T, Hosokawa D, Shinozaki K (1997) Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. Plant Cell 9:1859–1868

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell 15:63–78

Allen RD, Bernier F, Lessard PA, Beachy RN (1989) Nuclear factors interact with a soybean beta-conglycinin enhancer. Plant Cell 1:623–631

Bach TJ (1986) Hydroxymethylglutaryl-CoA reductase, a key enzyme in phytosterol synthesis? Lipids 21:82–88

Baker SS, Wilhelm KS, Thomashow MF (1994) The 5′-region of *Arabidopsis thaliana cor15a* has *cis*-acting elements that confer cold-, drought- and ABA-regulated gene expression. Plant Mol Biol 24:701–713

Beck JG, Mathieu D, Loudet C, Buchoux S, Dufourc EJ (2007) Plant sterols in "rafts": a better way to regulate membrane thermal shocks. FASEB J 21:1714–1723

Benveniste P (2004) Biosynthesis and accumulation of sterols. Annu Rev Plant Biol 55:429–457

Boter M, Ruiz-Rivero O, Abdeen A, Prat S (2004) Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and Arabidopsis. Genes Dev 18:1577–1591

Bouvier-Navé P, Husselstein T, Desprez T, Benveniste P (1997) Identification of cDNAs encoding sterol methyl-transferases involved in the second methylation step of plant sterol biosynthesis. Eur J Biochem 246:518–529

Bouvier-Navé P, Husselstein T, Benveniste P (1998) Two families of sterol methyltransferases are involved in the first and the second methylation steps of plant sterol biosynthesis. Eur J Biochem 256:88–96

Carland FM, Fujioka S, Takatsuto S, Yoshida S, Nelson T (2002) The identification of *CVP1* reveals a role for sterols in vascular patterning. Plant Cell 14:2045–2058

Carrington JC, Freed DD (1990) Cap-independent enhancement of translation by a plant potyvirus 5′ nontranslated region. J Virol 64:1590–1597

Chang WC, Lee TY, Huang HD, Huang HY, Pan RL (2008) PlantPAN: plant promoter analysis navigator, for identifying combinatorial *cis*-regulatory elements with distance constraint in plant gene group. BMC Genomics 9:561–574

Chappell J, Wolf F, Proulx J, Cuellar R, Saunders C (1995) Is the reaction catalyzed by 3-hydroxy-3-methylglutaryl Coenzyme A reductase a rate-limiting Step for isoprenoid biosynthesis in plants? Plant Physiol 109:1337–1343

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. Genes Dev 17:1043–1054

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Clouse SD (2000) Plant development: a role for sterols in embryogenesis. Curr Biol 10:R601–R604

Clouse SD (2002) Brassinosteroid signal transduction: clarifying the pathway from ligand perception to gene expression. Mol Cell 10:973–982

Davies BJ, Rine J (2006) A role for sterol levels in oxygen sensing in *Saccharomyces cerevisiae*. Genetics 174:191–201

Deckert J, Gresshoff PM (Unpublished) Soybean cyclin gene structure and expression during nodulation. Genbank Accession number Z26331

Diener AC, Li H, Zhou W-X, Whoriskey WJ, Nes WD, Fink GR (2000) STEROL METHYLTRANSFERASE 1 controls the level of cholesterol in plants. Plant Cell 12:853–870

Dufourc EJ (2008) The role of phytosterols in plant adaptation to temperature. Plant Signal Behav 3:133–134

Fujiwara T, Beachy RN (1994) Tissue-specific and temporal regulation of a beta-conglycinin gene: roles of the RY repeat and other *cis*-acting elements. Plant Mol Biol 24:261–272

Goldstein JL, Rawson RB, Brown MS (2002) Mutant mammalian cells as tools to delineate the sterol regulatory element-binding protein pathway for feedback regulation of lipid synthesis. Arch Biochem Biophys 397:139–148

Harker M, Holmberg N, Clayton JC, Gibbard CL, Wallace AD, Rawlins S, Hellyer SA, Lanot A, Safford R (2003) Enhancement of seed phytosterol levels by expression of an N-terminal truncated *Hevea brasiliensis* (rubber tree) 3-hydroxy-3-methylglutaryl-CoA reductase. Plant Biotechnol J 1:113–121

Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay SA (2000) Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science 290:2110–2113

Hartmann MA (1998) Plant sterols and the membrane environment. Trends Plant Sci 3:170–175

Hase Y, Tanaka A, Baba T, Watanabe H (2000) FRL1 is required for petal and sepal development in Arabidopsis. Plant J 24: 21–32

Hase Y, Fujioka S, Yoshida S, Sun G, Umeda M, Tanaka A (2005) Ectopic endoreduplication caused by sterol alteration results in serrated petals in Arabidopsis. J Exp Bot 56:1263–1268

Hemmerlin A, Gerber E, Feldtrauer JF, Wentzinger L, Hartmann MA, Tritsch D, Hoeffler JF, Rohmer M, Bach TJ (2004) A review of tobacco BY-2 cells as an excellent system to study the synthesis and function of sterols and other isoprenoids. Lipids 39:723–735

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database. Nucleic Acids Res 27:297–300

Holmberg N, Harker M, Gibbard CL, Wallace AD, Clayton JC, Rawlins S, Hellyer A, Safford R (2002) Sterol C-24 methyltransferase type 1 controls the flux of carbon into sterol biosynthesis in tobacco seed. Plant Physiol 130:303–311

Holmberg N, Harker M, Wallace AD, Clayton JC, Gibbard CL, Safford R (2003) Co-expression of N-terminal truncated 3-hydroxy-3-methylglutaryl CoA reductase and C24-sterol methyltransferase type 1 in transgenic tobacco enhances carbon flux towards end-product sterols. Plant J 36:12–20

Huang H, Mizukami Y, Hu Y, Ma H (1993) Isolation and characterization of the binding sequences for the product of the Arabidopsis floral homeotic gene AGAMOUS. Nucleic Acids Res 21:4769–4776

Hughes AL, Todd BL, Espenshade PJ (2005) SREBP pathway responds to sterols and functions as an oxygen sensor in fission yeast. Cell 120:831–842

Jayasimha P, Bowman CB, Pedroza JM, Nes WD (2006) Engineering pathway enzymes to understand the function and evolution of sterol structure and activity. Rec Adv Phytochem 40:211–251

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Kawaoka A, Kaothien P, Yoshida K, Endo S, Yamada K, Ebinuma H (2000) Functional analysis of tobacco LIM protein Ntlim1 involved in lignin biosynthesis. Plant J 22:289–301

Kim SY, Wu R (1990) Multiple protein factors bind to a rice glutelin promoter region. Nucleic Acids Res 18:6845–6852

Lescot M, Déhais P, Moreau Y, De Moor B, Rouzé P, Rombauts S (2002) PlantCARE: a database of plant *cis*-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res 30:325–327

Luo H, Song F, Goodman RM, Zheng Z (2005) Up-regulation of OsBIHD1, a rice gene encoding BELL homeodomain transcriptional factor, in disease resistance responses. Plant Biol (Stuttg) 7:459–468

Luo M, Tan K, Xiao Z, Hu M, Liao P, Chen K (2008) Cloning and expression of two sterol C-24 methyltransferase genes from upland cotton (*Gossypium hirsuturm* L.). J Genet Genomics 35:357–363

Manjunath S, Sachs MM (1997) Molecular characterization and promoter analysis of the maize cytosolic glyceraldehyde 3-phosphate dehydrogenase gene family and its expression during anoxia. Plant Mol Biol 33:97–112

Martínez-García JF, Huq E, Quail PH (2000) Direct targeting of light signals to a promoter element-bound transcription factor. Science 288:859–863

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Inoue E, Yamaya T, Takahashi H (2005) Identification of a novel *cis*-acting element conferring sulfur deficiency response in Arabidopsis roots. Plant J 42:305–314

Miyazono K, Miyakawa T, Sawano Y, Kubota K, Kang H-J, Asano A, Miyauchi Y, Takahashi M, Zhi Y, Fujita Y, Yoshida T, Kodaira K-S, Yamaguchi-Shinozaki K, Tanokura M (2009) Structural basis of abscisic acid signaling. Nature 462:609–614

Mohanty B, Krishnan SP, Swarup S, Bajic VB (2005) Detection and preliminary analysis of motifs in promoters of anaerobically induced genes of different plant species. Ann Bot (Lond) 96: 669–681

Neelakandan AK, Song Z, Wang J, Richards MH, Wu X, Valliyodan B, Nguyen HT, Nes WD (2009) Cloning, functional expression and phylogenetic analysis of plant sterol 24C-methyltransferases involved in sitosterol biosynthesis. Phytochemistry 70:1982–1998

Nes WD (2000) Sterol methyltransferase: enzymology and inhibition. Biochim Biophys Acta 1529:63–88

Nes WD, Venkatramesh M (1999) Enzymology of phytosterol transformations. Crit Rev Biochem Mol Biol 34:81–93

Nieto B, Forés O, Arró M, Ferrer A (2009) 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. Phytochemistry 70:53–59

Niu X, Helentjaris T, Bate NJ (2002) Maize ABI4 binds coupling element1 in abscisic acid and sugar response genes. Plant Cell 14:2565–2575

Ohgishi M, Oka A, Morelli G, Ruberti I, Aoyama T (2001) Negative autoregulation of the Arabidopsis homeobox gene *ATHB-2*. Plant J 25:389–398

Pastuglia M, Roby D, Dumas C, Cock JM (1997) Rapid induction by wounding and bacterial infection of an S gene family receptor-like kinase in *Brassica oleracea*. Plant Cell 9:1–13

Pichersky E, Bernatzky R, Tanksley SD, Breidenbach RB, Kausch AP, Cashmore AR (1985) Molecular characterization and genetic mapping of two clusters of genes encoding chlorophyll *a/b*-binding proteins in *Lycopersicon esculentum* (tomato). Gene 40:247–258

Posé D, Castanedo I, Borsani O, Nieto B, Rosado A, Taconnat L, Ferrer A, Dolan L, Valpuesta V, Botella MA (2009) Identification of the Arabidopsis *dry2/sqe1-5* mutant reveals a central role for sterols in drought tolerance and regulation of reactive oxygen species. Plant J 59:63–76

Rohmer M (2008) From molecular fossils of bacterial hopanoids to the formation of isoprene units: discovery and elucidation of the methylerythritol phosphate pathway. Lipids 43:1095–1107

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Schaeffer A, Bouvier-Navé P, Benveniste P, Schaller H (2000) Plant sterol-C24-methyl transferases: different profiles of tobacco transformed with SMT1 or SMT2. Lipids 35:263–269

Schaeffer A, Bronner R, Benveniste P, Schaller H (2001) The ratio of campesterol to sitosterol that modulates growth in Arabidopsis is controlled by STEROL METHYLTRANSFERASE 2;1. Plant J 25:605–615

Schaller H, Grausem B, Benveniste P, Chye ML, Tan CT, Song YH, Chua NH (1995) Expression of the *Hevea brasiliensis* (H.B.K.) Mull. Arg. 3-hydroxy-3-methylglutaryl-Coenzyme A reductase 1 in tobacco results in sterol overproduction. Plant Physiol 109:761–770

Schaller H, Bouvier-Navé P, Benveniste P (1998) Overexpression of an Arabidopsis cDNA encoding a sterol-C24(1)-methyltransferase in tobacco modifies the ratio of 24-methyl cholesterol to sitosterol and is associated with growth reduction. Plant Physiol 118:461–469

Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J, Xu D, Hellsten U, May GD, Yu Y, Sakurai T, Umezawa T, Bhattacharyya MK, Sandhu D, Valliyodan B, Lindquist E, Peto M, Grant D, Shu S, Goodstein D, Barry K, Futrell-Griggs M, Abernathy B, Du J, Tian Z, Zhu L, Gill N, Joshi T, Libault M, Sethuraman A, Zhang XC, Shinozaki K, Nguyen HT, Wing RA, Cregan P, Specht J, Grimwood J, Rokhsar D, Stacey G, Shoemaker RC, Jackson SA (2010) Genome sequence of the palaeopolyploid soybean. Nature 463:178–183

Sessa G, Steindler C, Morelli G, Ruberti I (1998) The Arabidopsis *Athb-8*, *-9* and *-14* genes are members of a small gene family coding for highly related HD-ZIP proteins. Plant Mol Biol 38:609–622

Shi J, Gonzales RA, Bhattacharyya MK (1996) Identification and characterization of an *S*-adenosyl-L-methionine Δ24-sterol C-methyltransferase cDNA from soybean. J Biol Chem 271: 9384–9389

Sitbon F, Jonsson L (2001) Sterol composition and growth of transgenic tobacco plants expressing type-1 and type-2 sterol methyltransferases. Planta 212:568–572

Stalberg K, Ellerstom M, Ezcurra I, Ablov S, Rask L (1996) Disruption of an overlapping E-box/ABRE motif abolished high transcription of the napA storage-protein promoter in transgenic *Brassica napus* seeds. Planta 199:515–519

Takaiwa F, Oono K, Wing D, Kato A (1991) Sequence of three members and expression of a new major subfamily of glutelin genes from rice. Plant Mol Biol 17:875–885

Tran LS, Quach TN, Guttikonda SK, Aldrich DL, Kumar R, Neelakandan A, Valliyodan B, Nguyen HT (2009) Molecular characterization of stress-inducible *GmNAC* genes in soybean. Mol Genet Genomics 281:647–664

Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An Arabidopsis *myb* homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell 5:1529–1539

Vauterin M, Frankard V, Jacobs M (1999) The *Arabidopsis thaliana dhdps* gene encoding dihydrodipicolinate synthase, key enzyme of lysine biosynthesis, is expressed in a cell-specific manner. Plant Mol Biol 39:695–708

Vik A, Rine J (2001) Upc2p and Ecm22p, dual regulators of sterol biosynthesis in *Saccharomyces cerevisiae*. Mol Cell Biol 21: 6395–6405

Wentzinger LF, Bach TJ, Hartmann MA (2002) Inhibition of squalene synthase and squalene epoxidase in tobacco cells triggers an up-regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Physiol 130:334–346

Westrate JA, Meijer GW (1998) Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolaemic and mildly hypercholesterolaemic subjects. Eur J Clin Nutr 52:334–343

Yamaguchi-Shinozaki K, Shinozaki K (1993) Arabidopsis DNA encoding two desiccation-responsive *rd29* genes. Plant Physiol 101:1119–1120

Yanagisawa S, Schmidt RJ (1999) Diversity and similarity among recognition sequences of Dof transcription factors. Plant J 17:209–214

Zhou W, Nes WD (2003) Sterol methyltransferase2: purification, properties, and inhibition. Arch Biochem Biophys 420:18–34