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Przemysław Wojtaszek, Faculty of Biology, Adam Mickiewicz University, Poland

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ORIGINAL RESEARCH PAPER

Methyl jasmonate-induced accumulation of metabolites and transcriptional responses involved in triterpene biosynthesis in *Siraitia grosvenorii* fruit at different growing stages

Kailun Zhang[1], Zuliang Luo[1], Yuhua Guo[1], Changming Mo[2], Dongping Tu[1], Xiaojun Ma[1,3]*, Longhua Bai[2]

[1] Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, No. 151 Malianwa North Road, Haidian District, Beijing 100193, China
[2] Guangxi Botanical Garden of Medicinal Plants, No. 189 Changgang Road, Nanning 530023, China
[3] Yunnan Branch Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, No. 138 Xuanwei Road, Jinghong 530023, China

* Corresponding author. Email: mayixuan10@163.com

Abstract

The cucurbitane-type triterpenoid glycosides, mogrosides, are the main active components of *Siraitia grosvenorii* fruit. Squalene and cucurbitadienol are among the intermediates of the biosynthetic pathway for the formation of cucurbitane-type triterpenoid backbones of mogrosides. It is recognized that the exogenous application of methyl jasmonate (MeJA) increases the accumulation of secondary metabolites in various plant species. Here, the effect of MeJA (50, 200, and 500 μM) on the accumulation of squalene and cucurbitadienol in the fruits of *S. grosvenorii* at 10, 20, and 30 days after flowering (DAF) was tested for the first time. Since mogroside II E is the main cucurbitane-type triterpenoid present at this time, its concentration was also determined. The results show that MeJA can indeed promote squalene and cucurbitadienol accumulation, the application of 500 μM MeJA at 30 DAF being optimal. The concentration of squalene and cucurbitadienol increased up to 0.43 and 4.71 μg/g dry weight (DW), respectively, both of which were 1.2-fold greater than that of the control. The content of mogroside II E increased by 15% over the untreated group. We subsequently analyzed the expression of key genes involved in the mogroside biosynthetic pathway, including the 3-hydroxy-3-methylglutaryl-coenzyme A reductase gene (*SgHMGR*), squalene synthetase gene (*SgSQS*), cucurbitadienol synthase gene (*SgCS*), and cytochrome P450 (*SgCYP450*) with quantitative real-time PCR. The results showed that transcriptional levels of these genes were upregulated following the treatment described above. Additionally, their responses in the presence of MeJA was related to the concentration and timing of MeJA treatment.

Keywords

Siraitia grosvenorii; methyl jasmonate (MeJA); squalene; cucurbitadienol; mogroside II E

Introduction

Siraitia grosvenorii (Cucurbitaceae) is a perennial vine widely distributed in southern China in the Guangxi Province. It is used as a zero-calorie sweetener, and historically, as a household remedy for colds, sore throats, and lung congestion [1,2]. The extracted mixture of mogrosides is about 300 times sweeter than sucrose and has been demonstrated to be "generally regarded as safe" (GRAS) by the U.S. Food and

Drug Administration (FDA) [1]. Of these, mogroside V is the main active ingredient [3] and its biosynthetic pathway is shown in Fig. 1a. Furthermore, mogrosides exhibit a wide range of pharmacological characteristics, e.g., anti-oxidant, immunomodulatory, liver-protecting, anti-fatigue and anti-microbial properties [4,5]. Thus, with an increased public awareness of healthy diets, *S. grosvenorii* fruit have increasingly been used in Chinese herbal medicine, dietary supplements, and soft drinks [4,6].

Isopentenyl diphosphate (IPP), an early precursor of mogrosides, is synthesized in plant cells by two parallel isoprenoid-generating pathways – the cytosolic mevalonate (MVA) and the plastidial 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway [7]. Mogrosides, as all triterpenoids, are considered to be formed by the MVA pathway [2]. The bitter mogroside II E is the biogenetic precursor of the sweet mogroside V and the principal product formed within the first 45 days of *S. grosvenorii* fruit growth [8]. As the fruit mature, mogroside II E disappears gradually, while mogroside V increases dramatically during the period of 50 to 70 days and after 85 days its content remains stabile.

Squalene (2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22-hexaene) is an acyclic triterpenoid containing six isoprene units. In fact, squalene is present in all eukaryotic cells, however, some species are recognized as important accumulators of this compound. These include wheat germ, rice bran, shark liver, and olive oils, etc. [9], as well as *S. grosvenorii* seed oil [10]. Studies have shown that squalene has a number of pharmacological attributes, such as antioxidant (used as moisturizer and emollient in cosmetic industry) [11] and anti-inflammatory properties [9], as well as lowering

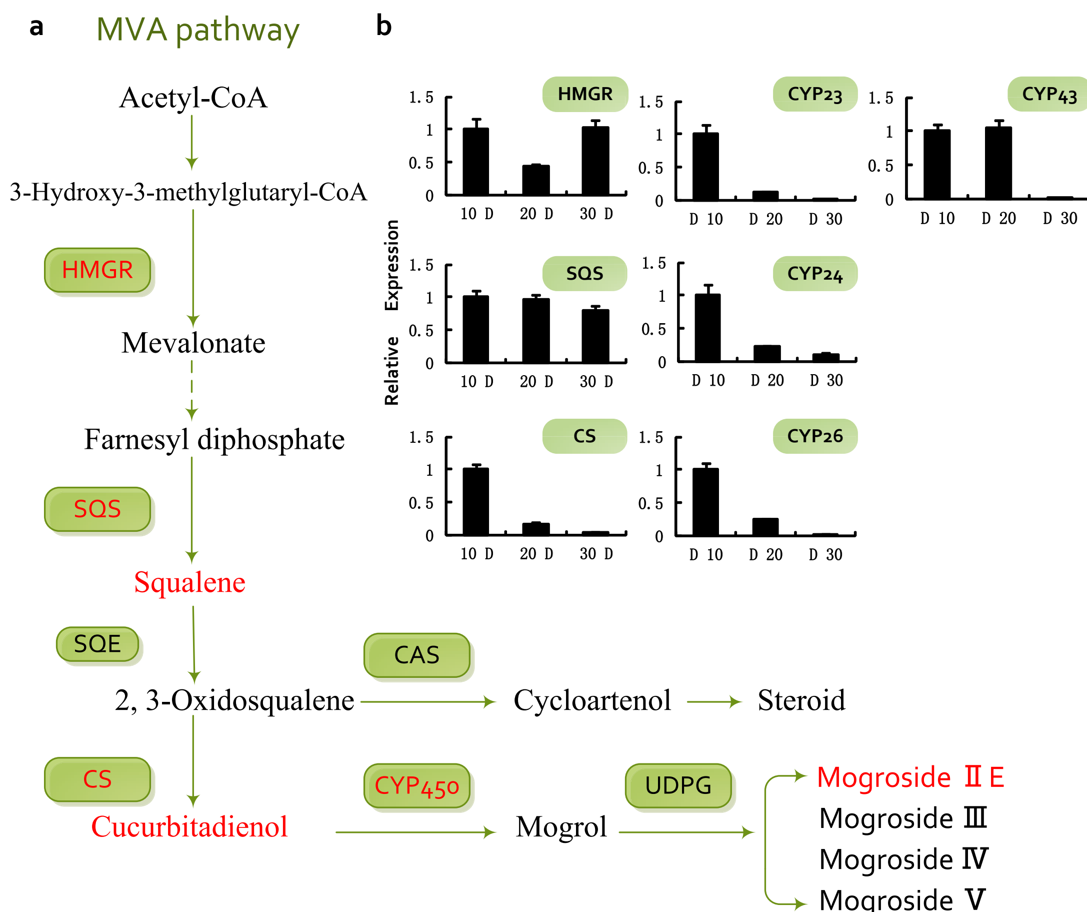

Fig. 1 Mogroside biosynthetic pathway along with relevant genes and components studied in this experiment. **a** Mogroside biosynthesis via mevalonate (MVA) pathway, enzymes and compounds investigated are shown in red, dashed arrows represent multistep pathways while solid arrows represent only one step. HMGR – HMG-CoA reductase; SQS – squalene synthase; SQE – squalene epoxidase; CS – cucurbitadienol synthase; CAS – cycloartenol synthase; CYP450 – cytochrome P450; UDPG – UDP-glucosyltransferase. **b** Expression of genes (*SgHMGR*, *SgSQ*, *SgCS*, *SgCYP23*, *SgCYP24*, *SgCYP26*, *SgCYP43*) in control conditions at 10, 20, and 30 DAF. Transcriptional levels of the latter two stages are normalized to that of the 10 DAF.

cholesterol levels [12], strengthening the body's immune system [13], and serving as biofuels derived from algae [14]. Squalene is also a general precursor for the biosynthesis of triterpenes in plants [15]. However, to date, little research has focused on its production by *S. grosvenorii* fruit, and therefore the study of squalene in *S. grosvenorii* fruit is of importance.

Cucurbitadienol is the simplest tetracyclic triterpene synthesized from squalene via oxidation and cyclization. Cucurbitadienol can form a variety of cucurbitane-type triterpenoid glycosides which occur mainly in the family Cucurbitaceae. These include mogrosides, as in *Siraitia grosvenorii*, and cucurbitacins in *Trichosanthes kirilowii* [16], *Cucumis sativus* [17], and *Cucurbita pepo* [18]. It has been known as the basic backbone of cucurbitane-type triterpenes. Yield of cucurbitadienol is extremely low in higher plants, comprising only about 3.9 mg/kg of fruit dry weight in *Siraitia grosvenorii* [19] and 0.5 g/kg of dried seeds of *Trichosanthes kirilowii* [20]. Consequently, finding ways to improve the cucurbitadienol concentration is highly important, as this compound can serve as an intermediate when attempting to increase the yield of the final products.

Methyl jasmonate (MeJA), a fragrant volatile compound, belongs to a group of highly investigated phytohormones ubiquitous in the plant kingdom [21]. MeJA is not only a vital regulator in plant defense responses against biotic and abiotic stresses [22–24], but also plays a role in plant physiological processes, such as fruit ripening [25], stomatal closure [26], etc. Moreover, research has indicated that exogenous application of MeJA to plant cell cultures or to the intact plant can stimulate the accumulation of a wide range of plant secondary metabolites [27], including terpenoids [28], flavonoids [29], and alkaloids [30] and up-regulate expression of the genes involved in their biosynthesis [31–33]. However, the effect of MeJA on cucurbitane-type terpenoids of *S. grosvenorii* fruit has not yet been investigated.

Some of the key enzymes involved in mogroside biosynthesis are: 3-hydroxy-3-methylglutaryl-coenzyme A reductase (SgHMGR, EC:1.1.1.34) which converts 2-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonic acid (MVA), which is regarded as the first committed step in the MVA pathway [34]. Squalene synthetase (SgSQS, EC:2.5.1.21) catalyzes the conversion of two molecules of farnesyl diphosphate (FPP) to squalene and it has been suggested that this is one of the important regulatory enzymes in squalene synthesis [35]. Cucurbitadienol synthase (SgCS, EC:3.4.99.33), belongs to the family of oxidosqualene cyclases (OSCs), and is responsible for the cyclization of 2,3-oxidosqualene to generate cucurbitadienol. SgCS was considered the first pathway-specific enzyme in the biosynthesis of cucurbitane-type triterpene glycosides [36]. Cytochrome P450 (SgCYP450) proteins are superfamily enzymes which convert cucurbitadienol into the mogrosides aglycone. Previously, our research group cloned the full-length sequences of *SgHMGR*, *SgSQS*, and *SgCS*, and it has been shown that these are single copy genes, whereas 85 *SgCYP450* unigenes were identified, four of which were chosen as candidates for cloning [2]. The aim of our project was to find an effective way of increasing the accumulation of metabolites in order to improve the final product yield. Consequently, we set out to test the effectiveness of MeJA, a commonly used phytohormone considered to be a potentially good elicitor of this process.

In the present work, we investigated the effect of different concentrations of MeJA on the squalene, cucurbitadienol, and mogroside II E concentrations of *S. grosvenorii* fruit at different stages of growth in order to find the optimal treatment for obtaining the greatest yield of these compounds. In addition, the expressions of key genes (*SgHMGR*, *SgSQS*, *SgCS*, *SgCYP450*) involved in the response to MeJA were also determined.

Material and methods

Plant material and MeJA treatments

The fruit of 10 plants of *S. grosvenorii* grown under identical conditions were chosen from the collection of Guangxi Botanical Garden of Medicinal Plants, Nanning,

Guangxi autonomous region, China. Since MeJA is insoluble in water, it was dissolved in ethanol to form concentrated stock solutions. Fruit of *S. grosvenorii* plants at 10, 20, and 30 days after flowering (DAF) were dipped in MeJA solution of different concentrations (50, 200, 500 μM, respectively) for about 60 s until their surface became completely wet (at 8 a.m.). MeJA was dissolved in ethanol and the final concentration of ethanol was 2% (v/v). The control fruits were also treated with aqueous ethanol (2%). Each sample comprised three fruit as biological replicates. The fruit were sampled at 0, 2, 6, 10, 24, and 48 h post MeJA treatment. After the fruit had been peeled and de-seeded, the pulp was subsequently cut into small pieces and immediately frozen in liquid nitrogen and stored at −80°C.

Extraction procedures

The powdered sample (0.5 g) was suspended in 20 mL of methanol with KOH (6%, w/v) and kept for 10 minutes at room temperature before ultrasonic extraction was used to abstract active components. After the extraction, the solution was filtered and diluted with 20 mL of distilled water. The mixture was poured into a separation funnel together with 20 mL of *n*-hexane. The *n*-hexane layer was collected and the aqueous layer was re-extracted with hexane three times. Hexane extracts were pooled and Na_2SO_4 was added to remove the remaining water. The solvent was then evaporated to dryness under reduced pressure by rotary evaporator at 40°C. The residue was dissolved in 1 mL of methanol, and subsequently, gas chromatography–mass spectrometry (GC-MS) was applied.

GC-MS analysis of squalene and cucurbitadienol

GC-MS technique was applied to determine concentrations of squalene and cucurbitadienol by using a 7890B GC System (Agilent Technologies Inc., China) coupled to a 5977A inert MSD with Data System. A capillary column with 5% phenyl and 95% dimethylpolysiloxane (30 m × 0.25 mm i.d. × 0.25 μm film thickness) from Agilent Technologies was used for the separation. The mass spectrometer was operated in electron ionization mode (EI) at 70 eV. The initial oven temperature was 100°C and held for 2 min then ramped up by 20°C/min increments to 260°C, and finally heated to 300°C at a rate of 10°C/min and held for 8 min. Helium was used as the carrier gas and the carrier flow rate was 1 mL/min. The volume injected was 2 μL in 1:10 split mode. The temperature of both the ion source and MS transfer line was 250°C. All the samples were analyzed in selective ion monitoring mode (squalene: m/z 69, 81, 341, 410; cucurbitadienol: m/z 426, 274, 259, 134). Squalene and cucurbitadienol were identified by the retention time and mass spectra of their individual reference substance. Their concentrations were calculated by the one-point curve method using the external standards of squalene and cucurbitadienol. Authentic squalene was purchased from National Institutes for Food and Drug Control (Beijing, China) and authentic cucurbitadienol was provided by Dr. Zhou Yuan (Chinese Academy of Agricultural Sciences, Beijing, China).

HPLC analysis of mogroside II E

A 0.5-g sample was dissolved in 25 mL of methanol and subsequently extracted by ultrasonic treatment. The mixture was cooled to room temperature and additional methanol was added to replenish the lost solvent. Following filtration, the solution was evaporated and the dry residue was dissolved in 5 mL of methanol before being introduced to the HPLC equipment Agilent 1100 with UV detector at 203 nm to determine the content of mogroside II E. The ZORBAZ SB-C18 column (4.6 mm × 250 mm, 5 μm) was used at a temperature of 20°C. Other parameters were as follows: mobile phases: 0.05% phosphoric acid dissolved in water (A)-acetonitrile (B); gradient elution: 0–8 min, 3–13.5% B; 8–35 min, 13.5–35% B; 35–45 min, 35% B; run time: 45 min; flow: 0.8 mL/min; volume of injection: 10 μL. The concentration was calculated by

Tab. 1 Primes used in this experiment.

Genes	Primer	Sequences
SgHMGR	Forward	5′-TAGGCTCCAAAGTATCCG-3′
	Reverse	5′-CAGTTTACAGCAGCAGGTT-3′
SgSQS	Forward	5′-CTGAGACACCCAGATGACT-3′
	Reverse	5′-GAGGGCTCGCAGAACAAGA-3′
SgCS	Forward	5′-TGAGAAATGGTTGAAGAGC-3′
	Reverse	5′-CCATTTTCTACTTCCTTTCCAT-3′
SgCYP23	Forward	5′-GGAGCACGAGGCATTTCTA-3′
	Reverse	5′-CAACCATAAGCGTCCACCC-3′
SgCYP24	Forward	5′-GATTCTACGGCGATATTCCTT-3′
	Reverse	5′-AATGGATGAAGTATGACCTGAA-3′
SgCYP26	Forward	5′-TTTGTACTGCTGTCTTTGCTTCA-3′
	Reverse	5′-GTTTGGAAGAGCATGGTTTTATT-3′
SgCYP43	Forward	5′-CAACCTAGCGTCGAAGTCAAA-3′
	Reverse	5′-TAGCCAATCTATCGTCTACAACC-3′
SgUBQ5	Forward	5′-ATAAAAGACCCAGCACCACATTC-3′
	Reverse	5′-CCCTTGCCGACTACAACATCC-3′

the one-point curve method using the external standards of mogroside II E. Authentic mogroside II E (98.5%) was extracted by Dr. Fenglai Lu (Guangxi Institute of Botany, Chinese Academy of Sciences, Guangxi, China).

qRT-PCR analysis

Total RNA was isolated using the Trizol reagent (Invitrogen, USA) and its quality and concentration were tested through agarose gel electrophoresis and a Nano Drop 2000 spectrophotometer (Thermo Scientific, USA). A PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time; TaKaRa, Dalian, China) was used to remove remaining DNA residues and to convert the total RNA into cDNA used as template. Later, transcription levels of genes (*SgHMGR*, *SgSQS*, *SgCS*, *SgCYP23*, *SgCYP24*, *SgCYP26*, *SgCYP43*) were determined by quantitative real-time PCR performed using the AB 7500 Fast Real-Time PCR System (Applied Biosystem, USA). Starting the reaction followed the standard protocol of SYBR Premix Ex Taq™ II (Tli RNaseH Plus; TaKaRa, Dalian, China): an initial denaturation at 95°C for 30 s, 40 cycles of annealing at 95°C for 5 s and extension at 60°C for 34 s. It has been shown that *S. grosvenorii* ubiquitin (*UBQ5*) gene is the most suitable reference gene for spatio-temporal expression analysis [37], therefore *SgUBQ5* was used as an internal control in our experiment. The gene expression of MeJA-elicited samples was normalized to that of the unstimulated sample. Relative gene expression was calculated according to the $2^{-\Delta\Delta C_T}$ method [38]. The primers used in this experiment are listed in Tab. 1.

Statistical analysis

The values of squalene, cucurbitadienol, and mogroside II E concentrations, as well as the transcriptional levels of genes, were presented as mean ± standard error (*SE*) and the data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test to examine the significant differences between various treatments using the SPSS software (version 16.0) at $p < 0.05$ and $p < 0.01$.

Results

Variation in squalene concentration during fruit developmental stages

The concentration of squalene under control conditions and MeJA treatments at 10, 20, and 30 DAF were analyzed by GC-MS (Tab. 2). Squalene accumulation decreased at 20 DAF (2.1-fold) and increased at 30 DAF (1.1-fold) compared to 10 DAF under control conditions. When squalene concentration was tested at 24 and 48 h following the application of three concentrations of MeJA (50, 200, and 500 μM) to *S. grosvenorii* fruit, in most cases (except when *S. grosvenorii* fruit were treated with 50 and 500 μM MeJA at 10 DAF), the results revealed enhanced squalene accumulation compared to that of the control. The optimal concentration of MeJA was 200 μM. At 20 DAF, the concentration of squalene was about 2.5-fold greater at 48 h (0.40 μg/g dry weight,

Tab. 2 The concentrations of squalene and cucurbitadienol (µg/g dry weight) in *S. grosvenorii* fruit treated with MeJA at 10, 20, and 30 DAF.

	Control			50 µM		200 µM		500 µM	
	0 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
10 DAF MeJA									
Squalene	0.34 ±0.02	0.37 ±0.03	0.47 ±0.04	0.19 ±0.02	0.39 ±0.03	0.57 ±0.03*	0.64 ±0.03**	0.27 ±0.04	0.33 ±0.04
Cucurbitadienol	12.33 ±1.32	11.46 ±0.94	11.66 ±1.50	6.18 ±0.86	11.05 ±1.14	17.02 ±1.61**	11.64 ±1.16	8.84 ±0.60	11.42 ±1.26
20 DAF MeJA									
Squalene	0.17 ±0.01	0.16 ±0.02	0.17 ±0.03	0.21 ±0.01*	0.17 ±0.03	0.23 ±0.02	0.40 ±0.03*	0.23 ±0.01	0.39 ±0.02**
Cucurbitadienol	4.80 ±0.67	6.84 ±0.66	5.40 ±0.72	7.22 ±0.81	2.89 ±0.42	10.16 ±0.86**	6.34 ±0.97*	10.69 ±1.18*	6.69 ±0.70*
30 DAF MeJA									
Squalene	0.36 ±0.07	0.23 ±0.03	0.29 ±0.06	0.27 ±0.04	0.48 ±0.02	0.38 ±0.05**	0.64 ±0.04**	0.21 ±0.06	0.43 ±0.03**
Cucurbitadienol	3.86 ±0.42	3.70 ±0.53	4.15 ±0.51	1.76 ±0.20	3.16 ±0.31	3.29 ±0.25	3.86 ±0.28	2.59 ±0.73	4.71 ±0.80*

Values are expressed as mean ±*SE* ($n = 3$). Asterisks denote significant variance: * represents $p < 0.05$, ** stands for $p < 0.01$.

DW) after applying 200 µM MeJA compared to that of control, attaining the highest value for all the treatments. Furthermore, 200 µM MeJA also increased the concentration of squalene at 10 DAF (1.9-fold) and 30 DAF (1.8-fold).

Variation in cucurbitadienol concentration during fruit developmental stages

The concentration of cucurbitadienol was also determined under control conditions and following MeJA treatment (Tab. 2). The concentration of cucurbitadienol decreased gradually and values at 20 and 30 DAF were about 2.6-fold, and 3.2-fold less than at 10 DAF, respectively. The highest concentration of cucurbitadienol was obtained after applying 500 µM MeJA at 20 DAF (10.69 µg/g DW at 24 h), which was 2.2-fold greater than the control value. Another three treatments also showed an increase in cucurbitadienol accumulation compared to the control: applying 200 µM MeJA at 10 DAF (1.4-fold at 24 h) and 20 DAF (2.1 and 1.3-fold at 24 and 48 h, respectively) and 500 µM MeJA at 30 DAF (1.2-fold at 48 h). However, there were no striking changes to the concentrations of cucurbitadienol following other MeJA treatments. Moreover, it would appear that 50 µM MeJA had little effect on the accumulation of cucurbitadienol and 20 DAF was a better time for MeJA application.

Variation in mogroside II E concentration during fruit developmental stages

Mogroside II E was the major component detected during the first 30 days of *S. grosvenorii* fruit growth. Therefore, the concentration of mogroside II E was measured throughout this period in order to establish whether MeJA can enhance its accumulation. The results showed that the concentration of mogroside II E increased 19% at 20 DAF and then diminished by 40% at 30 DAF compared to 10 DAF (Fig. 2c). In the case of MeJA-treated fruit, the accumulation of mogroside II E did not change significantly for most treatments except following the application of 500 µM MeJA at 30 DAF. The value at 48 h was 15% higher than the control (Fig. 2f).

Changes in the expression of seven genes during fruit developmental stages

The expression of seven genes (*SgHMGR*, *SgSQS*, *SgCS*, *SgCYP23*, *SgCYP24*, *SgCYP26*, *SgCYP43*) during the first 30 days was determined by qRT-PCR (Fig. 1b). Results showed that the expression of the *SgHMGR* gene dropped by about half at 20 DAF and was subsequently roughly equal to its initial value at 30 DAF compared to 10 DAF, whereas the expression of the *SgSQS* gene remained broadly unchanged throughout this period. The transcriptional level of *SgCS* was about 5.9-fold lower at 20 DAF and 30.0-fold lower at 30 DAF than at 10 DAF. The expression level of three out of the

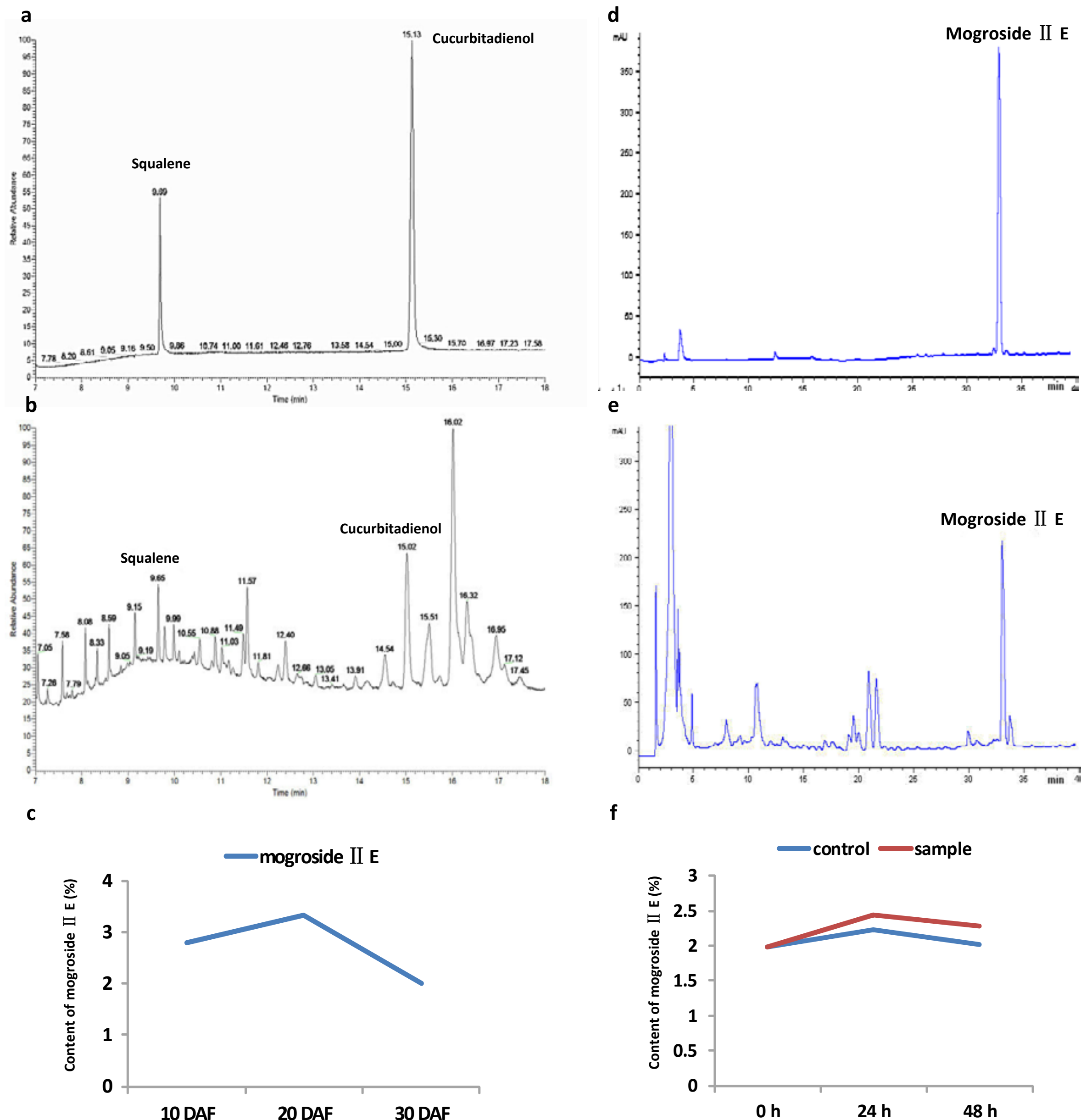

Fig. 2 Chromatographic analysis of metabolites [squalene, cucurbitadienol (GC-MS), and mogroside II E (HPLC/UV)]. **a–c** GC-MS chromatogram of squalene and cucurbitadienol standards (**a**), extract (**b**), concentration of mogroside II E at 10, 20, and 30 DAF in control conditions (**c**). **d–f** HPLC/UV chromatogram of mogroside II E standard (**d**), extract (**e**), concentration of mogroside II E when treated with 500 µM MeJA at 30 DAF (**f**).

four analyzed SgCYP450 encoding genes (*SgCYP23*, *SgCYP24*, *SgCYP26*) decreased during these 30 days, while the transcriptional level of *SgCYP43* remained constant at 20 DAF and then diminished dramatically at 30 DAF compared to 10 DAF.

The expression of these genes was then investigated at 0, 2, 6, 10, 24 h following the application of 50, 200, and 500 µM MeJA in order to study the effect of MeJA on the expression of these key genes. It was found that gene expression was affected by MeJA. As shown in Fig. 3, the expression of *SgHMGR*, *SgSQS*, *SgCS*, and *SgCYP23* genes was upregulated following treatment with 200 µM MeJA at 20 DAF and all three concentrations of MeJA at 30 DAF (except for *SgHMGR* upon treatment with 500 µM MeJA). In this respect, 200 µM might be considered the optimal concentration for induction by MeJA. Besides the treatments described above, expression of *SgCYP26* was also enhanced at 10 DAF following the application of 50 and 200 µM MeJA, and thus differed from the expression of the other genes. For example, for *SgCYP24*, application of MeJA at 20 DAF was better than at 10 and 30 DAF since the transcriptional level of *SgCYP24* gene increased at this stage for all three concentrations of MeJA treatment.

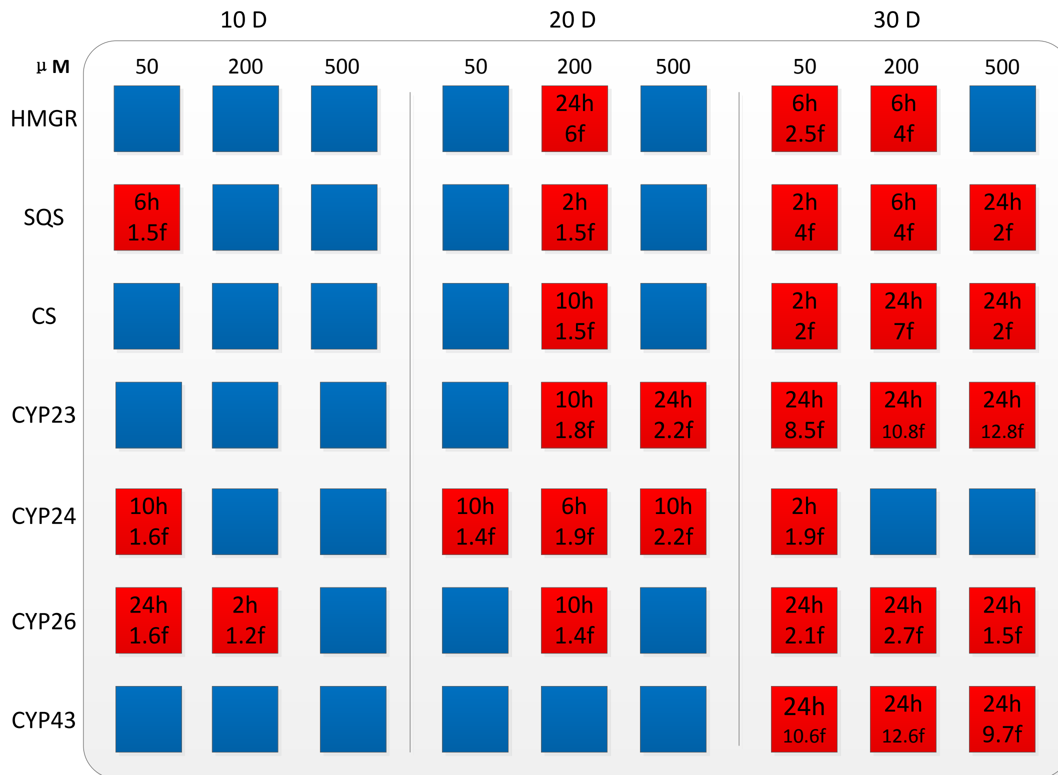

Fig. 3 Transcriptional levels of the seven genes (*SgHMGR*, *SgSQS*, *SgCS*, *SgCAS*, *SgCYP23*, *SgCYP24*, *SgCYP26*, *SgCYP43*) following the exogenous application of MeJA. Colors of the squares represent the effects of MeJA on seven genes at 10, 20, 30 DAF with 50, 200, and 500 µM MeJA, blue and red represent no significant effect and promotion effect, respectively. In the red squares, the figures on the first line are the time points following MeJA treatments when gene expression reaches its highest level and the figures on the second row are the corresponding multiples increased compared to the control.

Furthermore, *SgCYP43* gene expression could only be upregulated at 30 DAF. The highest values for the expression of the four *SgCYP450* genes were almost all reached at 24 h following the application of MeJA at 30 DAF. Of these genes, the transcriptional levels of *SgCYP23* and *SgCYP43* increased by a much greater extent than those of the other two genes, the expressions of *SgCYP23* and *SgCYP43* being 8–13 times greater than the control, whereas the transcriptional levels of *SgCYP24* and *SgCYP26* were only about 1–3 times greater.

Discussion

The concentrations of metabolites (squalene, cucurbitadienol, and mogroside II E) and expression of corresponding genes were determined at 10, 20, and 30 DAF under control conditions. Inconsistent changes in squalene concentration and *SgSQS* gene expression indicated that changes in squalene accumulation might result from its conversion to downstream products. The content of cucurbitadienol mirrors the changes of the level of the transcript of *SgCS*. Moreover, the concentration of mogroside II E increased at 20 DAF and then decreased at 30 DAF and this closely resembles the results of a previous report [39]. This indicates that mogroside II E is synthesized mainly during the first 20 days, and that the reduction in mogroside II E concentration thereafter might be explained by a reduction in the concentration of cucurbitadienol and/or expression of *SgCYP450* genes. The changes in *SgSQS* and *SgCS* expression were at odds with another study performed by Tang et al., which showed that expression of these two genes were upregulated [2]. This might be due to the different developmental stages of *S. grosvenorii* fruit selected for these two experiments (the latter was performed at 3, 50, and 70 DAF).

Many reports have demonstrated that the concentration of triterpene glycosides can be increased by the application of exogenous MeJA, and this is always accompanied by the upregulation of expression of corresponding genes [40,41]. Studies of the effect of MeJA on fruit mainly concentrated on post-harvest fruit quality [42–44]. In this experiment, we anticipated that treating *S. grosvenorii* fruit with MeJA in the field during its early growing stages would also regulate the synthesis of important intermediates which could contribute to a greater yield of final products. The results indicated that the stimulus indeed had a positive effect on the concentrations of squalene and cucurbitadienol. The treatments required to reach the maximum increments for these two components were different: concentration of squalene was 2.5-fold greater than the control when 200 μM MeJA was applied at 20 DAF, whereas accumulation of cucurbitadienol was 2.2-fold greater than the control after application of 500 μM MeJA at 20 DAF. Also, the transcriptional levels of the corresponding genes, *SgSQS* and *SgCS*, were upregulated by the application of MeJA in each case (Fig. 3). Subsequently, we investigated the concentration of the main mogroside (mogroside II E) throughout the 30 days, and found that applying 500 μM MeJA to the fruit at 30 DAF caused an increase in the accumulation of mogroside II E. In this case, the concentration of squalene and cucurbitadienol, as well as the expression of five of the seven genes (*SgSQS*, *SgCS*, *SgCYP23*, *SgCYP26*, *SgCYP43*) increased, indicating that the application of MeJA can improve the accumulation of cucurbitane-type triterpenoid glycosides and that this might be related to the increased concentration of intermediates and the increased expression of the genes involved.

It is generally recognized that treatment with MeJA causes reprogramming of gene expression in higher plants subject to specific factors [21]. A number of factors may contribute to this, such as cultivation conditions, growth stage of the culture, concentration of MeJA and its time of application, etc., all of which indicate a complex network of MeJA regulation [21,45–47]. Consequently, in this experiment we also evaluated the effect of MeJA on the expression of critical genes involved in the mogroside biosynthetic pathway. The results indicate that the concentration and time of application of MeJA are two important factors which affect the MeJA signal that is perceived by the fruit of *S. grosvenorii*. A concentration of 200 μM MeJA is recommended for improved the expression of these genes. Moreover, the timing of the application of MeJA is critical [48,49]. Our experimental results showed that treating *S. grosvenorii* fruit with MeJA at 30 DAF was better than at 10 or 20 DAF since six of the seven groups at 30 DAF showed a positive response to the stimulus (Fig. 3). This might be due to the relatively slow growth rate at 30 DAF compared to 10 and 20 DAF [50], which would be beneficial for the accumulation of further biochemical compounds and the regulation of these active genes. In *S. grosvenorii*, *HMGR* and *SQS* genes are encoded by single genes which are homologous to the *HMGR* gene in *Cucumis melo* and to the *SQS* gene in *Gynostemma*, both plants belonging to family Cucurbitaceae. The *CS* gene is present in *S. grosvenorii*, *Cucurbita pepo*, and *Cucumis sativus* (all members of Cucurbitaceae). The *SgCS* gene is single copied, whereas this is not the case for the latter two species. The transcriptional levels of *SgHMGR* and *SgSQS* can be upregulated by MeJA, as has also been reported elsewhere: treatment with MeJA can enhance the expression of *CaHMGR* gene in *Cyanotis arachnoidea* [51] and the expression of *SQS* gene in *Poria cocos* [52]. Furthermore, of the four *SgCYP450* genes, it seems that *SgCYP23* and *SgCYP43* are more likely to be candidate genes involved in mogroside synthesis, since expression of these genes was enhanced to a greater extent than was that of the other two (*SgCYP24* and *SgCYP26*). Our experiment has established a method for investigating the effect of MeJA on the metabolites of *S. grosvenorii* fruit. The results showed that the application of exogenous MeJA could lead to the augmentation of accumulated mogrosides, which in turn, could contribute to the identification of MeJA-responsive transcription factors involved in the mogroside synthetic pathway.

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