Phytochemistry Letters 17 (2016) 194–200

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

SNPs of dammarenediol synthase gene were associated with the accumulation of ginsenosides in *DAMAYA ginseng*, a cultivar of *Panax ginseng* C. A. Mey.

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ARTICLE INFO

Article history:
Received 25 April 2016
Received in revised form 8 July 2016
Accepted 17 July 2016
Available online xxx

Keywords:
Ginseng
SNP
Ginsenoside
Dammarenediol synthase
Gene mutation

ABSTRACT

Context: Ginsenosides are regarded as the major active ingredients of ginseng, the dried taproot of *Panax ginseng* C. A. Mey. Our previous study has shown that several single nucleotide polymorphisms (SNPs) loci were identified in dammarenediol synthase (DS) gene, a key enzyme in the biosynthetic process of ginsenoside.
Objective: The current study aims to investigate the possible influence of SNPs on the content of ginsenosides in *DAMAYA* ginseng, a cultivar of *P. ginseng*.
Materials and methods: We selected a particular group of *DAMAYA ginseng* samples that were harvested in the same place of origin for SNP analysis, and then analyzed 18 ginsenosides in these samples with ultrahigh pressure liquid chromatography (UHPLC) coupled with time-of-flight mass spectrometer system (TOF/MS).
Results: We found that these SNPs are associated with the accumulation of active ingredients ginsenosides Rf, Rg1 and F1. Moreover, OPLS-DA analysis implied that SNP loci of 279 might play a role in influencing the content level of ginsenosides. Further bioinformatics analysis showed that Leu83aa mutant might cause structural and functional changes of DS gene, which could in turn result in the content change of dammarane ginsenosides.
Discussion and conclusion: Our findings suggest that specific SNPs of DS gene might be associated with the accumulation of some ginsenosides in *DAMAYA* ginseng and thus can be used for varietal characterization, molecular identification, qualitative evaluation of ginseng-type medicinal materials, or gene engineering and ginseng breeding.

1. Introduction

Ginseng is the dry taproot of *Panax ginseng* C.A. Meyer and the most commonly used medicinal herb in Asian countries. More than a dozen farm cultivars are grown in Northeast part of *China,* such as *Jilin* and *Liaoning* Provinces (Mao et al., 2014; Lee et al., 2015). The bioactive compounds in Chinese herbal medicines play a pivotal role in the therapeutic effect of these medicines in clinic (Huang et al., 2014). Ginsenosides are triterpene saponins produced by *P. ginseng* and generally regarded as the major bioactive component of this herb (Lee et al., 1997; Sunwoo et al., 2014). As modern research indicates (Han et al., 2011), dammarenediol synthase (DS) plays a very important role in the biosynthetic pathway of ginsenoside, and is a key component in obtaining dammarene-type ginsenoside. The first committed step in ginsenoside synthesis is the cyclization of 2, 3-oxidosqualene to dammarenediol II catalyzed by the DS of the oxidosqualene cyclase group, and ginsenoside are then synthesized from dammarenediol II after hydroxylation by cytochrome P450 (Tansakul et al., 2006) and glycosylation by glycosyltransferase (Jung et al., 2014). Single nucleotide polymorphism (SNP) refers to polymorphism in genome induced by substitution, insertion or deletion of single base that widely exists in gene sequence, and involves in regulating the level of gene expression and the activity of the expressed gene product (Jo et al., 2014). Therefore, SNP in DS gene may cause

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http://dx.doi.org/10.1016/j.phytol.2016.07.030
1874-3900/

change in the level and activity of DS and in turn affect the production of dammarenediol II and consequently the content of ginsenoside. The identification of ginseng cultivars has been reported with a wealth of technical and methods in recent years (Mihalov et al., 2000; Park et al., 2006; Kim et al., 2007; Wang et al., 2011). However, the association between DS gene mutation and the accumulation of ginsenosides has never been reported. In this study, *DAMAYA ginseng*, a typical farm cultivar, was used as materials to establish possible relationship between DS gene mutations and ginsenosides content. In our previous study (Wu et al., 2015), we have reported a number of stable SNPs in DS gene fragments of ginseng using several cultivars of *P. ginseng*. In the current study, we analyzed the SNPs stably existing in DS gene fragments of *DAMAYA ginseng* from our previous results, and further investigated the association between these SNPs and the accumulation of active ingredients of ginseng. The result shows that these SNPs might associate with the accumulation of active ingredients ginsenosides Rg1, Rf, and F1, bioinformatics analysis also explained the possible molecular mechanism of this phenomenon.

2. Materials and methods

2.1. Main reagents and instruments

EasyPure Plant RNA Reagent Kit and 5 K DNA Ladder (5000, 3000, 2000, 1500, 1000, 800, 500, 300 bp) were purchased from Beijing TransGen Biotech Co., Ltd., 2 × PCR PrimeSTAR HS (Premix) was purchased from Taraka Biotech Co., Ltd., Thermo Maxima 1 st strand cDNA synthesis Kit for reverse transcription was purchased from Thermo Scientific Biotech Co., Ltd. Veriti 96-Well Fast Thermal Cycler (US Applied Biosystems), Tissuelyser II Tissue Homogenizer (German QIAGEN), Eppendorf 5424 R bench top high speed refrigerated centrifuge (German Eppendorf), Gel Doc XR+ Vilber Lourmat (US BIORAD), Agilent 1200 UHPLC Coupled with Agilent 6230 Accurate Mass Time-of-Flight Mass Spectrometer (Agilent, USA), and Waters ACQUITY UPLC BEH shield RP18 column (2.1 mm × 100 mm, 1.7 μM, Waters corporation, USA) were employed in this study.

2.2. Materials

Samples of DAMAYA ginseng from different production places and growing years were obtained from the main cultivation zone of *P. ginseng* in China such as *Jilin* and *Liaoning* Provinces, etc., with GPS location information recorded. After cleaned with water, the samples were stored at −80 °C in the State Key Laboratory of Quality Research in Chinese Medicine (Macau University of Science and Technology). The detailed information of these samples is listed in Table 1.

2.3. Discovery of SNPs with nested PCR and direct sequencing

Nested PCR and direct sequencing were employed to discover SNPs from the samples as described in our previous study (Wu et al., 2015). In brief, the total RNA of *P. ginseng* were extracted with the EasyPure Plant RNA Reagent Kit and then reversely transcribed to cDNA according to the instructions of Thermo Maxima 1 st Strand cDNA Synthesis Kit. Nested PCR amplification primer of DS gene of *P. ginseng* were designed by primer design software Primer 5.0, and synthesized by Beijing Liuhehua Greater Gene Science & Technology Co., Ltd. (Beijing, China). A pair of outer primers P1 (5′-GACACCACATACCAACAAGAAGA-3′) and P2 (5′-ACTGAAGCCA-GAAGCTGGAA-3′) were used to obtain a DNA template including the coding sequence of DS gene; two pairs of inside primers, i.e.: upstream primers, [P3 (5′-AGACTTAAGAATGTGGA AGCTGAAG-3′) and P4 (5′-AGCAAGGTGATGTTTAAACTCATCA-3′)] and downstream primers [P5 (5′-ACAGGAAATGGGGAAAAAGCT-3′) and P6 (5′-ATTTGGAGACGATACTAGTGTGGAA-3′)] were also designed. There are over 100 bp overlapping between sequences amplified by the upstream and downstream primers that can be used to construct a longer coding sequence of DS gene segement. Primers of P1 and P2 were chosen for the first round of amplification; two PCR tubes containing the amplified product from the first round were added with primer pair P3 and P4 or primer pair P5 and P6, respectively to perform the second round of amplification, and the obtained products were directly sequenced by Sanger method at the Guangzhou Sequencing Branch of the Shenzhen BGI Technology Corporation (Shenzhen, China). Similarity of sample sequences was analyzed by DNAMAN7.0 biostatistical software, and then consensus sequence was generated for homologous comparison with the reference sequences of DS gene of *P. ginseng* in GenBank (Accession No.: GU183405.1, AB265170.1, JN596111.1, BD420539.1). The confirmed sequences were further analyzed with DNAMAN to calculate the genetic diversity between samples and then to find out SNP.

2.4. Chemical analysis of DAMAYA ginseng

The content of eighteen kinds of ginsenosides, i.e. Rg1, Re, F11, Rf, S-Rh1, Rg2, Rb1, R-Rh1, Rc, F1, R0, Rb2, Rb3, Rd, F2, S-Rg3, R1, and R-Rg3 in the methanol extraction of the samples were analyzed against the standard curve of these ginsenosides established from reference standards in an Agilent 1290 ultrahigh pressure liquid chromatography (UHPLC) coupled with Agilent 6230 Accurate Mass Time-of-Flight Mass Spectrometer system (TOF/MS) (Agilent technologies, USA) and a ACQUITY UPLC BEH shield RP18 column (2.1 mm × 100 mm, 1.7 μm, Waters corporation, USA). The raw data of the UHPLC-TOF/MS was processed by Agilent MassHunter Qualitative Analysis B.06.00 (Agilent technologies, USA). The injection volume was 1 μl. The column temperature was maintained at 40 °C. The separation was made with the mixture of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) at a flow rate of 0.35 ml/min in a gradient manner as

Table 1
Information of *DAMAYA* ginseng samples used in this study.

Sample name	Sample code	Place of collection	Growing year	Quantity
Cultivated *DAMAYA* ginseng	DMYJY4	Ginseng Field of Jiayi Local Products Co., Ltd. at Changbai County, Jilin Province	4	7
Cultivated *DAMAYA* ginseng	DMYHJ4	Ginseng Field of Hongjiu Ginseng Industry Co., Ltd. at Fusong County, Jilin Province	4	10
Linxia[a] *DAMAYA* ginseng	DMYLX6	Linxia Ginseng Field at Xintunzi Town, Fusong County, Jilin Province	6	4
Linxia *DAMAYA* ginseng	DMYLX10		10	2
Linxia *DAMAYA* ginseng	DMYLX15		15	3
Total				26

[a] Linxia denotes under forest, where the seeds of *DAMAYA* ginseng were planted in a wild woods to mimic the natural growing environment of wild ginseng.