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Methyl jasmonate as a control factor of the *synthase squalene* gene promoter and ginsenoside production in American ginseng hairy root cultured in shake flasks and a nutrient sprinkle bioreactor

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ABSTRACT

Hairy root cultures of *Panax quinquefolium* (L.) produce triterpenoid saponins: ginsenosides with broad medical applications. The crucial enzyme in the process of ginsenoside biosynthesis is squalene synthase (SSq). In this study a 741 bp fragment of the *P. quinquefolium SSq* gene, consisting of a proximal promoter, 5′UTR (5′ untranslated region) and 5′ CDS (coding DNA sequence) was isolated. An analysis of an isolated fragment with *in silico* tools indicated a lack of tandem repeats, miRNA binding sites and CpG/CpNpG elements. However, the proximal promoter contained potential *cis*-elements, mediating the response to multiple external stimuli, including light, heat-stress and drought. Among them, two CGTCA motifs could be involved in the response to methyl jasmonate (MeJA) treatment. To evaluate the functional significance of MeJA on *P. quinquefolium SSq* expression, quantitative RT-PCR experiments were performed at different elicitor concentration. Additionally, the effect of methyl jasmonate on ginsenoside biosynthesis was examined at 5, 50, 100, 250, 500 μM l^{-1} concentrations. Experiments were performed in shake flasks and a nutrient sprinkle bioreactor offering better oxygenation rate and potential for future scaling-up of the biosynthesis process. The saponin content was determined using HPLC. In shake flask and bioreactor culture, the maximum yield (respectively 27.33 mg g^{-1} d.w. and 51.0 mg g^{-1} d.w.) of the sum of six examined ginsenosides was achieved in modified Gamborg B-5 medium containing 250 μM l^{-1}methyl jasmonate after seven days of elicitation. Rb1 (20(*S*)protopanaxadiol-3-[O-β-D-glucopyranosyl(l → 2)-β-D-glucopyranoside]-20-*O*-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranoside) and Re (20(*S*)-protopanaxatriol-6-[O-α-L-rhamnopyranosyl(l → 2)-β-D-glucopyranoside]-20-*O*-β-D-glucopyranoside) metabolites quantitatively dominated both in shake flask and bioreactor cultures. The level of Rb1 increased 5.6-fold and achieved 14.35 mg g^{-1} d.w. of hairy roots cultivated in flasks. In bioreactor cultures, this metabolite achieved 24.77 mg g^{-1} d.w. increasing its content 2.6-fold compared to control. The level of Re increased 1.8-fold and reached 4.92 mg g^{-1} d.w. in flask cultures. In bioreactor cultures the content of this metabolite was 6.23 mg g^{-1} d.w. The highest productivity of ginseng saponins (15.37 mg g^{-1}d.w. l^{-1} day^{-1}) was noted in bioreactor hairy root cultures after 7 days elicitation of 250 μM l^{-1} MeJA.

Abbreviations: CDS, coding DNA sequence; d.w., dry weight; f.w., fresh weight; MeJA, methyl jasmonate; SSq, squalene synthase; TBE, Tris-borate-EDTA electrophoresis buffer; 5'UTR, 5' untranslated region; Rb1, 20(*S*)protopanaxadiol-3-[O-β-D-glucopyranosyl(l→2)-β-D-glucopyranoside]-20-*O*-β-D-glucopyranosyl(1→6)-β-D-glucopyranoside; Rb2, 20(*S*)-protopanaxadiol-3-[O-β-D-glucopyranosyl(L-2)-β-D-glucopyranoside]-20-[O-α-L-arabinopyranosyl(l→6)-β-D-glucopyranoside]; Rb3, (3β,12β)-3-[(2-*O*-β-D-Glucopyranosyl-β-D-glucopyranosyl) oxy]-12-hydroxydammar-24-en-20-yl 6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside; Rc, 20(*S*)-protopanaxadiol-3-[O-β-D-glucopyranosyl(l→2)-β-D-glucopyranoside]-20-*O*-α-L-arabinofuranosyl (l→6)-β-D-glucopyranoside; Rd, 20(*S*)-protopanaxadiol-3-[O-β-D-glucopyranosyl(l→2)-β-D-glucopyranoside]-20-(O-β-D-glucopyranoside; Rg3, (3β,12β)-12,20-Dihydroxydammar-24-en-3-yl 2-*O*-β-D-glucopyranosyl-β-D-glucopyranoside; Rh2, (3β,12β)-12,20-Dihydroxydammar-24-en-3-yl-β-D-glucopyranoside; Re, 20(*S*)-protopanaxatriol-6-[O-α-L-rhamnopyranosyl (l→2)-β-D-glucopyranoside]-20-*O*-β-D-glucopyranoside; Rg1, 20(*S*)-protopanaxatriol-6,20-di-*O*-β-D-glucoside; Rg2, (3β,6α,12β)-3,12,20-Trihydroxydammar-24-en-6-yl 2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside; Rh1, (3β,6α,12β)-3,12,20-Trihydroxydammar-24-en-6-yl-β-D-glucopyranoside; Ro, 1-*O*-[(3β)-3-{[2-*O*-(β-D-Glucopyranosyl)-β-D-glucopyranuronosyl]oxy}-28-oxoolean-12-en-28-yl]-β-D-glucopyranose

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1. Introduction

Panax quinquefolium (L.) Alph.Wood (syn. *Panax quinquefolius* (L.), *Aralia quinquefolia* (L.) Decne. & Planch), or American ginseng, a member of the *Araliaceae* family, is widely used as a component of herbal medicines in North America and in Eastern Asia (Pengelly and Bennett, 2011; Shin et al., 2015). The American ginseng genome is large, being approximately 1.58-times larger than the genome of the related *Panax ginseng* and 31.30-times larger than the genome of the model plant *Arabidopsis thaliana* (Obae and West, 2012). The significant size of the *P. quinquefolium* genome, 4914 Mbp has drawn out the completion of genome sequencing process and subsequent structural and functional genomics analysis. Until now complete sequences of about 156 kbp have been obtained only for *P. quinquefolium* chloroplast DNA (Han et al., 2016; Kim et al., 2016), as well as some partial data regarding *P. quinquefolium* genomic DNA (Joly et al., 2017). However, the gathering and analysis of *P. quinquefolium* transcriptome data has been faster, at least in relation to root tissue (Sun et al., 2010; Wu et al., 2013; Cao et al., 2015). As a results, the data concerning the genomic sequences of *P. quinquefolium* are insufficient. These DNA fragments include 5′ regulatory elements of genes known as promoters (Hernandez-Garcia and Finer, 2014; Porto et al., 2014; Biłas et al., 2016). Knowledge of promoter structure provides fundamental data characterizing the factors controlling gene expression rate in response to particular biotic and abiotic stimuli. However, the regulation of the ginseng biosynthesis pathway at the level of gene expression is not completely known (Kim et al., 2009a, 2009b; Wu et al., 2010). The action of the regulatory mechanisms could be elucidated by identifying the promoter sequences of the genes encoding key enzymes in the biosynthetic pathway of ginseng saponins and by identifying the transcription factors which regulate promoter activity.

Great interest has surrounded *P. quinquefolium* due to the large number of clinically and pharmacologically-beneficial effects possessed by the plant extracts, whose components act on the nervous and cardiovascular systems (Nah, 2014; Lee and Kim, 2014). In addition, they have been found to possess anti-diabetic, hepato-protective activity and stress-reducing activities, and have been found to offer benefits to immune system (Im and Nah, 2013; Tung et al., 2012; Lakshmi et al., 2011; Christensen 2009). The powdered roots of ginseng and their extracts are often included in dietary supplements, healthy food and energy drinks (Qu et al., 2009; Shen et al., 2003).

The primary biologically-active constituents of American ginseng are triterpene saponins called ginsenosides. These secondary metabolites are divided into three groups based on their structure: the Rb group, Rg group and Ro group. Saponins of the Rb and Rg groups belong to dammarane-type ginsenosides. The Rb group is composed of a number of protopanaxadiol derivatives including: Rb1, Rb2(20(*S*)-protopanaxadiol-3-[O-β-D-glucopyranosyl(L-2)-β-D-glucopyranoside]-20-[O-α-L-arabinopyranosyl (l → 6)-β-D-glucopyranoside]), Rb3 ((3β,12β)-3-[(2-*O*-β-D-Glucopyranosyl-β-D-glucopyranosyl)oxy]-12-hydroxydammar-24-en-20-yl 6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside), Rc (20(*S*)-protopanaxadiol-3-[O-β-D-glucopyranosyl(l → 2)-β-D-glucopyranoside]-20-*O*-α-L-arabinofuranosyl (l → 6)-β-D-glucopyranoside), Rd (20(*S*)-protopanaxadiol-3-[O-β-D-glucopyranosyl(l → 2)-β-D-glucopyranoside]-20-(O-β-D glucopyranoside), Rg3 ((3β,12β)-12,20-Dihydroxydammar-24-en-3-yl 2-*O*-β-D-glucopyranosyl-β-D-glucopyranoside) and Rh2 ((3β,12β)-12,20-Dihydroxydammar-24-en-3-yl-β-D-glucopyranoside). The Rg group includes protopanaxatriol derivatives, such as: Rg1 (20(*S*)-protopanaxatriol-6,20-di-*O*-β-D-glucoside), Rg2 (Rg2; (3β,6α,12β)-3,12,20-Trihydroxydammar-24-en-6-yl 2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside), Re and Rh1 ((3β,6α,12β)-3,12,20-Trihydroxydammar-24-en-6-yl-β-D-glucopyranoside) while the Ro group is represented by only one oleanane-type ginsenoside – known as metabolite Ro (1-*O*-[(3β)-3-{[2-*O*-(β-D-Glucopyranosyl)-β-D-glucopyranuronosyl]oxy}-28-oxoolean-12-en-28-yl]-β-D-glucopyranose) (Wang et al., 2012). Six of these triterpene saponins derived from *P. quinquefolium, viz.* Rb1, Rb2, Rc, Rd, Rg1, and Re are regarded to be the major ingredients used in Pharmacy.

Due to their wide range of medical uses, the market for ginseng root products is growing. However, covering the demand for the material from natural sources is problematic because of the special issues related to field-cultivation, which is a long-term process that lasts for at least three years before the roots can be harvested. Moreover, the tillage requires many agronomic treatments including a special preparation of seeds before sowing or providing special conditions which limit the direct sunlight exposition. Therefore, the entire cultivation process is difficult and expensive (Hankins 2009; Peterson 2014). For these reasons, the biotechnological technique of *in vitro* cultures appears to be a vital alternative to field cultivation. Although, the productivity of ginsenosides in *in vitro* cultures has increased significantly, due to developments in media composition or culture process optimization, the ginseng saponin content is still lower than that of the roots of field-cultivated plants (Szymańska et al., 2013). In addition to improving the *in vitro* culture conditions, the ginsenoside productivity rate can be enhanced by increased expression of genes encoding for enzymes of critical importance for the biosynthesis process (Fig. 1). One of such enzymes is squalene synthase (SSq) which catalyses the first enzymatic step in sterol and triterpenoid biosynthesis from common metabolites of the isoprenoid pathway. The enzyme condenses two molecules of farnesyl pyrophosphate (FPP) in the form of squalene (Kim et al., 2011). Kim et al. (2015) noted that overexpression of the *synthase squalene* gene in *P. ginseng* induced the downstream ginsenoside pathway genes, including *squalene epoxidase* (*SE*), β-*amyrin synthase* (β-*AS*), finally resulting in a dramatic increase of ginsenosides concentration in adventitious roots of transgenic *P. ginseng*.

A relatively simple method for boosting the gene expression rate and thus raising secondary metabolite production is the supplementation of plant cultures by signaling compounds such as jasmonates or salicylic acid, commonly known as elicitors. Their application in the elicitation process can lead to the increased accumulation of secondary metabolites in plant tissues (Shabani et al., 2009; Ruiz-May et al., 2009; Zabala et al., 2010; Ram et al., 2013; Sun et al., 2013). For example methyl jasmonate is used successfully in *in vitro* cultures of medicinal plants to improve the production of biologically-active phytochemicals such as: paclitaxel (Lenka et al., 2015), vincristine, vinblastine (Zhang et al., 2015), vindoline, catharanthine, ajmalicine (Zhou et al., 2015) or triterpenoids (James et al., 2013). Many reports have demonstrated that the effectiveness of elicitation depends on the complex interactions between the elicitor and the plant cell. Additionally, there are some factors such as elicitor type, dose and treatment schedule which can affect these interactions and the response to the elicitor (Vasconsuelo and Boland 2007). Moreover, the optimal concentration of the elicitor may be specific for each plant species and should be determined individually (Frankfater et al., 2009; Ruiz-May et al., 2009; Ram et al., 2013).

Several studies describe the influence of exogenous elicitors such as: jasmonates, cobalt nitrate, hydrogen peroxide or N,N'-dicyclohexylcarbodiimid on enhancing ginsenoside productivity in suspension cultures of *P. quinquefolium* (Biswas et al., 2016), *P. notoginseng* (Wang and Zhong, 2002; Wang et al., 2005) and adventitious and hairy root cultures of *P. ginseng* (Kim et al., 2005; Kim et al., 2009a,b; Huang et al., 2013; Kim et al., 2013); however, similar studies regarding hairy root cultures of American ginseng are not available. The probable technical application of transformed *P. quinquefolium* transformed roots as a source of ginsenosides will require media optimization and scaling up and this can be performed using nutrient sprinkle bioreactors. Therefore, the present study examines the effects of MeJA treatment on ginsenoside accumulation in hairy root cultures of *P. quinquefolium* in nutrient sprinkle bioreactor. Previous studies (Palazón et al., 2003b; Kim et al., 2002) confirm that the transition of *in vitro* culture from shake flasks to bioreactor is the first and in many cases the most difficult step in scaling up, often resulting in reduced productivity. Therefore, the improved ginsenoside yield observed in shake flasks should be evaluated in bioreactor cultures to facilitate the future scaling up of