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Selection of high ginsenoside producing ginseng hairy root lines using targeted metabolic analysis

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Abstract

To develop an experimental system for studying ginsenoside biosynthesis, we generated thousands of ginseng (*Panax ginseng* C.A. Meyer) hairy roots, genetically transformed roots induced by *Agrobacterium rhizogenes*, and analyzed the ginsenosides in the samples. 27 putative ginsenosides were detected in ginseng hairy roots. Quantitative and qualitative variations in the seven major ginsenosides were profiled in 993 ginseng hairy root lines using LC/MS and HPLC-UV. Cluster analysis of metabolic profiling data enabled us to select hairy root lines, which varied significantly in ginsenoside production. We selected hairy root lines producing total ginsenoside contents 4–5 times higher than that of a common hairy root population, as well as lines that varied in the ratio of the protopanaxadiol to protopanaxatriol type ginsenoside. Some of the hairy root lines produce only a single ginsenoside in relatively high amounts. These metabolites represent the end product of gene expression, thus metabolic profiling can give a broad view of the biochemical status or biochemical phenotype of a hairy root line that can be directly linked to gene function.

Keywords: *Panax ginseng*; Araliaceae; Hairy root ginsenoside biosynthesis; Metabolic profiling

1. Introduction

Plant functional genomics involves generation of transgenic and mutant plants in association with multi-parallel analysis of gene products such as mRNA and protein. In the last few years, significant technological progress has been made that will facilitate the generation and characterization of genetic diversity in plant systems. Recently, metabolic profiling, which provides a means of screening variation in metabolite content that cannot be scored by visual inspection, has been developed (Fiehn et al., 2000; Raamsdonk et al., 2001; Roessner et al., 2001). Metabolites represent the end products of gene expression thus their profiling can give a broad view of the biochemical status or biochemical phenotype of an organism that can be directly linked. to gene function (Raamsdonk et al., 2001; Roessner et al., 2001). Therefore, metabolic profiling is emerging as a powerful tool for functional genomics (Fiehn et al., 2000; Sumner et al., 2002; Trethewey, 2001).

Plants produce a diversity of secondary metabolites that play important biological roles in their environmental adaptation and provide humans with dyes, flavors, drugs and many other useful chemicals (Croteau et al., 2000). However, both the regulation and biosynthetic

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pathways of various plant secondary metabolites are still poorly understood (Dixon, 1999; Pichersky and Gang, 2000). Mutation approaches have been successfully applied in screening various types of mutations in the biosynthesis of secondary metabolites and cloning the corresponding genes (Borevitz et al., 2000; Haralampidis et al., 2001; Huang et al., 2001; Winkel-Shirley, 2002). However, this strategy is subject to limitations in several plant species for which transformation systems have not been established. Furthermore, it is not always easy to apply conventional mutation approaches to plant secondary metabolism, because the rich accumulation of many plant secondary metabolites can be restricted to plant species that are genetically intractable (Croteau et al., 2000; Pichersky and Gang, 2000). The application of an activation tagging system to such genetically intractable plant species to isolate genes involved in secondary metabolism was reported by van der Fits et al. (2001). They screened activation-tagged calli of *Catharathus roseus* and isolated a gene encoding ORCA3, an AP2 domain transcriptional regulator for several genes involved in the biosynthesis of terpene indole alkaloids (van der Fits and Memelink, 2000). Recently, Goossens et al. (2003) investigated metabolic profiles using GC/MS and combined them with cDNA-AFLP-based transcript profiling of jasmonate-elicited tobacco Bright yellow (BY-2) cell to yield an extensive inventory of known and novel genes involved in tobacco secondary metabolism. The challenge now is to develop experimental systems to explore the functional genomics of secondary metabolism in those plant species that are a rich source of natural products, but are genetically intractable.

Korean ginseng (*Panax ginseng* C.A. Meyer) is a typical medicinal plant, which has been widely used as a traditional medicine since ancient times, owing to its stimulative and tonic properties. The ginsenosides, triterpene saponins, are suggested to be the major active components of ginseng roots. However, despite the considerable commercial interest in ginsenosides, little is known about the genes and biochemical pathways of ginsenoside biosynthesis (Haralampidis et al., 2002). Here, we produced ginseng hairy root lines and profiled their ginsenoside content using LC/MS and HPLC-UV systems. We describe an experimental system in which hairy root lines can be used for functional genomic analysis of plant secondary metabolism.

2. Results and discussion

2.1. Generation of ginseng hairy roots

We generated 2220 ginseng hairy root lines using *Agrobacterium rhizogenes* strains A4 and R-1000 and *A. rhizogenes* strain R-1000 including activation tagging vector, pKH01 (Table 1). Ginseng hairy roots have different morphological phenotypes and were classified into three different groups based on their root morphology (Fig. 1). The first group has a low branching phenotype and, with the exception of the newly developing part, shows slow growth rate and brownish color (Fig. 1(a)). The second group has a typical hairy root phenotype with a high degree of branching and a rapid growth rate (Fig. 1(b)). Two different phenotypes were distinguished within this group by their degree of curling at the end of the branching root. The third group has a callus-like phenotype with little branching (Fig. 1(c)). In the hairy roots of this group, all of the roots, especially the main roots, are thickened but have the root phenotype. Some of the hairy roots show a typical callus-like phenotype.

Phenotypic variation was observed in both hairy roots generated by *A. rhizogenes* strains including activation tagging vectors and wild-type *A. rhizogenes* strains. These results indicate that phenotypic variation in hairy root lines may not result from the transcriptional activation by enhancer elements introduced into the cell but rather from the gene introduced into the plant genome by T-DNA. There have been several reports indicating that ginsenoside production differs among ginseng hairy root lines (Bulgakov et al., 1998; Mallol et al., 2001). Our results indicate that each hairy root line equates to a genetically modified root, with distinct biochemical and/or physiological phenotypes. Hairy roots have the following advantages as material for functional genomic analysis: (1) They produce high levels of secondary metabolites, which are detectable in the roots of wild-type plants (Ahn et al., 1996; Bonhomme et al., 2000; Bulgakov et al., 1998; Li et al., 2001; Mallol et al., 2001; Nakano et al., 1998); (2) A hairy root system does not require plant regeneration. Generated hairy root lines are subsequently maintained as primary transformants without regeneration steps and used for further analysis. It is also possible to regenerate whole plants from selected hairy roots when transformed seeds are needed (Choi et al., 2004); (3) Hairy roots are differentiated organs and therefore, are genetically much more stable than suspension culture cells or callus.

Table 1
Production of ginseng hairy roots

Agrobacterium strain	Vector	No. of hairy root lines	No. of analyzed hairy root lines
A. rhizogens A4	pRiA4	757	56
A. rhizogens R1000	pRiA4	363	44
A. rhizogens R1000	pRiA4 + pKH01	1100	893
Total		2220	993

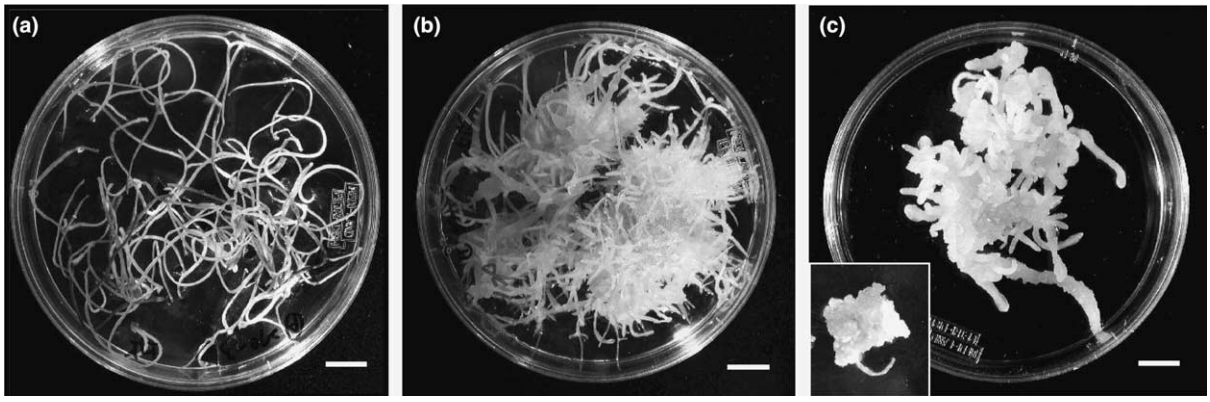
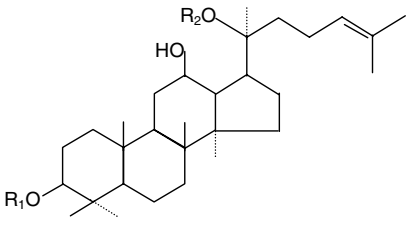


Fig. 1. Phenotypes of ginseng hairy roots. The first group (a) is the low branching hairy root type, which has a slow growth pattern and brownish color (except for the newly developing part). The second group (b) has the typical hairy root phenotype with high branching and rapid growth. The third group (c) has a callus-like phenotype with little branching. The inset view in panel (c) shows a typical callus-type transformed tissue. Bar = 1 cm.

Here, we demonstrate the possibility that activation tagging can be applied to hairy roots for functional genomic analysis of secondary metabolism. To facilitate the generation of activation tagging ginseng hairy root lines, an efficient and appropriate selection marker should be used, since kanamycin severely inhibits ginseng root growth. In addition, new activation tagging vectors with enhancer elements and only *rol*B genes should be designed to minimize inter-line genetic variation between many T-DNA encoding genes.

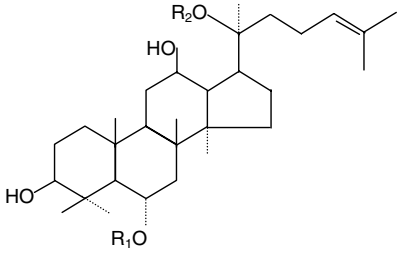
2.2. Ginsenoside profiling in ginseng hairy root lines

Quantitative and qualitative variations in ginsenoside content among ginseng hairy root lines was determined using LC/MS and HPLC-UV analyses. In addition, seven ginsenosides (Rb_1, Rb_2, Rc, Rd, Re, Rf and Rg_1) **1**–**7** (see Fig. 2) were used as ginsenoside standards for analytical conditions, with 20 other ginsenoside candidates were detected using selected ion chromatogram (SIC) profiling by comparison with mass spectral data (Fuzzati et al., 1999; Miao et al., 2002; Park, 1996). Twenty putative ginsenosides were further deleted by analysis of the fragmentation patterns of triterpene aglycone and hexose conjugations using tandem mass spectrometry (LC/SSI/MS/MS). The tandem mass conditions of isolation and collision induced dissociation for molecular ions of interest was optimized with standards compounds of 7 ginsenosides and applied to the identification of 20 putative ginsenoside candidates. The resulting fragment ions are produced from the isolated parent ion as the most abundant molecular ions in every full scanning mass spectrum and utilized in structural elucidation. All MS/MS results, including retention time and the molecular weight of the observed ions are summarized in Table 2. Including the 7



Ginsenoside		R1	R2
Rb1	1	Glc^2-Glc	Glc^6-Glc
Rb2	2	Glc^2-Glc	Glc^6-Ara(p)
Rc	3	Glc^2-Glc	Glc^6-Ara(f)
Rd	4	Glc^2-Glc	Glc

Protopanaxadiol type ginsenosides 1-4



Ginsenoside		R1	R2
Re	5	Glc^2-Rha	Glc
Rf	6	Glc^2-Glc	H
Rg1	7	Glc	Glc

Protopanaxatriol type ginsenosides 5-7

Fig. 2. Structural formula of ginsenosides **1**–**7**. Different ginsenosides are synthesized by adding one or several monosaccharides to R1 and R2 of the aglycones.

Table 2
Ginsenosides **1**–**7** as putative ginsenosides observed by negative-ion HPLC/SSI/MS and HPLC/SSI/MS/MS in ginseng hairy root [*m*/*z* with relative abundance (%) in parentheses]

Peak no.	Ginsenosides	Mol. weight	Rt (min)	Observed LC/MS	Observed LC/MS/MS of $[M-H]^-$
1	Di-glucopyranosyl protopanaxatriol	801.03	3.43	799.9(100)$[M-H]^-$; 637.8(24)$[M-glc-H]^-$	637.8(100)$[M-glc-H]^-$; 554.1(46)$[M-glc-C6H11-H]^-$; 475.9(68)$[M-2glc-H]^-$
2	Ginsenoside F1	638.89	3.70	637.7(100)$[M-H]^-$; 475.6(6)$[M-glc-H]^-$	475.9(100)$[M-glc-H]^-$
3	Ginsenoside Ro	957.12	3.74	956.4(100)$[M-H]^-$; 794.1(16)$[M-glc-H]^-$	794.0(100)$[M-glc-H]^-$; 750.7(3)$[M-glc-CO2-H]^-$; 732.1(2)$[M-glc-CO2-H2O-H]^-$;631.5(3)$[M-2glc-H]^-$; 613.9(2)$[M-2glc-H2O-H]^-$; 595.2(3)$[M-2glc-2(H2O)-H]^-$; 570.3(2)$[M-2glc-CO2-H2O-H]^-$
4	Quinquenoside R1	1151.35	3.85	1150.4(100)$[M-H]^-$; 1090.7(5)$[M-AcO-H]^-$; 988.6(0.5)$[M-glc-H]^-$; 946.3(46)$[M-glcAc]^-$; 459.6(1)$[M-glcAc-3glc-H]^-$	1108.5(72)$[M-CH3CO]^-$; 1092.3(100)$[M-Ac-H]^-$; 988.5(2)$[M-glc-H]^-$; 946.3(2)$[M-glc-H]^-$; 946.3(10)$[M-glcAc-H]^-$; 928.1(17)$[M-glcAc-H2O-H]^-$; 784.1(7) $[M-glcAc-glc-H]^-$
5	Malonyl ginsenoside Rb2/Rb3/Rc	1165.34	4.50	1164.3(100)$[M-H]^-$; 1032.1(83)$[M-ara-H]^-$	1164.4(24)$[M-H]^-$; 1120.2(7)$[M-CO2-H]^-$; 1072.2(13)$[M-2(CO2)-H]^-$; 988.5(100)$[M-ara-CO2-H]^-$; 970.4(26)$[M-ara-CO2-H2O-H]^-$; 928.3(22)$[M-ara-malonyl-H2O-H]^-$; 910.3(22)$[M-ara-malonyl-2(H2O)-H]^-$
6	Malonyl ginsenoside Rb1	1195.36	5.00	1194.5(100)$[M-H]^-$; 1150.2(7)$[M-CO2-H]^-$;988.3(33)$[M-CO2-glc-H]^-$; 946.1(16)$[M-malonyl-glc-H]^-$	1150.5(100)$[M-CO2-H]^-$; 1132.4(79)$[M-CO2-H2O-H]^-$; 946.1(6)$[M-malonyl-glc-H]^-$; 784.9(10)$[M-malonyl-2glc-H]^-$; 765.8(4)$[M-malonyl-glc-H2O-H]^-$
7	Ginsenoside Re[a] (**5**)	947.17	5.70	946.3(100)$[M-H]^-$; 800.5(1)$[M-rha-H]^-$;	800.0(100)$[M-rha-H]^-$; 783.7(56)$[M-glc-H]^-$; 765.7(8)$[M-glc-H2O-H]^-$; 637.7(90)$[M-rha-glc-H]^-$; 619.7(28)$[M-rha-glc-H2O-H]^-$; 475.4(41)$[M-$ rha–2glc–$H]^-$
8	Ginsenoside Rg1[a] (**7**)	801.03	6.04	800.1(100)$[M-H]^-$; 637.8(24)$[M-glc-H]^-$;475.5(3)$[M-2glc-H]^-$;	637.5(100)$[M-glc-H]^-$; 620.6(3)$[M-glc-H2O-H]^-$; 475.6(6)$[M-2glc-H]^-$
9	Malonyl ginsenoside Rb2/Rb3/Rc	1165.34	6.20	1164.3(19)$[M-H]^-$; 1120.2(5.3)$[M-CO2-H]^-$; 1032.1(100)$[M-ara-H]^-$; 988.3(18)$[M-ara-CO2-H]^-$; 970.3(100)$[M-ara-CO2-H2O-H]^-$	1164.4(14)$[M-H]^-$; 1120.2(6)$[M-CO2-H]^-$; 1076.2(9)$[M-2(CO2)-H]^-$; 988.5(100)$[M-ara-CO2-H]^-$; 970.4(20)$[M-ara-CO2-H2O-H]^-$; 928.3(8)$[M-ara-malonyl-H2O-H]^-$; 910.3(22)$[M-ara-malonyl-2(H2O)-$ $H]^-$; 784.1(4)$[M-ara-malonyl-glc-H]^-$
10	Malonyl ginsenoside Rd	1033.22	7.90	1032.1(2)$[M-H]^-$; 988.5(100)$[M-CO2-H]^-$; 946.5(7)$[M-malonyl-H]^-$; 784.1(1)$[M-malonyl-glc-H]^-$; 621.6(1)$[M-malonyl-2glc-H]^-$; 459.5(1)$[M-malonyl-3glc-H]^-$	1032.1(5)$[M-H]^-$; 970.1(5)$[M-CO2-H2O-H]^-$; 945.3(100)$[M-malonyl-H]^-$; 928.2(35)$[M-malonyl-H2O-H]^-$; 784.0(100) $[M-malonyl-glc-H]^-$; 765.9(4) $[M-malonyl-glc-H2O-H]^-$
11	Panax saponin C	794.98	15.20	794.0(100)$[M-H]^-$	618.3(13) $[M-CO2-rha-H]^-$; 587.7(38) $[M-CO2-glc-H]^-$; 569.7(100) $[M-CO2-glc-H2O-H]^-$
12	Ginsenoside Ra3 or Notoginsenoside R4	1241	15.27	1276.9(6)$[M + Cl]^-$; 1240.6(100)$[M-H]^-$; 928.1(6)$[M-xyl-glc-H2O-H]^-$; 622.5(35)$[M-xyl-3glc-H]^-$; 459.3(6)$[M-xyl-4glc-H]^-$	1108.4(63)$[M-xyl-H]^-$; 1078.2(88)$[M-glc-H]^-$; 945.8(25)$[M-xyl-glc-H]^-$; 928.3(50)$[M-xyl-glc-H2O-H]^-$; 784.0(100)$[M-xyl-2glc-H]^-$

13	Ginsenoside Rf[a] (**6**)	801.03	17.87	800.1(100)[M–H]$^-$	637.5(100)[M–glc–H]$^-$; 475.6(6)[M–2glc–H]$^-$
14	Ginsenoside Ra1 or Ra2	1211.41	24.50	1210.7(100)[M–H]$^-$; 1078.4(2)[M–xyl–H]$^-$; 946.2(0.7)[M–xyl–ara–H]$^-$; 784.0(3)[M–xyl–ara–glc–H]$^-$; 766.1(5)[M–xyl–ara–glc–H2O–H]$^-$; 622.5(7)[M–xyl–ara–2glc–H]$^-$; 604.5(16)[M–2H]$^-$	1078.3(72)[M–xyl–H]$^-$; 1049.5(4)[M–glc–H]$^-$; 917.1(4)[M–xyl–glc–H]$^-$; 898.6(100)[M–xyl–glc–H2O–H]$^-$; 784.0(4) [M–xyl–ara–glc–H]$^-$
15	Ginsenoside Ra3 or Notoginsenoside R4	1241	26.81	1240.8(100)[M–H]$^-$; 1108.6(6)[M–xyl–H]$^-$; 1078.4(100)[M–glc–H]$^-$; 946.2(2)[M–xyl–glc–H]$^-$; 784.0(10)[M–xyl–2glc–H]$^-$;766.1(15)[M–H]$^-$; 622.5(23)[M–xyl–3glc–H]$^-$	1108.4(100)[M–xyl–H]$^-$; 1078.2(47)[M–glc–H]$^-$; 945.8(16)[M–xyl–glc–H]$^-$; 784.0(6) [M–xyl–2glc–H]$^-$
16	Ginsenoside Rb1[a] (**1**)	1109.32	28.25	1108.6(100)[M–H]$^-$; 946.1(0.5)[M–glc–H]$^-$; 784.1(5)[M–2glc–H]$^-$	946.3(100)[M–glc–H]$^-$; 784.0(32)[M–2glc–H]$^-$; 621.6(8)[M–3glc–H]$^-$
17	Ginsenoside Rg2	785.03	28.82	820.13(5)[M + Cl]$^-$; 784.2(100)[M–H]$^-$; 61 637.7(3) [M–rha–H]$^-$; 475.5(1) [M–rha–glc–H]$^-$	638.5(44)[M–rha–H]$^-$; 9.7(100)[M–rha–H2O–H]$^-$; 475.7(31)[M–rha–glc–H]$^-$
18	Ginsenoside Rh1	638.89	32.00	673.9(100)[M + Cl]$^-$; 637.8(90)[M–H]$^-$; 475.8(6)[M–glc–H]$^-$	475.9(100) [M–glc–H]$^-$
19	Ginsenoside Ra1 or Ra2	1211.41	40.65	1210.7(100)[M–H]$^-$; 1078.4(0.6)[M–xyl–H]$^-$; 1047.7(2)[M–glc–H]$^-$; 946.0(2)[M–xyl–arap–H]$^-$; 784.0(4)[M–xyl–ara–glc–H]$^-$; 766.0(8)[M–xyl–ara–glc–H2O–H]$^-$; 622.0(3)[M–xyl–ara–2glc–H]$^-$; 604.6(13)[M–2H]$^-$; 538.3(9)[M–xyl–2H]$^-$; 459.9(0.4)[M–xyl–ara–3glc–H]$^-$	1078.3(100)[M–xyl–H]$^-$; 766.1(100)[M–xyl–ara–glc–H2O–H]$^-$
20	Ginsenoside Rc[a] (**3**)	1079.29	40.70	1078.5(100)[M–H]$^-$; 946.1(1)[M–araf–H]$^-$	946.2(100)[M–araf–H]$^-$; 910.9(55)[M–araf–2(H2O)–H]$^-$; 783.9(100)[M–araf–glc–H]$^-$; 765.9(15)[M–araf–glc–H2O–H]$^-$; 622.5(15)[M–araf–2glc–H]$^-$
21	Ginsenoside Rb2[a] (**2**)	1079.29	48.72	1078.4(100) [M–H]$^-$	946.3(100)[M–arap–H]$^-$; 915.9(57)[M–glc–H]$^-$; 784.1(36)[M–arap–glc–H]$^-$; 765.9(13)[M–arap–glc–H2O–H]$^-$; 622.4(13)[M–arap–2glc–H]$^-$
22	Ginsenoside Rb3	1079.29	51.32	1078.4(100) [M–H]$^-$; 784.3(95)[M–glc–xyl–H]$^-$; 766.1(9)[M–glc–xyl–H2O–H]$^-$; 621.7(7)[M–2glc–xyl–H]$^-$; 459.7(1)[M–3glc–xyl–H]$^-$	1060.4(5.4)[M–H2O–H]$^-$; 946.1(23)[M–xyl–H]$^-$; 915.9(7)[M–glc–H]$^-$; 784.1(100)[M–xyl–glc–H]$^-$
23	Ginsenoside Rd[a] (**4**)	947.17	63.72	946.3(100) [M–H]$^-$	784.0(100)[M–glc–H]$^-$; 766.0(8)[M–glc–H2O–H]$^-$; 621.7(39)[M–2glc–H]$^-$; 460.5(4)[M–3glc–H]$^-$
24	Ginsenoside Rs1/Rs2	1122.23	63.71	1121.4(100)[M–H]$^-$; 945.9(11)[M–Ac–ara–H]$^-$; 930.5(36)[M–AcO–ara–H]$^-$; 784.0(8)[M–glcAc–ara–H]$^-$; 766.0(8)[M–glcAc–ara–H2O–H]$^-$; 621.6(10)[M–glcAc–ara–glc–H]$^-$; 459.6(6)[M–glcAc–ara–2glc–H]$^-$	1120.4(100)[M–H]$^-$; 1078.5(30)[M–Ac–H]$^-$; 1062.7(33)[M–AcO–H]$^-$; 988.1(1)[M–ara–H]$^-$; 916.2(4)[M–glcAc–H]$^-$
25	Ginsenoside Rs1/Rs2	1122.23	72.07	1121.4(10)[M–H]$^-$; 946.3(100)[M–Ac–ara–H]$^-$; 930.0(1)[M–AcO–ara–H]$^-$; 917.3(1)[M–glcAc–H]$^-$; 784.8(2)[M–glcAc–ara–H]$^-$; 766.0(6)[M–glcAc–ara–H2O–H]$^-$; 753.8(3)[M–glcAc–glc–H]$^-$; 621.1(5)[M–glcAc–ara–glc–H]$^-$; 459.3(0.4)[M–glcAc–ara–2glc–H]$^-$	1078.5(34)[M–Ac–H]$^-$; 946.3(100)[M–Ac–ara–H]$^-$; 784.1(7)[M–glcAc–ara–H]$^-$
26	Ginsenoside Rg3	785.03	74.10	784.0(100) [M–H]$^-$; 621.6(15) [M–glc–H]$^-$	621.5(100)[M–glc–H]$^-$; 459.5(35)[M–2glc–H]$^-$
27	Ginsenoside Rh2	622.89	77.00	621.9(100)[M–H]$^-$; 1243.5(45)[2M–H]$^-$	538.4(86)[M–C6H11–H]$^-$; 459.8(100)[M–glc–H]$^-$

ginsenosides confirmed using comparisions with standard compounds, a total of 27 ginsenosides was detected from ginseng hairy root by LC/MS.

The peak area of SIC for each ginsenoside was normalized using the SIC of aloesin as an internal standard (Fig. 3(a)). The ginsenoside Re5 was detected at the highest level in most of the hairy roots, followed by Rb_1 **1**, while Rd4 and Rf6 were detected at very low levels. Except for the seven ginsenosides **1**–**7** used as standards, most of the ginsenosides were detected at trace levels, with the exception of ginsenoside **1**–**7** such as Rg_2, Ro, Rs_1/Rs_2 and the quinquenoside R1 which were found at higher levels in several hairy root lines (data not shown).

To quantify the seven ginsenosides using UV spectrometry, the HPLC system was adjusted to the optimum condition in order to obtain a retention time for ginsenoside similar to that obtained by LC/MS profiling (Fig. 4(a)). We confirmed that the standard deviation of the quantitative data set of each ginsenoside detected by UV spectrometry was similar to that of the relative response value derived from the SIC peak area detected by LC/MS (data not shown). These results indicate that

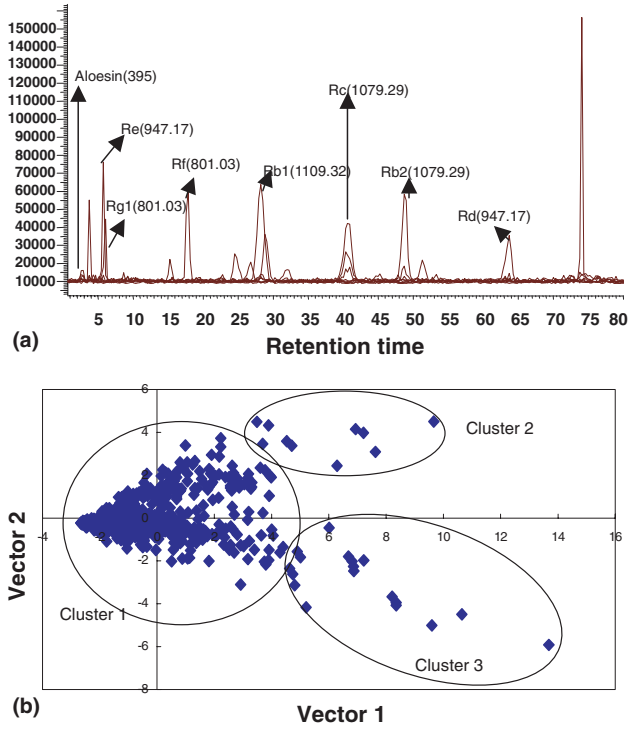


Fig. 3. LC/MS profiling of ginsenosides and principal component analysis of metabolic profiles of ginseng hairy roots. (a) The SICs corresponding to the seven ginsenosides are obtained by HPLC/SSI/MS of hairy root extracts, and the each SIC area of them was used for statistical analysis. The peaks corresponding to the seven ginsenosides are indicated. (b) Clusters found after PCA of ginsenoside profile data from 603 ginseng hairy root lines. The distances between hairy root samples were calculated as described in Section 3 by log-transformation. PCA vectors span a 7-dimensional space to give the best sample separation, with each point representing a linear combination of the seven ginsenosides from an individual sample. Vectors 1 and 2 were chosen for best visualization of differences between hairy root lines and include 87.9% of the total information content derived from ginsenoside variance. Each data point represents an independent sample. Hairy roots were sorted into three clusters as marked.

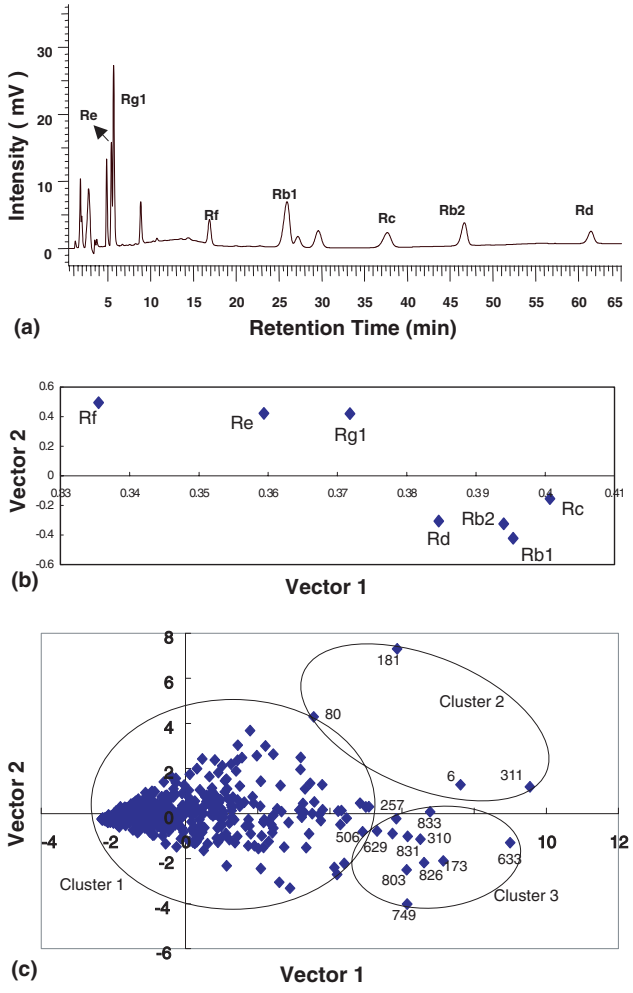


Fig. 4. HPLC-UV profiling of ginsenosides **1**–**7** and principal component analysis of metabolic profiles of ginseng hairy roots. (a) HPLC-UV analysis of crude extracts from ginseng hairy roots. The peaks corresponding to the seven ginsenosides **1**–**7** are indicated. (b) The impact of individual ginsenosides on clustering results. The contribution of individual ginsenosides to the PCA vector calculation is computed by linear combination. Vector 2 predominantly separates hairy roots, which have different ratios of Rb (Rb_1, Rb_2, Rc and Rd) to Rg (Re, Rf and Rg_1) group ginsenosides. (c) Clusters found after PCA of ginsenoside profile data from 463 ginseng hairy root lines. The distances between hairy root samples were calculated as described in Section 3 and Fig. 3 legends. Vectors 1 and 2 were chosen for the best visualization of differences between hairy root lines with a 69.7% cumulative proportion. Each data point represents an independent sample. Most of the samples were sorted into one cluster, and 15 samples were separated as outliers and sorted into two clusters. Cluster 1 includes most of the hairy root lines. Hairy roots in clusters 2 and 3 have high levels of ginsenoside content and show variation in the Rb/Rg ratio.

both detection methods are applicable for profiling ginsenoside content in different plant tissue samples.

The induction and establishment of ginseng hairy roots by infection of *A. rhizogenes* and the detection and quantification of major ginsenosides have been performed previously (Bulgakov et al., 1998; Mallol et al., 2001; Shu et al., 1999). However, to our best knowledge, this is the first report in which 27 ginsenosides have been detected in ginseng hairy roots and is also the first demonstration of ginsenoside profiling in a large population of genetically transformed ginseng root lines.

2.3. Principal component analysis and hierarchical cluster analysis of ginsenoside accumulation in ginseng hairy root lines

To facilitate the analysis of ginsenoside profile data from 603 hairy roots and the selection of ginseng hairy roots with variations in ginsenoside accumulation, two pattern analysis methods, hierarchical component analysis (HCA) and principal cluster analysis (PCA), were applied. PCA uses an *N*-dimensional vector approach to separate samples on the basis of the cumulative correlation of all metabolite data and then identifies the vector (eigenvector) that yield the greatest separation among samples. The results from the chosen vector can be displayed in two or three dimensions. As applied to our data, hairy roots in the same cluster could be regarded as having a specific metabolic phenotype. Fig. 3(b) illustrates PCA of 603 hairy roots for seven ginsenosides. This indicated that most of the samples were clustered in one group and a few samples were distinguished as outliers. Although hairy roots were generated from three different *Agrobacterium* (Table 1), they were not grouped into discrete clusters. These results indicate that PCA of LC/MS data cannot discriminate between wild type hairy roots and activation tagged hairy roots. This is not surprising since in activation tagged hairy roots, most of the enhancer elements will not have an effect on gene expression. Therefore, we attempted pattern analysis for all ginseng hairy roots to identify the hairy root lines that varied in ginsenoside production. Outlier samples were re-confirmed by HCA. Based on these results, we can generate three clusters, as shown in Fig. 3(b).

In this analysis, we used only seven ginsenosides **1**–**7** as standard controls, although 27 different types of ginsenoside were detected in hairy roots. Thus, we wondered whether the profile of these seven ginsenosides was representative of all ginsenosides in hairy roots. To determine this, we extracted relative MS values of 27 ginsenosides in 42 hairy roots selected from 3 clusters, and performed PCA analysis. We also applied the PCA method to profile 20 ginsenosides (excluding the 7 standard ginsenosides) from 42 selected hairy root lines. The results indicated that hairy roots selected from three different clusters were reclassified into the same pattern (data not shown), which suggests that the seven standard ginsenosides **1**–**7** are sufficient for profiling the total ginsenoside pattern in ginseng hairy roots.

On the basis of these results, it appears that clustering of hairy roots by the PCA method depends on the quantity rather than the quality of each ginsenoside. In terms of ginsenoside quantification, the HPLC-UV approach is more powerful than the LC/MS method. Thus, we applied the HPLC-UV system to check the absolute content of the seven standard ginsenosides **1**–**7** in 74 selected hairy root lines; 17 lines from cluster 2 and the boundary, 47 from cluster 3 and the boundary and 10 from cluster 1. In addition, the absolute ginsenoside contents of 390 activation tagged ginseng hairy root lines were obtained. Finally, from the total 464 ginseng hairy root lines, the seven ginsenosides **1**–**7** were profiled using HPLC-UV and PCA. After applying PCA, the results from the chosen vectors were displayed in two dimensions (Fig. 4). As seen previously by LC/MS analysis, hairy root samples were classified into three clusters (Fig. 4). Most of the samples constituted one cluster and 15 samples were separated as outliers. PCA also can be used to analyze which metabolites exert the largest influence on the basic vector calculation. When PCA was applied to the seven ginsenosides **1**–**7** among 603 hairy roots, the ginsenosides **1**–**7** were clearly classified into two clusters and were identical to the ginsenoside types from two different aglycones (Fig. 4(b)). PCA vector 2 was strongly influenced by Rg group ginsenosides. This suggests that the outlier samples may differ in their Rb/Rg ratio. Of the 15 outlier samples, only 4 were separated into cluster 2 and the other 11 samples were assigned to cluster 3. Table 3 summarizes the ginsenoside content and Rb/Rg ratio of selected hairy root lines. All of the samples classified into clusters 2 and 3 had a higher ginsenoside content than the samples in cluster 1. Hairy root lines in cluster 2 have low Rb/Rg ratio whereas those in cluster 3 have a high Rb/Rg ratio.

2.4. Selection of ginseng hairy root lines showing variation in ginsenoside content

The ginsenoside content of hairy roots of clusters classified by PCA analysis are summarized in Table 3 and Fig. 5(a). PCA analysis allowed us to select hairy root lines with significant variation in the accumulation of ginsenosides, especially total ginsenoside content, as well as in Rb/Rg ratio. Hairy root lines 311 and 633 have the highest total ginsenoside content with at approximately 4–5 times higher than that of the hairy roots in cluster 1 (Fig. 5(b)). We also selected hairy root lines that varied in Rb/Rg ratio. Hairy root line 181 (cluster 2) has a typical, low Rb/Rg ratio, whereas line 749 in cluster 3 has a high Rb/Rg ratio (Fig. 5(c)). In most hairy root lines, ginsenoside Re5 was detected at

Table 3
Content of seven ginsenosides in selected ginseng hairy roots

Hairy root line	Ginsenoside content (mg/g dry weight)							Rb/Rg ratio[a]	Total content
	Rb_1 (**1**)	Rb_2 (**2**)	Rc (**3**)	Rd (**4**)	Re (**5**)	Rf (**6**)	Rg_1 (**7**)		
6	2.89	1.05	0.73	0.53	5.17	0.42	0.94	0.80	11.73
80	0.55	0.20	0.24	0.15	6.21	0.20	1.59	0.14	9.13
181	0.47	0.34	0.28	0.21	2.08	0.99	1.20	0.30	5.55
311	3.42	0.93	1.65	0.45	9.10	0.19	1.38	0.60	17.12
173	2.71	1.49	1.31	0.48	2.81	0.12	1.02	1.52	9.94
257	3.75	0.82	1.05	0.23	6.11	0.21	0.34	0.88	12.51
310	3.63	1.55	0.71	0.32	4.85	0.25	0.42	1.13	11.72
506	1.40	2.39	0.25	0.11	1.00	0.21	1.25	1.68	6.60
629	4.21	0.74	0.60	0.49	2.96	0.22	0.87	1.49	10.10
633	5.13	0.92	0.83	0.89	4.63	0.25	1.27	1.27	13.91
749	5.47	1.33	0.68	0.55	3.55	0.06	0.27	2.07	11.90
803	6.05	0.74	0.94	0.38	4.04	0.05	0.76	1.67	12.96
826	5.87	0.81	0.60	0.59	2.52	0.19	0.91	2.17	11.50
831	2.54	0.86	1.52	0.37	2.31	0.20	0.85	1.58	8.66
833	2.84	0.86	1.05	0.48	2.93	0.21	1.46	1.13	9.83
167	0.93	0.45	0.27	0.19	1.77	0.22	1.64	0.51	5.48
317	0.96	0.25	1.57	0.07	3.00	0.07	0.05	0.91	5.96
332	0.60	0.17	0.13	0.08	8.06	0.04	0.20	0.12	9.27

[a] Ratio of Rb group ginsenoside (Rb_1, Rb_2, Rc and Rd **1**–**4**) to Rg group ginsenoside (Re, Rf and Rg_1 **5**–**7**).

the highest level, followed by Rb_1 **1** and Rg_1 **7**. Ginsenosides Rb_2 **2** and Rc **3** were detected at a low level and Rd4 and Rf6 were detected at very low or trace levels. Some of the hairy roots exhibited variation in ginsenoside accumulation pattern, with a specific ginsenoside being accumulated at a high level but others being pre-

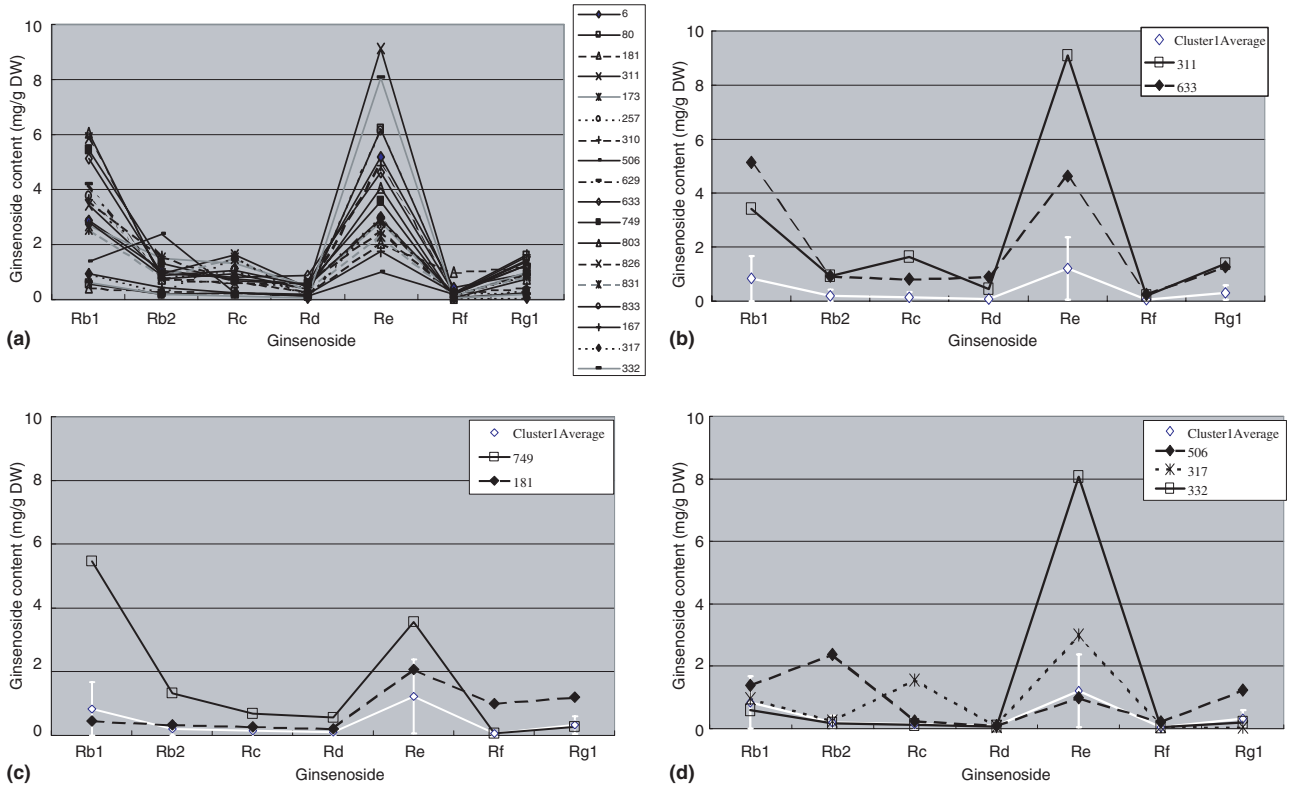


Fig. 5. Ginsenoside content of selected ginseng hairy root lines. (a) Eighteen hairy root lines which have ginsenosides at a high level and different ginsenoside patterns were selected by pattern analysis of ginsenoside profile data from 464 hairy root lines. (b) Hairy root lines 311 and 633 have a ginsenoside content level 4–6 times higher than average. (c) Hairy root lines 181 and 749 have typical low and high Rb/Rg ratios, respectively. (d) Hairy root lines which have high level ginsenoside especially in ginsenoside Re (line 332), Rb2 (line 506) and Rc (line 317). See Table 3 for the ginsenoside content in selected hairy root lines. Cluster 1 average was calculated from 449 hairy root lines classified in cluster 1 (Fig. 4(c)).

sent at a level that was average for cluster 1. For example in line 332, ginsenoside Re **5** specifically accumulated at 6.5 times the average level for cluster 1 (Fig. 5(d)). In addition, hairy root lines 506 and 317 had high levels of ginsenosides Rb_2 **2** and Rc **3**, respectively (Fig. 5(d)).

Metabolites representing the end products of gene expression thus metabolic profiling can provide a broad view of the biochemical status or biochemical phenotype of an organism that can be directly linked to gene function (Raamsdonk et al., 2001; Roessner et al., 2001). Ginsenosides, which are triterpene glycosides (saponins), are synthesized via the isoprenoid biosynthetic pathway in the cytosol (Haralampidis et al., 2002; Jung et al., 2003). A triterpene aglycone of the ginsenoside, protopanaxadiol, was synthesized from the 2,3-oxidosqualene by a dammarenediol synthase (DS), and was then modified at the C-6 position resulting in protopanaxatriol (Haralampidis et al., 2002). This modification reaction should normally be mediated by a cytochrome P450. Finally, different ginsenosides are synthesized by adding one or several monosaccharides to these triterpene aglycones by glycosyltransferase (GT). GT activity has been shown to correlate with saponin production in root cultures of *G. paniculata* (Herold and Henry, 2001) but little is known about the genes and biochemical pathways that regulate ginsenoside biosynthesis (Haralampidis et al., 2002). Thus, ginseng hairy roots with differential ginsenoside content can be used to identify and characterize genes involved in ginsenoside biosynthesis. For example, hairy root lines 311 and 633 which accumulate ginsenosides at high levels could be useful to identify the genes such as a OSC, P450, GTs and transcription factors that regulate ginsenoside biosynthesis. Hairy root lines 181 and 749, which show variation in Rb/Rg ratios, can be used to investigate cytochrome P450, which is involved in the modification of the C-6 position of protopanaxadiol. Similarly, hairy root lines 506 and 332, which have a high content of ginsenosides Rb_2 **2** and Re **5**, respectively, can be used as a source to study GTs, which are involved in Rb_2 **2** and Re **5** biosynthesis. The uses of hairy root lines will, furthermore, facilitate the design of the microarray experiments for selection of candidate genes involved in ginsenoside biosynthesis from EST collections (Jung et al., 2003).

Here, we have demonstrated that metabolic profiling by LC/MS and HPLC-UV could be used for the detection of qualitative and quantitative variation in ginsenoside patterning and content. On the basis of HCA and PCA with the ginsenoside profile dataset, we determined a means of selecting specific lines with significant variations in ginsenoside pattern. Selected hairy root lines could be used to mine genes related to secondary metabolites and their production/regulatory pathways. Hairy root lines can be produced from many plants that generate secondary metabolites. Therefore, this approach could be applied to other plants to elucidate the biosynthesis of various metabolites.

3. Experimental

3.1. Bacterial strains and plant materials

Korean ginseng, *Panax ginseng* C.A Meyer, cv. Chunpoong developed by KT&G (Korea Tobacco and Ginseng) was used as the plant material. After removing the seed coat, the ginseng seeds were sterilized with 70% ethanol for 30 min and then with 0.4% sodium hypochlorite for 15 min, following which they were rinsed with autoclaved water. Sterilized seeds were germinated on 1/2 MS medium and cotyledons and hypocotyls from 3-week-old in vitro cultured ginseng seedlings were sliced to less than 1 cm. These were used as materials for co-cultivation with *Agrobacterium rhizogene* s. *A. rhizogenes* strains A4 or R-1000 were purchased from KCTC and used as host strains for hairy root development.

3.2. Induction and generation of ginseng hairy root lines

Ginseng tissues were dipped in *Agrobacterium* suspension for 30 min, transferred to a solidified 1/2 MS medium with 0.4% Gelrite, and cultured at 27 °C in the dark for 2 days. Tissues were washed with 1/2 MS liquid medium with cefotaxime (800 mg/l) and transferred to the selection medium, 1/2 MS medium with of cefotaxime (400 mg/l) and kanamycin(100 mg/l). The selection medium was changed every 3 weeks to eliminate *Agrobacterium*. To generate activation tagged ginseng hairy roots, a vector, pKH01, which is based on pCAMBIA2300 and includes the tetramer enhancer of the 35S promoter, was constructed. This activation tagging vector, pKH01, was introduced into *A. rhizogenes* R-1000 and the enhanced element was co-introduced into the ginseng genome with wild type T-DNA.

To establish independent hairy root lines, a hairy root was cut from each of the tissue segments infected with *Agrobacteriu* m. When selecting two hairy root lines from a particular tissue segment, we selected them from different infection sites of a segment. Selected hairy roots were cultured on hormone-free 1/2 SH agar medium at 25 °C in the dark. Hairy root lines were sub-cultured at 4-week intervals.

3.3. Chemicals and reagents

Ginsenosides, protopanaxadiol glycosides (Rb_1 **1**, Rb_2 **2**, Rc **3** and Rd **4**; Rb group) and protopanaxatriol glycosides (Re **3**, Rf **6** and Rg_1 **7**; Rg group), were purchased from INDOFINE Chemical Company, Inc (Somerville, NJ, USA) at a purity verified to be >99% by HPLC–MS/MS. These compounds were used as

reference compounds. A stock solution of a mixture of reference compounds was prepared using methanol (100μg/ml of each compound) and stored at −15 °C.

3.4. Extraction and analysis of ginsenosides

Ginsenosides were extracted with 80% MeOH (1 ml) from freeze-dried ginseng hairy root tissue (20 mg) and sonicated for 1 h at 40 °C. The methanol extract was centrifuged at 1000 rpm for 10 min and evaporated. The dried pellet was re-extracted with water-saturated *n*-BuOH. After evaporating the *n*-BuOH phase to dryness, a weighed pellet was dissolved to a final concentration of 2 μg/μl.

To profile ginsenosides using an HPLC/UV/MS system, ginsenoside extract (40 μg) were individually subjected to HPLC separation using a reversed phase, C18, 5 um, 4.6 × 250 mm column (Capcell PAK, Shiseido, Japan) followed by gradient elution with H_2O : CH_3CN, 70:30 to 66:34 for 65 min and then washed with 100% CH_3CN for 95 min at a flow rate of 0.9 ml/min. All mass spectra profiling was performed using the 3DQ LC/Ion Trap system (Hitachi High Technologies America, Inc., San Jose, CA, USA) equipped with a sonic spray ionization interface (SSI). Mass spectrometer conditions were optimized in order to achieve maximum sensitivity with 1% Triethylamine (TEA) buffer solution. Calibration of the mass range (200–1200 Da) was performed in positive ion mode, using the standard calibration procedure and compounds (TBA, Reserpine, Bredkinin).

Full scan mass spectra were acquired from 300 to 1200 amu at unit mass resolution. For stepwise fragmentation experiments, data-dependent scanning was selected and the wideband activation turned off. The normalized collision energy was set to 50%. Seven ginsenosides **1**–**7** were quantified by UV absorption at 203 nm. The ginsenoside content of the samples subjected to the same separation method as that used for mass spectral profiling was calculated using the external standard method, and then expressed as mg ginsenoside/g DW (dry weight). Calibration curves were established with the seven ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf, and Rg_1 **1–7** at concentrations of 6.25–100 μg/ml methanol.

3.5. Cluster analysis

Principle component analysis (PCA) and hierarchical cluster analysis (HCA) were performed with the SAS software program (V8, SAS Institutes Inc., USA). PCA clustering was applied to differentiate the individual hairy root lines following variation in targeted component composition. PCA was undertaken based on the total correlation matrix derived from the peak area of SIC of each putative ginsenoside. A scatter diagram of the first two components, in which a single sample is represented by a point, was plotted to provide a two-dimensional graphical display of the data. We applied a hierarchical clustering algorithm to samples using the Pearson correlation coefficient.

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