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The effect of elicitors on oleanolic acid accumulation and expression of triterpenoid synthesis genes in *Gentiana straminea*

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Abstract

Gentiana straminea is native to the Qinghai-Tibet plateau, where it is exposed to extremes of cold and strong UV-B radiation. Here we showed that low temperature, but not UV-B radiation, affected the accumulation of the triterpenoid oleanolic acid. Neither of these stresses altered the expression of known triterpenoid synthesis genes. However, the application of methyl jasmonate (MeJA), but not salicylic acid (SA), significantly enhanced the accumulation of oleanolic acid and up-regulated the triterpenoid synthesis genes, especially the expression of *βAS,* the gene encoding β-amyrin.

Additional key words: β-amyrin synthase, low temperature, methyl jasmonate, salicylic acid, UV-B radiation.

Introduction

Plants produce a wealth of secondary metabolites, including alkaloids (Magdi *et al*. 2007), flavonoids (Li *et al.* 2007), and terpenoids (Kim *et al.* 2005a,b,c). The synthesis of these compounds can be affected by a number of external factors (Ebel *et al.* 1986). With respect to some medicinal plants, environmental conditions are known to affect production of valuable secondary metabolites (Zindorn 2009). In particular, UV-B radiation has been shown to influence the balance between terpenoids and flavonoids in peppermint (Dolzhenko *et al*. 2010). Both the accumulation of certain secondary metabolites and the expression of genes involved in their synthesis can also be elicited by the application of the phytohormone methyl jasmonate (MeJA; Choi *et al*. 2005, Kim *et al*. 2007).

Gentiana straminea Maxim is a Chinese herbal medicinal plant native to the Qinghai-Tibet plateau, a high altitude region characterized by frequent extremes of cold temperature, high rainfall, and strong UV-B radiation. Its medicinal value is due to the compounds oleanolic acid, loganic acid, gentiopicroside, swertiamarin, and roburic acid (Chen *et al.* 2005). Oleanolic acid (OA) is a pentacyclic triterpenoid associated with anti-hepatitis, anti-inflammatory, and anti-carcinoma activity (Laus 2004). Several enzymes in the *Centella asiatica* triterpenoid synthesis pathway have been isolated (Kim *et al*. 2005a,b,c), and the pathway itself has been explored in some detail (Mangas *et al*. 2008).

We have previously described the isolation of a full length cDNA encoding β-amyrin synthase (βAS) in *G. straminea* and showed that it catalyses 2,3-oxido-squalene to form β-amyrin (Liu *et al*. 2009). β-amyrin is thought to act as a precursor for oleanolic acid. Our aim was to study the effect of a number of external factors on the accumulation of OA and on the expression of various genes taking part in the triterpenoid synthesis pathway.

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Abbreviations: βAS - β-amyrin synthase; CYS - cycloartenol synthase; FPS - farnesyl diphosphate synthase; GPPS - geranyl diphosphate synthase; HMGR - 3-hydroxy-3-methylglutaryl-CoA (HMG) reductase; HMGS - HMG synthase; IPPI - isopentenyl-diphosphate isomerase; LUS - lupeol synthase; MS - Murashige and Skoog ; SE - squalene epoxidase; SS - squalene synthase.
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Materials and methods

Both embryonic calli with high frequency of plant regeneration and regenerated plants of *Gentiana straminea* Maxim were used (Fig. 1*A*,*B*). The calli were subcultured on Murashige and Skoog (1962; MS) medium supplemented with 30 g dm^{-3} sucrose and 2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D). After 14 d, they were transferred onto MS medium without hormones to rest for 2 d. Then they were exposed for 7 d at low temperature of 4 °C, irradiated for 30 min daily for 7 d with 70 μW cm^{-2} UV-B, or transferred onto MS medium containing either 50 μM MeJA or 50 μM salicylic acid (SA). Control (without any treatment) and calli treated as mentioned above were air dried, ground to powder, suspended in ten volumes of methanol, and exposed to 60 min ultrasonic homogenization. The material was then centrifuged (10 000 *g* for 10 min) and the supernatant filtered through a 0.45 μm membrane. HPLC separation of the components of the supernatant was carried by a method mentioned in Liu *et al*. (2009).

The calli and regenerated plants were treated with 50 μM MeJA for 21 d to analyze the expression of genes involved in triterpenoid synthesis. RNA was extracted from both calli and regenerated plants, using the *TRIzol* reagent (*Invitrogen*, Carlsbad, CA, USA) following manufacturer's instructions. After spectrophotometric quantification, RNA was reversed to cDNA and then cDNA was used as a template for a set of semi-quantitative reverse transcriptase - polymerase chain reactions (sqRT-PCRs) and quantitative (q) RT-PCR to assess the expression of triterpenoid pathway related genes. The primers for sqRT-PCR were designed from the sequences of orthologues in other plant species, and those for qRT-PCR were designed from the cDNA fragment sequences of *G. straminea*. The qRT-PCR was performed by using a *SYBR Green I* real-time PCR detection system with *actin* gene as an internal reference as described Wang *et al*. (2003).

In order to assess the growth and OA content of the calli, ten calli with diameter of 0.5 cm were measured at 1, 2, 4, 7, 10, 14, and 21 d after different treatments. In all statistical tests, data were the means of three independent biological replicates. All data were statistically analyzed by *t*-test using the *Statistica 6.0* software and values with $P \leq 0.05$ were interpreted as indicating statistically significant differences.

Results

The embryonic *G. straminea* calli with high frequency plant regeneration were used for analyzing the growth and OA accumulation (Fig. 1*A*,*B*). The fresh mass of calli was measured and the values showed that the growth rate increased over the 21 d incubation period, starting slowly but accelerating from 7 to 21 d after culture establishment (Table 1). The growth was unaffected by either the presence of MeJA or SA, or by treatment with UV-B, but growth was slow under the low temperature (Table 1).

The OA content of *G. straminea* calli mostly increased gradually over time (Table 1) especially during period of rapid growth from 7 to 21 d after transfer onto MS medium. OA accumulation was unaffected by either SA or UV-B radiation, but was limited at low temperature (Table 1). The presence of MeJA, however, increased OA content of calli treated for 21 d to 115.2 μg g^{-1}(f.m.) which was three times higher than that in untreated calli (Table 1).

Table 1. The fresh mass and oleanolic acid content of *G. straminea* calli 0 - 21 d after different treatments. Means of three assays ± SE. Values followed by the same letters were not significantly different at $P < 0.05$.

Treatment	0 d	1 d	2 d	4 d	7 d	10 d	14 d	21 d
	fresh mass [mg]							
Control	187.2±13.2a	194.3±35.7a	197.5±24.7a	234.6±34.5a	310.5±73.2a,b	404.4±45.9a,b	726.8±65.4b	998.5±58.4c
MeJA	182.5±27.5a	192.5±26.8a	205.2±13.4a	238.3±19.2a	306.2±65.3a,b	470.5±19.8a,b	656.2±82.8b	1027.0±39.4c
SA	202.3±18.5a	198.1±34.3a	196.4±34.2a	234.2±34.3a	324.4±43.7a,b	396.6±39.2a,b	748.7±56.8b	1142.0±58.9c
UV-B	212.4±13.4a	228.3±24.6a	230.8±32.5a	206.3±26.8a	317.6±27.6a,b	374.8±45.2a,b	750.5±22.5b	1072.0±30.7c
Cold	205.7±26.5a	208.5±29.6a	230.2±17.8a	224.8±15.8a	216.2±24.6a	196.2±45.7a	215.3±34.1a	238.4±32.9a
	content of oleanolic acid [μg g^{-1}(f.m.)]							
Control	19.2± 6.2a	22.3± 3.2a	25.2± 2.4a	30.2± 6.6b	37.2± 3.3b	40.2± 5.3b	32.3± 3.5b	31.2± 2.9b
MeJA	18.6± 5.4a	38.2± 7.6b	88.1±11.6c	93.6± 7.3c	106.5± 6.3c	114.0±23.2c	112.6± 3.8c	115.2± 3.9c
SA	17.3± 6.7a	21.1± 5.3a	23.2± 1.7a	31.4± 2.3b	35.2± 3.1b	33.2± 6.5b	33.1± 7.7b	38.9± 3.0b
UV-B	23.3± 7.5a	22.3± 9.5a	24.3± 2.1a	26.6± 1.3a	24.3± 1.8a	32.7± 2.7b	29.6± 1.3a	35.2± 9.6b
Cold	13.2± 2.8a	9.8± 2.4a	13.5± 1.1a	11.3± 3.2a	10.3± 2.5a	14.1± 5.4a	15.4± 2.8a	13.1± 2.7a

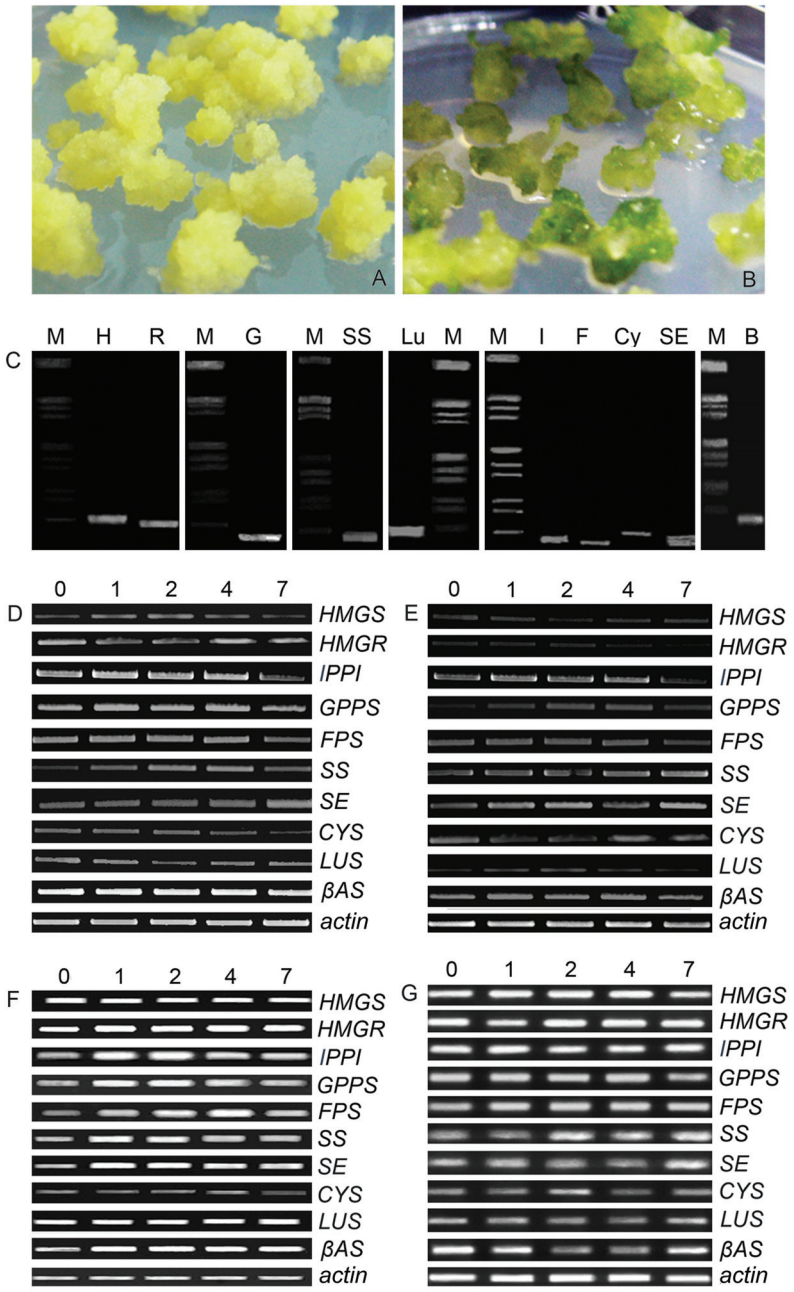

Fig. 1. The materials used for gene fragment amplification and sqRT-PCR. Two types materials were used: the embryonic calli with high regeneration frequency (*A*) and regenerated plants of *G. straminea* (*B*). The fragments of genes in the pathway amplified in *G. straminea* calli (*C*). Ten genes were amplified: M - size marker; H - *HMGS*; R - *HMGR*; G - *GPPS*; SS - *SS*; Lu - *LUS*; I - *IPPI*; F - *FPS*; Cy - *CYS*; SE - *SE*; B - *βAS*. The sqRT-PCR of triterpenoid synthesis genes in *G. straminea* calli treated for 0, 1, 2, 4, and 7 d with UV-B radiation (*D*), low temperature (*E*), MeJA (*F*), and SA (*G*).

Ten *G. straminea* gene fragments were amplified by using RT-PCR (Fig. 1*C*) and their sequences were used to design primers for qRT-PCR. The sqRT-PCR analysis suggested that the expression of none of the triterpenoid synthesis pathway was affected by UV-B irradiation, low temperature, or SA, but several of them, especially expresssion of gene coding β-amyrin synthase (*βAS*), were up-regulated in the presence of MeJA (Fig. 1*D*,*E*,*F*,*G*).

Since the above results showed that some genes involved in triterpenoid synthesis were up-regulated by MeJA treatment, a detailed analysis was carried out for the calli and regenerated plants. In the calli, the expression of the genes coding HMG-CoA-reductase (*HMGR*), isopentenyl-diphosphate isomerase (*IPPI*), geranyl diphosphate synthase (*GPPS*), farnesyldiphosphate synthase (*FPS*), squalene synthase (*SS*), and squalene

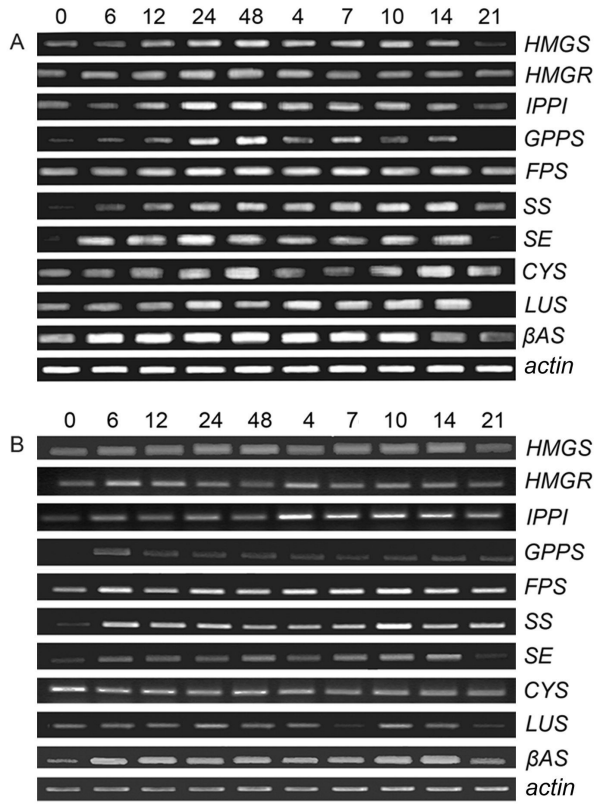

Fig. 2. The expression of genes involved in triterpenoid synthesis in *G. straminea* calli (*A*) and regenerated plants (*B*) treated with 50 μM MeJA for 0, 6, 12, and 48 h and 4, 7, 10, 14, and 21 d were analyzed by using sqRT-PCR (*actin* was used as the internal control).

epoxidase (*SE*) was maintained at a high level from 24 h to 14 d following the addition of MeJA to the medium, whereas expression of gene coding HMG-CoA synhase (*HMGS*) was not noticeably altered (Fig. 2*A*). The expression of *FPS* increased over the first 12 h reaching a peak of 11.4-fold increased expression (Table 2). *IPPI* and *SE* expression was similarly induced by MeJA with the highest expression at 48 h (Table 2). In branch pathway, *βAS* expression was induced by MeJA already after 6 h, peaked at 24 h, and maintained at high level for 10 d (Fig. 2*A*, Table 2). Its expression level at 24 h was almost 38-fold higher than that of background (Table 2), so increase was more remarkable than that of *FPS*, *IPPI*, and *SE*. However, neither gene coding cycloartenol synthase (*CYS*) nor lupeol synthase (*LUS*) responded clearly (Fig. 2*A*, Table 2). Similarly as in the calli, the expression of the triterpenoid synthesis pathway genes was up-regulated in regenerated plants after MeJA treatment especially that of *βAS* was up-regulated significantly peaking at 14 d (Fig. 2*B*). The transcription of *CYS* and *LUS* was little affected by MeJA (Fig. 2*B*).

Table 2. Real-time quantitave RT-PCR based assessment of gene expression in *G. straminea* calli treated with 50 μM MeJA for 0 - 21 d. Means ± SE of three assays. Values followed by the same letters were not significantly different at $P < 0.05$.

Genes	0 h	6 h	12 h	24 h	48 h	4 d	7 d	10 d	14 d	21 d
HMGS	1±0.43a	0.84±0.25a	1.27±0.38a	1.48±0.19a	1.53±0.16a	1.14±0.24a	1.19±0.31a	1.49±0.02a	1.21±0.35a	0.96±0.06a
FPS	1±0.22a	5.46±0.34ab	9.44±2.32b	11.4±1.49b	9.82±0.94b	10.6±2.89b	9.59±1.24b	8.36±1.23ab	9.82±0.54b	7.14±1.02ab
IPPI	1±0.12a	4.11±0.56ab	6.51±1.23ab	12.2±1.07b	19.7±2.19b	7.19±1.12ab	8.23±2.05ab	7.78±0.34ab	7.63±1.53ab	3.89±1.98ab
SE	1±0.08a	10.3±2.58b	9.92±3.24b	11.6±1.32b	13.2±2.33b	9.35±2.64b	7.87±2.26ab	10.1±3.22b	8.28±0.78ab	2.62±1.23ab
LUS	1±0.18a	0.86±0.25a	0.97±0.13a	1.35±0.21a	1.02±0.49a	1.31±0.45a	1.18±0.28a	1.38±0.09a	1.50±0.34a	0.88±0.46a
βAS	1±0.25a	12.3±1.54b	19.1±3.2b	38.2±5.82c	15.1±3.14b	15.9±3.52b	12.8±1.79b	14.8±0.69b	19.7±3.54b	16.3±2.43b

Discussion

The accumulation of secondary metabolites is affected by several environmental factors, such as UV-B radiation (Dolzhenko *et al*. 2010), low temperature (Zobayed *et al*. 2005), and drought (Liu 2008). Here we have studied rare plant *G. straminea* which has poor germination in low altitude environment (Tan *et al*. 1997). We chose to monitor the effect of low temperature and UV-B radiation as these are the two commonest environmental stresses encountered by *G. straminea* in the nature. It was shown that the accumulation of oleanolic acid was unaffected by UV-B but affected by low temperature (Table 1), consistent with the recent research that low temperature rather than UV-B radiation is the key driver of secondary metabolite composition in high altitude plants (Zindorn 2009).

It has been noted that the exogenous supply of either MeJA or SA affects the accumulation of secondary metabolites. In particular, in grape cell cultures, MeJA treatment enhances the accumulation of sesquiterpenes and proanthocyanidins and this enhancement is reversed by a subsequent application of SA (D'Onofrio *et al*. 2009). Here, we have shown that the accumulation of oleanolic acid was induced by MeJA treatment but there was no response to exogenously supplied SA. The accumulation

of secondary metabolites *via* up-regulation of the related enzyme genes under MeJA treatment were demonstrated (Nims *et al*. 2006). Here, the content of oleanolic acid in *G. straminea* was increased in the presence of MeJA *via* up-regulation of *HMGR*, *IPPI*, *GPPS*, *FPS*, *SS*, *SE,* and especially of *βAS* (Tables 1, 2, Fig. 2*A*,*B*).

In summary, we have demonstrated that the accumulation of the triterpenoid oleanolic acid can be induced in *G. straminea* calli and regenerants by exogenous MeJA. The up-regulation of the gene encoding βAS in particular suggests that this enzyme may be decisive in the synthesis of oleanolic acid. Since the *G. straminea* calli can be readily regenerated, transformation of this species should be possible. Therefore it is a potential of increasing triterpenoid content in *G. straminea* plants over-expressing *βAS*.

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