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ORIGINAL ARTICLE

Metabolic dynamics and physiological adaptation of *Panax ginseng* during development

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Abstract

Key message **The dynamics of metabolites from leaves to roots of *Panax ginseng* during development has revealed the tissue-specific and year-specific metabolic networks.**

Abstract Being an essential Oriental medicinal plant, ginseng (*Panax ginseng* Meyer) is a slow-growing perennial herb-accumulating pharmaceutically active metabolites such as ginsenosides in roots during growth. However, little is known about how ginseng plants survive in the harsh environments such as winter cold and summer heat for a longer period and accumulates those active metabolites as the plant grows. To understand the metabolic kinetics in both source and sink organs such as leaves and roots of ginseng plant, respectively, and to assess the changes in ginsenosides biosynthesis during ginseng growth, we investigated the metabolic profiles from leaves and roots of 1-, 4-, and 6-year-old field-grown ginseng plants. Using an integrated non-targeted metabolomic approach, we identified in total 348 primary and secondary metabolites, which provided us for the first time a global metabolomic assessment of ginseng during growth, and morphogenesis. Strikingly, the osmoprotectants and oxidized chemicals were highly accumulated in 4- and 6-year-old ginseng leaves suggested that ginseng develop a wide range of metabolic strategies to adapt unfavorable conditions as they mature. In 6-year-old plants, ginsenosides were decreased in leaves but increased in roots up to 1.2- to sixfold, supporting the view that there is a long-distance transport of ginsenosides from leaves to roots as ginseng plants mature. Our findings provide insights into the metabolic kinetics during the development of ginseng plant and this could complement the pharmacological importance of ginseng and its compounds according to their age.

Keywords *Panax ginseng* · Development · Ginsenosides · Metabolomics · Osmoprotectants

Introduction

Ginseng (*Panax ginseng* Meyer) is an adaptogenic herb in the *Araliaceae* family that has been cultivated in East Asia for thousands of years. The ginseng roots were shown to have antitumor, antidiabetic, antiaging, immune and improving cognitive function and are rich in secondary metabolites (Kim et al. 2015). Among the known metabolites in ginseng roots, triterpene saponins (also called ginsenosides) are nearly exclusively found in ginseng and widely recognized as the main active ingredient that contributes to its pharmacological efficacy. The backbone of ginsenosides are dammarenediol, which is being cyclized from squalene mostly via the mevalonic acid pathway (MVP) in the cytosol, including the protopanaxadiol (PPD) and the protopanaxatriol (PPT) groups (Oh et al. 2014; Kim et al. 2015).

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Yu-Jin Kim and Sung Chul Joo contributed equally to the work.

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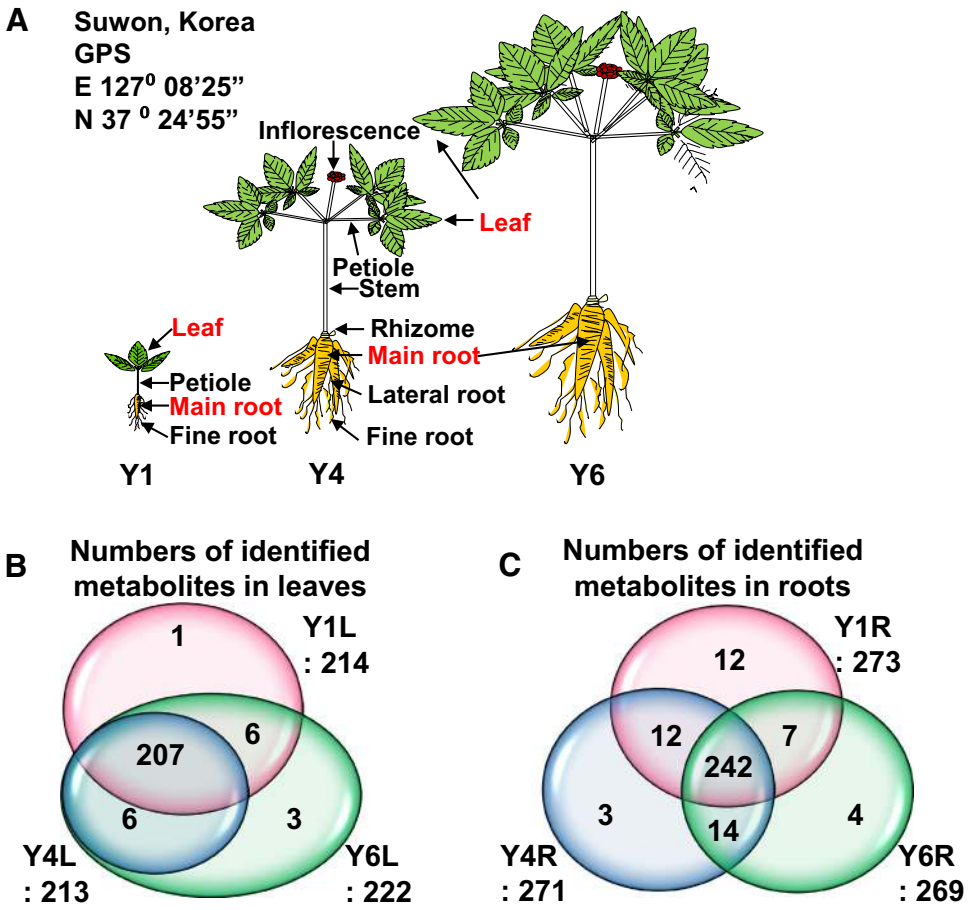

Fig. 1 Geographic location, morphology, and detection of metabolites in different ages and tissues of *Panax ginseng*. **a** Different morphologies of ginseng plants during development. Comparison of the number of metabolites identified from leaves (**b**) and roots (**c**). The drawing and painting were produced using PPT software by Kim and Joo based on Table S1

Ginseng is a slow-growing perennial herbaceous plant. The aboveground part shows distinct morphologies during vegetative development in different growth years. First-year (Y1) ginseng plant produces a single compound leaf without stem and in subsequent years until the fourth (Y4) to sixth (Y6) growth years, the plant develops more leaves that are attached to petioles on the stem (Fig. 1a). Ginseng plant starts to flower in the third year, and usually produce seeds from the fourth year. The tap root grows slowly during the first 3 years, and increases in diameter from the fourth year to sixth year, and then it can be harvested. A Y6 ginseng root is about 7–10 cm in length, about 3 cm in diameter, and contains several lateral roots and abundant fine roots. It is regarded as the mature ginseng because it has the best mass and shape as well as the highest amounts of secondary metabolites (Lin et al. 2010; Kim et al. 2015). During the ginseng cultivation, one major challenge is that ginseng plants are easily suffered from environmental stresses such as high light, salinity, and fungal infection, thus making them less survivable at Y6 (Shin et al. 2016). Intriguingly, ginseng roots accumulate more ginsenosides as they get older, whereas the leaves accumulate the maximal amount of ginsenosides during the first or second growth year (Wang et al. 2006; Shi et al. 2007). We hypothesized that more accumulated metabolites in older roots may be attributable to the high pharmacological value of Y6 roots. Systematic understanding on how ginseng plants synthesize, transport and accumulate metabolites in the leaves and roots during their growth remains scanty.

Recently, various metabolomics platforms including nuclear magnetic resonance spectroscopy (NMR) and liquid chromatography–mass spectrometry (LC/MS) have been successfully used to identify cultivation age of ginseng (Shin et al. 2007, 2016; Lin et al. 2010; Kim et al. 2011; Yang et al. 2012; Shan et al. 2014; Xiao et al. 2015; Liu et al. 2017a). Metabolic profiling using LC/MS was successfully applied to discriminate *Panax* species or organs (Dan et al. 2008; Yang et al. 2013; Chen et al. 2014; Mao et al. 2014; Park et al. 2014; Qiu et al. 2016). However, these studies mainly focused on the authentication of commercialized ginseng products and mostly targeted ginsenosides. Using gas chromatography–mass spectrometry (GC/MS), 11 fatty acids and 23 nonpolar primary metabolites were identified from *Panax* of different ages (Zhang et al. 2013a, b; Cui et al. 2015), and 91 and 109 metabolites were identified from 2- to 5-year-old roots and 1- to 5-year-old different tissues of *P. ginseng*, respectively (Cui et al. 2015; Liu et al. 2017b). A study using Fourier transform infrared spectral analysis

to discriminate ginseng leaves of 1- to 3-year-old suggested that the metabolome of ginseng leaves get alter depending on the age of the plant (Kwon et al. 2014). Despite these insights provided by abovementioned studies, fewer studies have focused on the metabolic kinetics of whole metabolites both in ginseng leaves and roots along different growth years.

To reveal the metabolic change along ginseng development, we examined metabolomic profiles in the source tissue leaves and the sink tissue roots from Y1, Y4, and Y6 plants, using an integrated GC/MS and ultra-performance liquid chromatography (UPLC)/MS/MS. We identified a total of 348 primary and secondary metabolites in ginseng tissues. Notably, the number of identified metabolites was at least three times higher than those of previous analyses of ginseng plant or related species. Remarkable dynamic changes of metabolites were observed in ginseng roots and leaves along the ginseng development, which were in good agreement with the developmental stage induced physiological and morphological changes. Moreover, our results suggested a flow of ginsenosides from the leaf to the root as ginseng matures, providing important insights into how ginsenosides accumulate in ginseng roots.

Results

Metabolic profiling of ginseng leaves and roots

To understand the global metabolic changes in ginseng during its development, we collected leaves and roots from Y1, Y4 and Y6 ginseng plants. Using an established metabolic profiling approach with GC/MS and UPLC/MS/MS, we identified 323 and 416 metabolites from leaves and roots, respectively; among them, 223 metabolites from leaves and 293 from roots had known structures while the remaining were unknown. From the total population, we assessed 348 metabolites with known structures, out of those 55 metabolites were from leaves and 125 were from roots specifically, and 168 metabolites were found common to both leaves and roots (Table S1, Fig. 1b). The number of identified metabolites was much higher than previous metabolic studies in ginseng plants, which facilitated further statistic analyses.

Those 348 metabolites of known structures have covered most of the known central metabolic pathways in plants, thus reflecting the final results of the corresponding physiological activities of ginseng leaves and roots in the tested three developmental stages. These metabolites were mapped onto eight metabolic super-pathways and their underlying 47 sub-pathways (Table S2), as defined by the Plant Metabolic Net (PMN) and Kyoto Encyclopedia of Genes and Genomes (KEGG). They included primary metabolites such as most amino acids and their derivatives, carbohydrates, lipids, cofactors, prosthetic groups and electron carriers (CPGECs), nucleotides, as well as 32 secondary metabolites.

Global metabolic changes in different growth years

To obtain a global view of the metabolic changes during ginseng development, principal component analysis (PCA) was performed on the identified metabolites. PCA showed a clear separation of metabolites among Y1, Y4, and Y6 leaves (Fig. 2a) and roots (Fig. 2b). In leaves, the first principal component (PC1), which accounted for 48.39% of the total variance, separated young leaves (Y1L) and mature leaves (Y4L and Y6L). Similarly, the second principal component (PC2), which accounted for the left 30.75% of the total variance, separated Y4L from Y6L. In roots, PC1 and PC2 that accounted for 40.97% and the left 22.26% of the total variance, respectively, resolved the classification of Y1R, Y4R, and Y6R. Finally, the metabolomes of Y1L and Y1R were distinguishable from the corresponding tissue samples of other growth years, consistent with the distinctive morphological and developmental features of first-year plants (Fig. 1a).

Hierarchical cluster analysis revealed considerable changes in the levels of a wide range of amino acids, carbohydrates, fatty acids, cofactors and secondary metabolites in leaves and roots across the ginseng development (Fig. 3, Tables S3, S4). The levels of many amino acids increased while the amount of several lipids and peptides decreased over growth years in both leaves and roots. Notably, the main difference in metabolites between leaves and roots were in secondary metabolites which showed decreased levels of phenylpropanoids and terpenoids in Y6L while increased levels of them in Y6R (Fig. 3).

Specific metabolic changes in different growth years of leaves and roots

Amino acid metabolism

From the detected amino acids, the amount of glutamate family amino acids (GFAAs) displayed the most significant changes in both leaves and roots (Figs. 3, 4, 5, 6; Tables 1, 2). In particular, the levels of the four nitrogen (N)-rich amino acids, Glu, Gln, Asp and Asn were highly increased in roots as plant aged. Gln showed the largest changes (17-fold), followed by Asn and Glu, which were more than fivefold in Y6R as compared with that in Y1R (Table S6). The increased GFAA metabolism seemed to be linked with the accumulation of polyamines and ornithine in leaves and roots. The obvious accumulation of polyamine in Y4R and Y6R is consistent with the previous report that polyamine stimulates ginseng root growth (Park et al. 1990). As ginseng grew, the levels of all of the

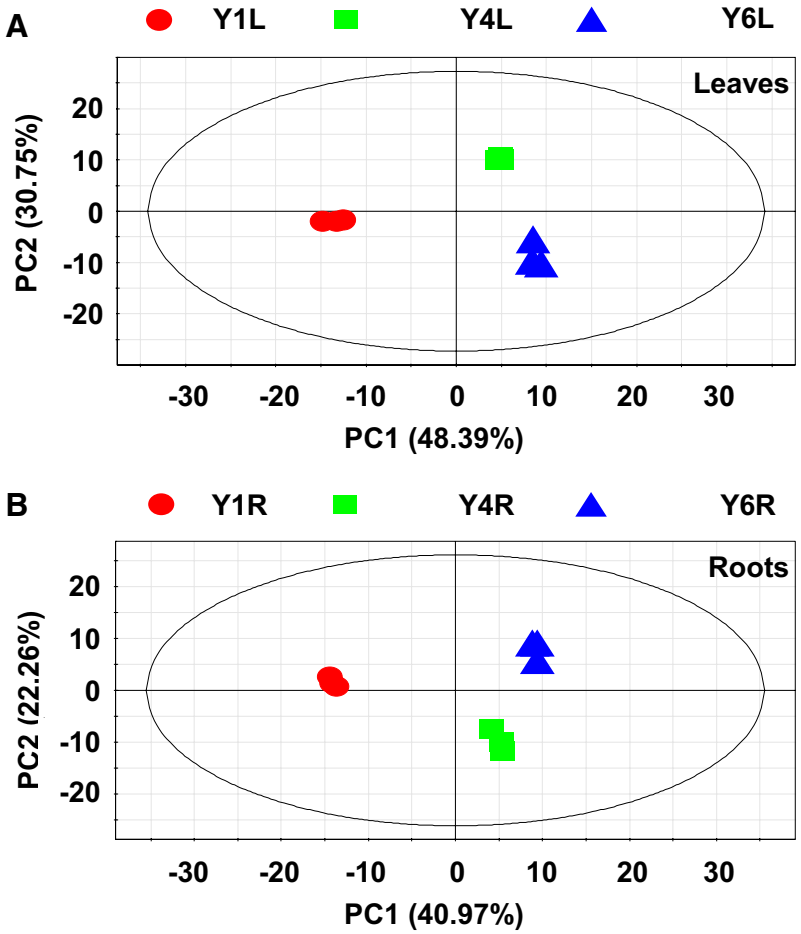

Fig. 2 PCA of the whole *P. ginseng* metabolome. PCA was conducted by MultiExperiment Viewer, on 223 and 293 annotated metabolites identified from leaves (**a**) and roots (**b**), respectively. **a** Two PCs explain 79.14% of variance that separates Y1L, Y4L and Y6L. **b** Two PCs explain 63.23% of variance that separates Y1R, Y4R, and Y6R. PC1 and PC2 scores are indicated as %. Circles, triangles, and squares indicate three biological replicates of Y1, Y4, and Y6 plants, respectively

serine family amino acids (SFAAs), except the precursor Gly, increased to threefold in roots and in leaves (Fig. 6). Consistent with the high accumulation of Ser in Y6L and Y6R, betaine, an osmoprotectant associated with drought/salt response in plants (Ros et al. 2014), was the highest accumulated metabolite in Y4L (tenfold) and Y6L (27-fold) as compared with that in Y1L (Tables 1; S3, S5).

Free amino acids, water soluble proteins, peptides, amines, free nucleotides, nucleic acid bases and alkaloids were highly accumulated in ginseng roots, which may facilitate the accumulation of metabolites as the ginseng root increases in size after 4 years (Fig. 4). High accumulation of N-containing metabolites in ginseng roots may contribute to the Y6R roots in terms of resisting roots, hence the better weight, shape, and quality of Y6R. Previously, peptides in ginseng root possibly have the function of potentially antilipolytic, and antidiabetic activities (Ando et al. 1980; Ye et al. 2016), thus we speculate that the high level of peptides in ginseng roots contributes to ginseng pharmacological activities.

Sugar and lipidic metabolites

The Y4L accumulated the highest level of sucrose (Fig. 3), suggesting a high demand of carbohydrates in leaves (source organ) of Y4-plants. Among the changes in leaves over the developmental time course, mannitol was heavily loaded and almost 50-fold higher in Y6L than in Y1L (Tables 1; S5). In addition, several other carbohydrates associated with the raffinose family of the oligosaccharide (galactinol, raffinose), as well as fructose and xylose showed a higher profile in Y4L and Y4R (Tables S3, S4). Similarly, fructose 6-phosphate, a metabolite with a central role in primary carbohydrate metabolism, accumulated strongly in Y4L (3.3-fold) and Y4R (3.6-fold) compared to first-year samples (Tables 1, 2), but decreased in Y6L and Y6R (Tables S3, S4). Interestingly, in contrast to highly accumulated mannitol that is derived from fructose 6-phosphate, the osmolyte sorbitol derived from glucose 6-phosphate was significantly lower in Y4L and Y6L (-5-fold) (Table S5).

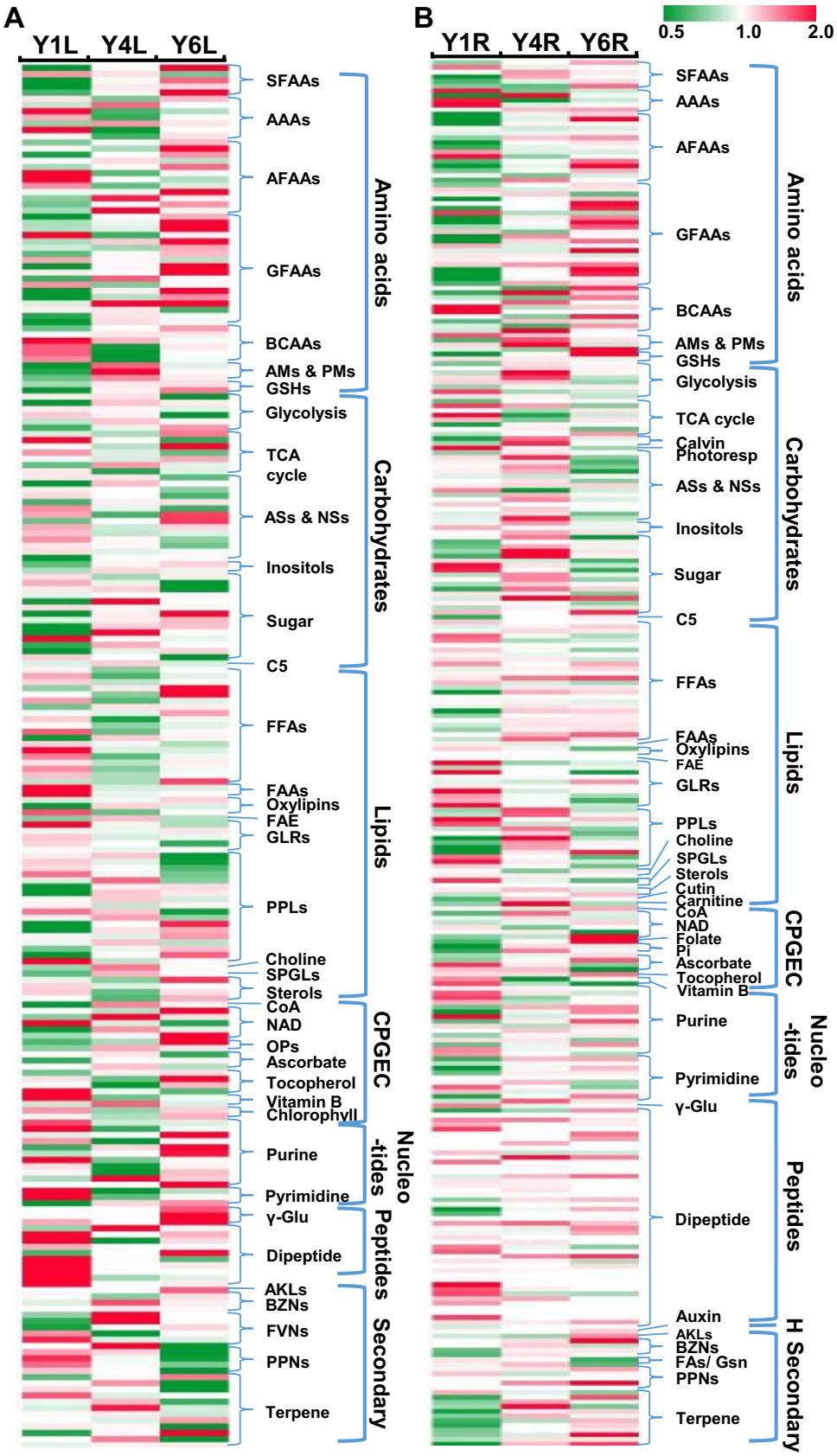

Fig. 3 Cluster analysis of the metabolomic data. **a** Heat map representation of 223 metabolites across leaves from Y1L, Y4L, and Y6L ginseng. **b** Heat map representation of 293 metabolites across leaves from Y1R, Y4R, and Y6R ginseng. All abbreviations are explained in Table S1, and details of metabolites are described in Tables S3 and S4

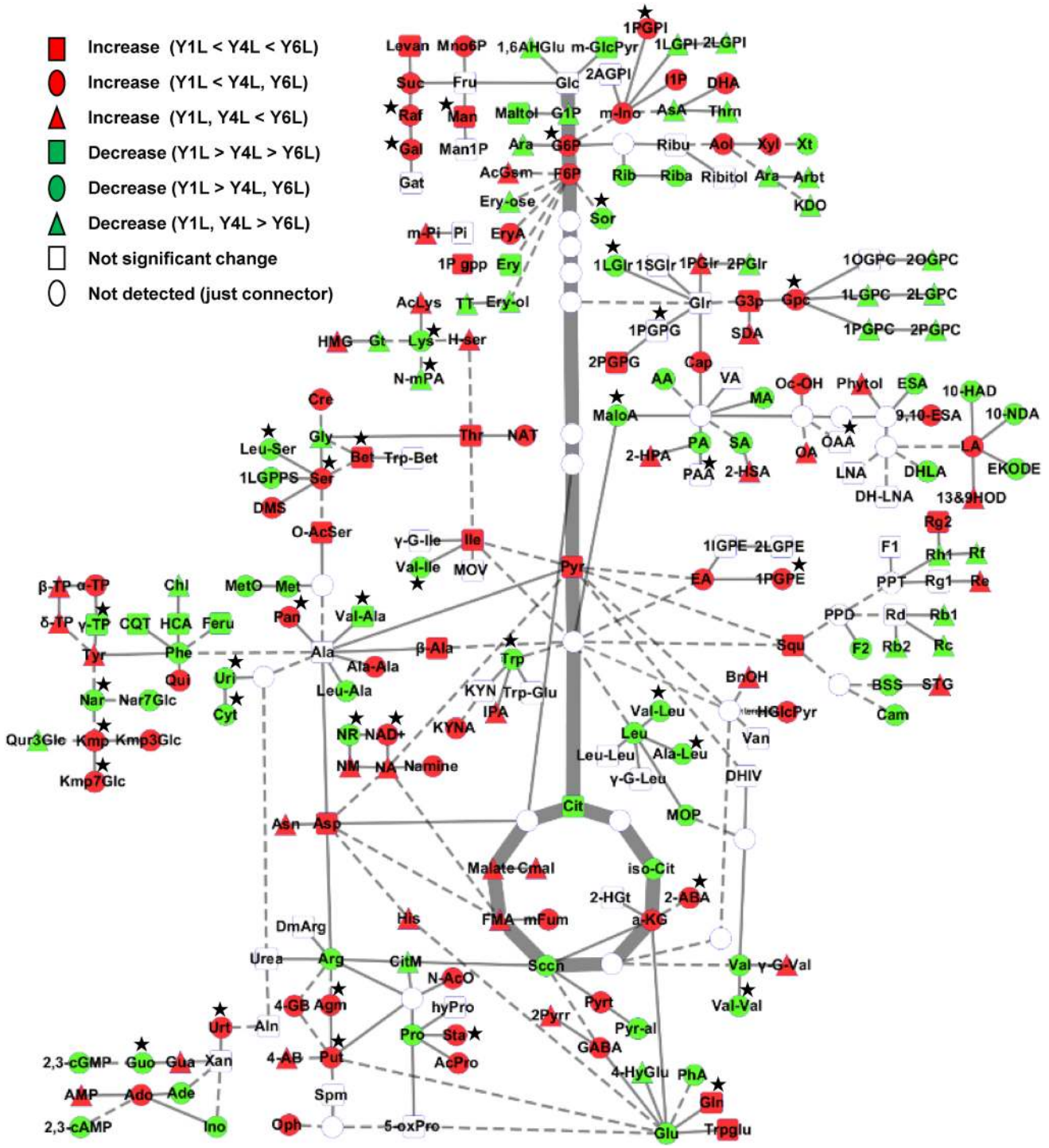

Fig. 4 Ginseng leaf metabolome. The identified 223 metabolites are mapped on a simplified metabolic network. Squares, circles and triangles denote metabolites detected in this study. Blank circles represent undetected metabolites that are used just for the connection of metabolites. The top 40 metabolites contributing significantly to the higher or lower mean value in Y4L compared with Y1L (indicated in Table S5) are marked with asterisks

Most of the free fatty acids, including 16:0 palmitate and 18:0 stearate, decreased in Y4L. However, the oxidized fatty acid intermediates, 2-hydroxypalmitate and 2-hydroxysterate accumulated in Y6L to 17.8- and 6.1-fold, respectively (Table S5), indicating that ginseng plants, particularly aboveground organs, may undergo an increased level of

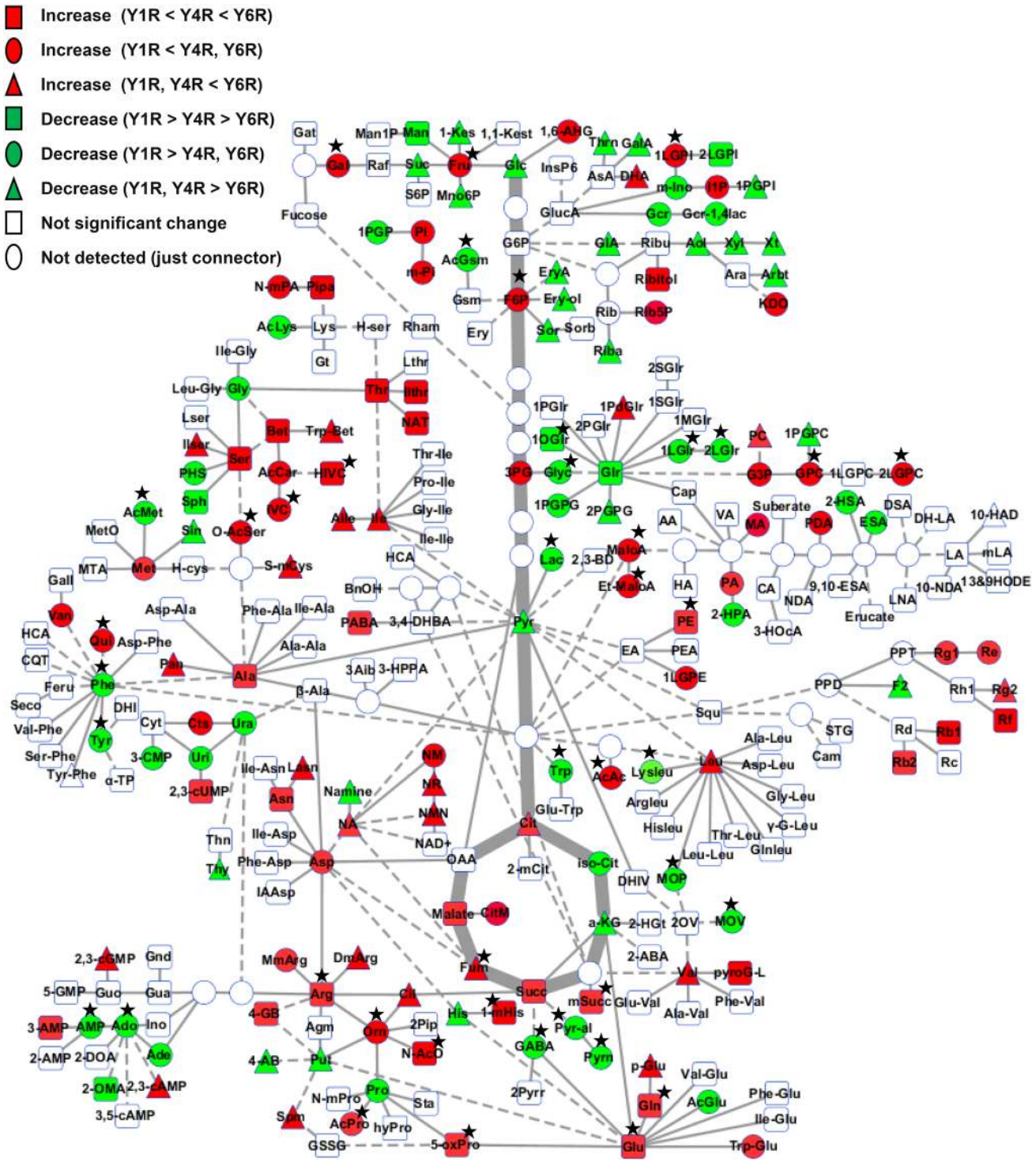

Fig. 5 Ginseng root metabolome. The identified 293 metabolites are mapped onto a simplified metabolic network. Squares, circles and triangles denote metabolites detected in this study. Blank circles represent undetected metabolites used just for connecting the metabolites. The top 40 metabolites that contribute significantly to the higher or lower mean value in Y4R compared with Y1R (indicated in Table S6) are marked with asterisks

oxidative stress. The reduction in linoleic acid phospholipids, which exist as the most abundant fatty acid in ginseng root (Zhang et al. 2013b), and the elevation of free fatty acids and other intermediates such as glycerophosphorylcholine (GPC) (Fig. 3; Tables 2; S6) support the notion that membrane lipids are remobilized for defense (Khajuria et al.

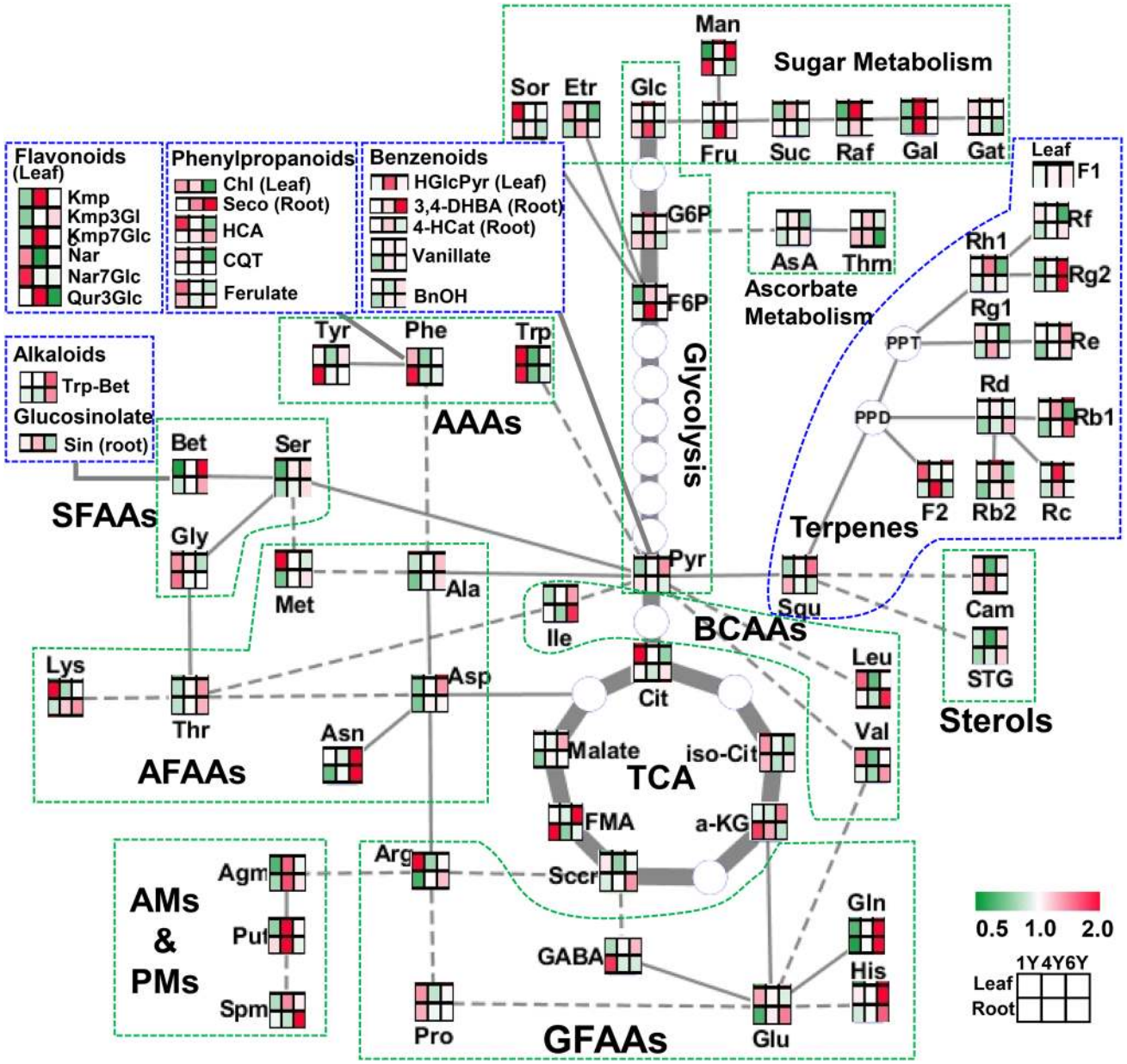

Fig. 6 Overview of connections between primary metabolism and major secondary metabolic pathways in *P. ginseng* leaves and roots during development. Heat maps of metabolite changes in primary metabolites (green dashed lines) including amino acids and carbohydrates, and carbon- and nitrogen-derived secondary metabolites (blue dashed lines), are displayed. Red and green colors denote higher and lower mean values as determined by nested ANOVA. Full names of the metabolites are in Table S1. (Color figure online)

2013) and suggest the existence of an active defense system in Y6 via reprogramming lipid metabolic pathways.

Cofactors and nucleotides

The metabolomics analysis has revealed that the amount of CPGEC accumulated in leaves as the ginseng grew. Nicotinamide riboside, the precursor of NAD or nicotinamide, accumulated in Y1L and highly decreased in Y4L (11-fold) and Y6L (17-fold) (Table 1; S5), possibly contributing to the escalation of NAD + in Y4L (4.3-fold) and further rise of nicotinamide (8.7-fold) and nicotinate (threefold) in Y6L than Y1L. The reduction of uracil (twofold) together with the increase of 3′-CMP (3.6-fold) during root growth (Table S6) possibly suggest the supply of pyrimidine nucleotides for the biosynthesis of phospholipids or polysaccharides for root growth and RNA and DNA synthesis (Katahira and Ashihara 2002).

Many antioxidant metabolites including vitamins B, C, and E showed obvious changes in ginseng leaves as plants

Table 1 Top 40 metabolites showing strong changes in Y4L compared with Y1L

Biochemical	Y4L/Y1L	*P* value	FDR-value	Super pathway	Sub pathway
Putrescine[a]	*10.41*	0.0007	0.0041	Amino acids	AMs and PMs
Betaine[a]	*10.18*	1E-06	0.0001	Amino acids	SFAAs
Glycerophosphorylcholine (GPC)[a]	*7.75*	0.0046	0.0132	Lipids	PPLs
2-Aminobutyrate[a]	*7.61*	0.004	0.0127	Amino acids	GFAAs
Raffinose[a]	*7.13*	5E-05	0.0009	Carbohydrates	Sugar
Urate[a]	*6.56*	0.0103	0.0245	Nucleotides	Purine
Mannitol[a]	*6.05*	0.0005	0.0035	Carbohydrates	Sugar
Agmatine[a]	*5.18*	0.0004	0.0032	Amino acids	AMs and PMS
1-Palmitoyl-GPI (16:0)[a]	*4.97*	0.0055	0.0149	Lipids	PPLs
Pantothenate (Vitamin B5)[a]	*4.92*	0.0021	0.0082	CPGEC	CoA
Galactinol[a]	*4.36*	0.0003	0.0025	Carbohydrates	Sugar
NAD + [a]	*4.27*	0.0002	0.0021	CPGEC	NAD
2-Palmitoylglycerophosphoglycerol[a]	*4.11*	0.0002	0.0021	Lipids	PPLs
Stachydrine[a]	*3.96*	0.0015	0.0067	Amino acids	GFAAs
Kaempferol[a]	*3.53*	0.0002	0.0019	Secondary	FLNs
Kaempferol 7-O-glucoside[a]	*3.44*	0.0004	0.0032	Secondary	FLNs
Serine[a]	*3.42*	0.0004	0.0032	Amino acids	SFAAs
Fructose 6-phosphate[a]	*3.27*	0.0078	0.0195	Carbohydrates	Glycolysis
1-Palmitoyl-GPE (16:0)[a]	*3.18*	0.0037	0.0121	Lipids	PPLs
Glutamine[a]	*3.13*	0.0002	0.002	Amino acids	GFAAs
Cytidine[a]	**−54.56**	7E-05	0.0011	Nucleotides	Pyrimidine
Oleamide[a]	**−14.09**	0.1083	0.1579	Lipids	FAAs
Valylleucine[a]	**−13.32**	0.0022	0.0083	Peptides	Dipeptide
Valylisoleucine[a]	**−11.65**	0.0138	0.0308	Peptides	Dipeptide
Nicotinamide riboside[a]	**−11.66**	3E-05	0.0006	CPGEC	NCTs
Naringenin[a]	**−10.98**	0.0354	0.0613	Secondary	FLNs
Alanylleucine[a]	**−10.35**	0.0011	0.0055	Peptides	Dipeptide
Tryptophan[a]	**−9.67**	4E-06	0.0002	Amino acids	AAAs
Malonate (propanedioate)[a]	**−9.50**	0.0013	0.0061	Lipids	FFAs
Leucylalanine[a]	**−8.46**	0.0073	0.0184	Peptides	Dipeptide
1-Linoleoylglycerol (18:2)[a]	**−7.94**	0.0002	0.0021	Lipids	GLRs
Delta-tocopherol[a]	**−7.31**	2E-05	0.0005	CPGEC	Tocopherol
Valylalanine[a]	**−6.69**	0.0004	0.0033	Peptides	Dipeptide
Leucylserine[a]	**−5.70**	0.0042	0.0131	Peptides	Dipeptide
Sorbitol[a]	**−5.44**	1E-06	8E-05	Carbohydrates	Sugar
Uridine[a]	**−5.36**	2E-07	5E-05	Nucleotides	Pyrimidine
Guanosine[a]	**−5.30**	4E-06	0.0002	Nucleotides	Purine
Valylvaline[a]	**−5.25**	0.007	0.0179	Peptides	Dipeptide
Lysine[a]	**−4.76**	0.0024	0.0086	Amino acids	AFAAs
Palmitic amide	**−4.65**	0.1052	0.1565	Lipids	FAAs

Italic and bold values indicate that the mean values are significantly higher and lower, respectively

[a]Indicates metabolites responsible for the separation by PLS-DA. *P* value and FDR-value indicate the significance and false discovery rate of difference of the relative metabolite levels between Y1L and Y4L, respectively

aged. Pantothenate (vitamin B5), which is important for coenzyme A (CoA) synthesis, accumulated remarkably in Y4L (fivefold) and Y6L (3.5-fold) as compared with that of Y1L (Tables 1; S5). Alpha-tocopherol, the major vitamin E in chloroplast membranes to quench reactive oxygen species (ROS) and protect lipid from peroxydation during photooxidation or oxidative stress (Munn`e and Alegre 2002), accumulated in Y4L (1.5-fold) and Y6L (1.6-fold). Furthermore, β-tocopherol accumulated to fourfold in Y6L (Table S5), indicating the activation of

Table 2 Top 40 metabolites showing strong changes in Y4R compared with Y1R

Biochemical	Y4R/Y1R	*P* value	FDR-value	Super pathway	Sub pathway
Malonate (propanedioate)[a]	*10.59*	0.0027	0.021	Lipids	FFAs
Beta-hydroxyisovaleroylcarnitine[a]	*10.31*	0.0148	0.0481	Amino acids	BCAAs
Glutamine[a]	*9.04*	5E-06	0.0007	Amino acids	GFAAs
2-Linoleoyl-GPC (18:2)[a]	*7.03*	0.0015	0.0158	Lipids	PPLs
Glycerophosphorylcholine (GPC)[a]	*6.50*	0.0008	0.0134	Lipids	PPLs
Quinate[a]	*6.48*	0.0002	0.0037	Amino acids	AAAs
O-Acetylserine[a]	*5.37*	0.0013	0.0147	Amino acids	SFAAs
Ethylmalonate[a]	*5.27*	0.0067	0.0326	Lipids	FFAs
1-Methylhistidine[a]	*4.22*	0.0028	0.0209	Amino acids	GFAAs
Phosphoethanolamine (PE)[a]	*4.00*	0.0065	0.0322	Lipids	PPLs
Isovalerylcarnitine (C5)[a]	*3.99*	0.0017	0.0159	Amino acids	BCAAs
Ornithine[a]	*3.89*	0.0087	0.0374	Amino acids	GFAAs
Glutamate[a]	*3.84*	0.0012	0.0151	Amino acids	GFAAs
N-Acetylproline[a]	*3.77*	0.0002	0.0036	Amino acids	GFAAs
Fructose[a]	*3.74*	0.0153	0.0494	Carbohydrates	Sugar
Arginine[a]	*3.73*	4E-05	0.0015	Amino acids	GFAAs
5-Oxoproline[a]	*3.71*	2E-05	0.0011	Amino acids	GSHs
Fructose 6-phosphate[a]	*3.58*	0.0119	0.0426	Carbohydrates	Glycolysis
Galactinol[a]	*3.56*	0.0135	0.0464	Carbohydrates	Sugar
N-delta-acetylornithine[a]	*3.35*	3E-05	0.0012	Amino acids	GFAAs
1-Linoleoyl-GPI (18:2)[a]	**−7.57**	2E-07	7E-05	Lipids	PPLs
1-Linoleoylglycerol (18:2)[a]	**−6.07**	0.0474	0.1208	Lipids	GLRs
4-Methyl-2-oxopentanoate[a]	**−5.15**	0.0019	0.0172	Amino acids	BCAAs
3-Methyl-2-oxovalerate[a]	**−5.01**	0.0006	0.0098	Amino acids	BCAAs
Pyridoxal[a]	**−4.75**	0.0103	0.0394	CPGECs	Vitamin B
2-Linoleoylglycerol (2-monolinolein)[a]	**−4.43**	0.0034	0.0226	Lipids	GLRs
Tryptophan[a]	**−4.38**	0.0102	0.0394	Amino acids	AAAs
Lysylleucine[a]	**−4.37**	0.0001	0.0035	Peptides	Dipeptide
N-Acetylglucosamine[a]	**−4.05**	7E-06	0.0007	Carbohydrates	Ass and NSs
AMP[a]	**−3.89**	3E-05	0.0015	Nucleotides	PURs
Fumarate[a]	**−3.70**	0.0681	0.1523	Carbohydrates	TCA
Phenylalanine[a]	**−2.65**	0.001	0.0134	Amino acids	AAAs
1-Oleoylglycerol (18:1)[a]	**−2.61**	0.0089	0.0378	Lipids	GLRs
N-Acetylmethionine[a]	**−2.50**	0.0182	0.0567	Amino acids	AFAAs
Tyrosine[a]	**−2.27**	0.0075	0.0343	Amino acids	AAAs
Adenosine[a]	**−2.25**	0.0002	0.0038	Nucleotides	PURs
Glycerate[a]	**−2.23**	0.0126	0.0441	Carbohydrates	PRP
Gamma-aminobutyrate (GABA)[a]	**−2.13**	0.0013	0.0149	Amino acids	GFAAs
Lactate[a]	**−2.11**	0.0145	0.0476	Carbohydrates	Glycolysis
Pyridoxine (vitamin B6)[a]	**−2.01**	0.0099	0.0399	CPGECs	Vitamin B

Italic and bold values indicate that the mean values are significantly higher and lower, respectively

[a]Indicates metabolites responsible for the separation by PLS-DA. *P* value and FDR-value indicate the significance and false discovery rate of difference of the relative metabolite levels between Y1R and Y4R, respectively

antioxidant defense mechanisms in aging ginseng leaves. Moreover, the increase in these metabolites could prevent the Y4L and Y6L from the accumulated oxidized fatty acids in the mature leaves.

Secondary metabolites

We observed abundant terpenoids, including 11 major ginsenosides as the ginseng ages, in correlation with a previous

study which used terpenoids to distinguish ginseng ages (Cui et al. 2015). In this study, we detected an increase of squalene, the precursor of ginsenosides derived from the mevalonic acid pathway, in Y4L (1.5-fold) and Y6L (2.3-fold) compared to Y1L, while there was no significant decrease of squalene content in root during growth. These findings were in consistent with the medicinal importance of ginseng roots, ginsenosides accumulated dramatically to 1.2- to sevenfold in root as the plant grew (Fig. 7; Table S4). Among the 40 different ginsenosides that have been identified and isolated from different tissues of *P. ginseng*, the major ginsenosides in roots and leaves are the PPD-type Rb1, Rb2, Rc, and Rd, and the PPT-type Re, Rg1 and Rf, respectively, which normally account for more than 80% of the total ginsenosides in *P. ginseng* (Kim et al. 2015). Our metabolomic analysis identified seven of these major ginsenosides as well as other PPD- and PPT-type ginsenosides such as F2, Rg2, Rh1, and F1.

Ginsenosides accumulated continuously in roots as plants grew, even though the reported total ginsenoside content and ginsenoside profiles vary among different studies (Kim et al. 2015). In our study, nearly all the detected ginsenosides in

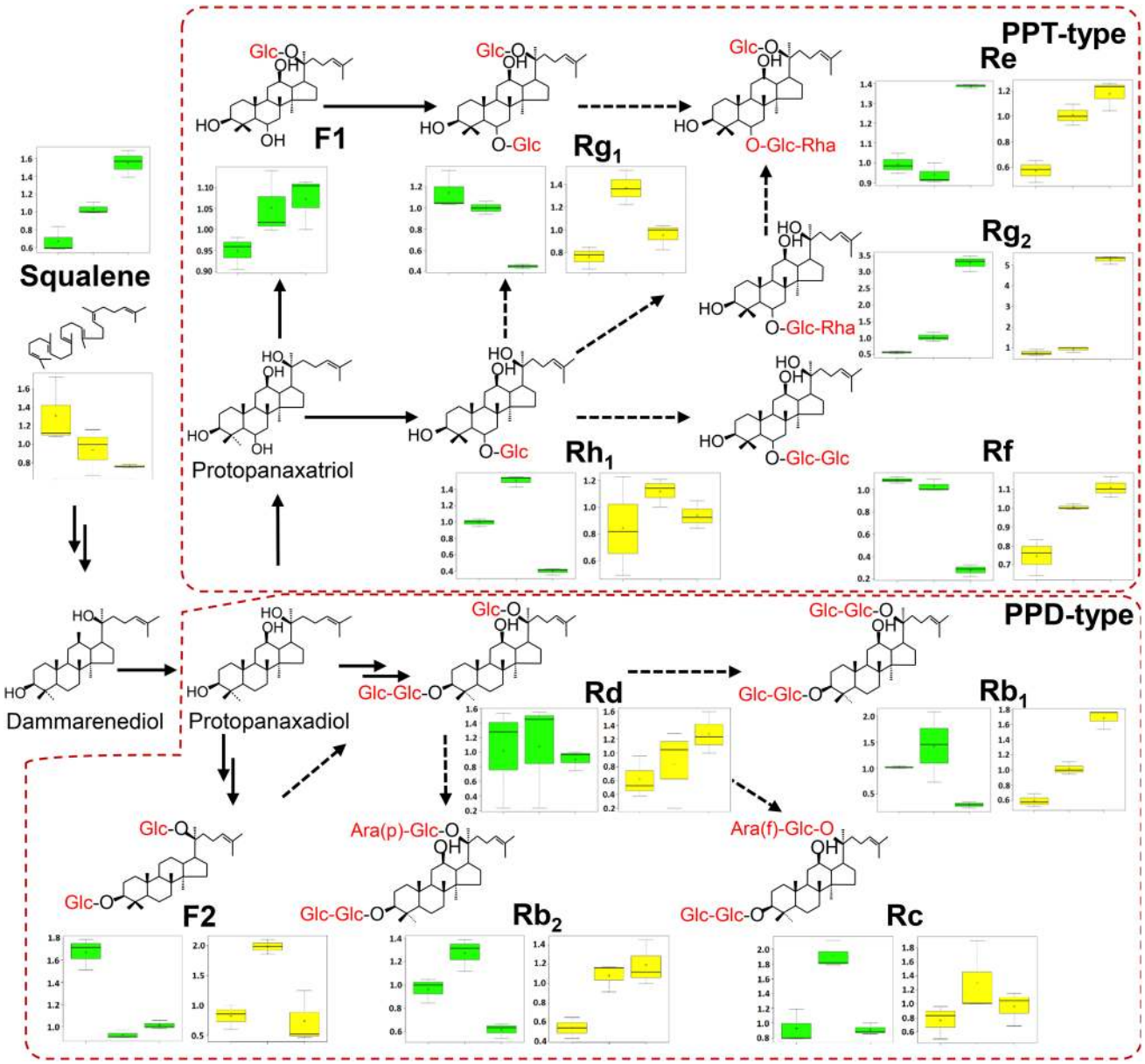

Fig. 7 Ginsenoside metabolic pathways and the changes of ginsenoside metabolites in different years. Dashed arrows represent putative pathways. Green and yellow graphs represent metabolic changes in leaves and roots, respectively. Data represent mean values of three samples for each growth stage (Y1, Y4, or Y6). Error bars represent SDs ($n=3$). (Color figure online)

roots increased over the time (Fig. 7). All of the ten detected ginsenosides accumulated significantly in Y4R, among which ginsenoside Rb1 and Rg2 continued to increase in Y6R. The level of ginsenoside Rg2 was sevenfold higher in Y6R than Y1R (Table S4). Rb1's increase in root and decrease in leaves during its growth was consistent with previous reports (Wang et al. 2006; Shi et al. 2007; Kim et al. 2012), confirming that Rb1 can be used as a marker for Y6R (Kim et al. 2012).

Leaves contained a reduced level of ginsenosides compared with roots (Fig. 7), which was consistent with previous reports (Wang et al. 2006; Shi et al. 2007). Most ginsenosides including Rb1, Rb2, Rg1, Rf, and Rh1 showed lower levels in Y6L than Y1L (Table S3), which was different from previous studies (Shi et al. 2007; Li et al. 2012). This variation in individual ginsenosides may be caused by different growth stage and harvest time, and/or soil environment (Kim et al. 2011; Chen et al. 2013). Interestingly, unlike other ginsenosides, the levels of Re and Rg2 increased two- and sixfold in Y6L from Y1L (Table S5; Fig. 7).

Phenolic compounds have been reported to have antioxidant activity in ginseng (Chung et al. 2012). Alkaloid (tryptophan betaine) and phenolics (five benzenoids and five phenylpropanoids) besides ginsenosides accumulated in Y4R or Y6R as compared with those in Y1R (Fig. 3, Table S4), suggesting that ginseng roots accumulate mostly carbon (C)-based secondary metabolites, this correlates to the high medicinal value of older ginseng roots. Here, we identified flavonols including kaempferols and quercetin, and flavanone naringenin in leaves, but not in roots. Kaempferol and kaempferol 7-*O*-glucoside were increased highly in Y4L as compared with those in Y1L, while quercetin 3-*O*-glucoside increased in Y4L but decreased highly in Y6L (Table S5), supporting the view that antioxidants are highly accumulated in old ginseng leaves to prevent oxidative stress (Agati et al. 2012). Consistent with the previous findings (Chung et al. 2012), the levels of benzenoids, such as vanillate, and benzyl alcohol, a type of phenolic acid, and antioxidant polyphenol were increased in Y4R or Y6R. However, most phenylpropanoids were not significantly changed in roots, except the high accumulation of secoisolariciresinol in Y6R (Table S4).

Discussion

Understanding metabolic changes during plant development allows us to reveal the biochemical pathways that control development and physiological status (Kooke and Keurentjes 2011). However, metabolic diversity in most medicinal plants that have nutritional, pharmaceutical and chemical importance is poorly characterized. Here we present the first global scenario of ginseng metabolism in association with growth, development, and morphology (Fig. 8), which demonstrates tissue-specific and year-specific metabolic networks within ginseng plants.

Dynamics of metabolites during development

Ginseng is a slow-growing medicinal plant that generally requires at least 4–6 years of cultivation before harvest. Previous metabolomic approaches using NMR spectroscopy

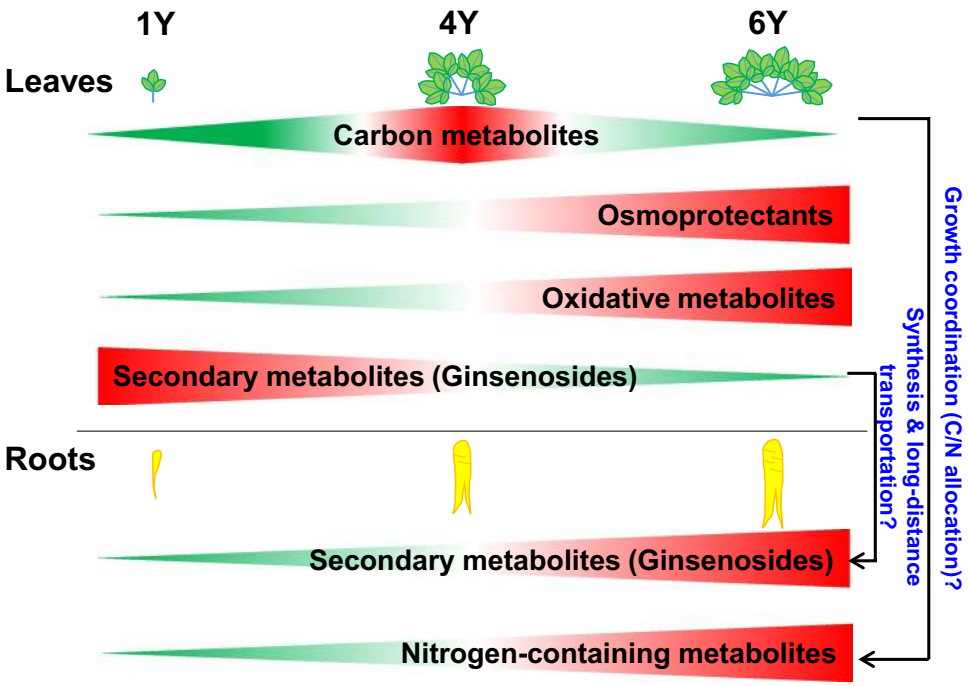

Fig. 8 A speculative model of metabolic changes in leaves and roots during the growth of *P. ginseng*. The drawing and painting were produced using PPT software by Kim

and UPLC-Q-Tof MS-based non-targeted metabolomic analysis have been mainly used to discriminate ginseng roots in different cultivated years, which lacked a clear identification of metabolites from ginseng plants (Lin et al. 2010; Kim et al. 2011, 2012; Kwon et al. 2014), let alone the dynamic patterns. In this study, we used non-targeted metabolomic profiling to identify various primary and secondary metabolites, with those identified metabolites we could clearly differentiate Y1 from Y4 and Y6 samples and leaves from roots (Fig. 2). Notably, we observed remarkable growth year dependent alterations in many metabolites such as N-containing metabolites, sugars and fatty acids in both leaves and roots of ginseng (Figs. 3, 4). Especially, the accumulation of amino acids and other N-containing metabolites such as ornithine, and polyamines in both leaves and roots as plants mature in Y4 and Y6 indicated that those metabolites are likely important for the increase of size and ginsenosides contents of roots. In addition, our non-targeted metabolomics analysis also identified vitamin precursors, stigmasterol and natural antioxidants. The levels of natural antioxidants and ginsenosides in roots increased as ginseng plants grow (Tables S3, S4), consistent with the view that older ginseng has more beneficial and pharmacological efficacy to human beings than the younger roots.

Dynamic C/N metabolism in roots and leaves

Changes in the level of soluble sugars were suggested to link plant metabolism with the environment, as sugar levels are influenced by both sink activities and biotic/abiotic stresses (Geigenberger 2011). Raffinose and galactinol, which were found to accumulate in both Y4L and Y4R in our study, were documented to be defense-response osmoprotectants under salt and drought stresses (Nishizawa et al. 2008). Sugar alcohols, mannitol and sorbitol have been linked to stress tolerance, especially tolerance against salinity and drought stresses (Chen et al. 2005). In Y6L, the dramatic increase of mannitol was sharp in contrast to the decrease of sorbitol (Table S5), which is also a stress-induced osmoprotectant. This observation implies that mannitol may be the preferred osmoprotectant in aged ginseng leaves. The increase of glycolysis components in Y4L and Y4R indicates active respiration for energy production, which was also supported by the high level of NAD^+ at this stage (Fig. 5; Tables 1, 2).

Y6R accumulated high levels of Glu, Gln, Asp, Asn and their derivates (Table S6), which are considered as the sensor of N status (Chen et al. 2005). However, these molecules decreased continuously in leaves as ginseng aged, implying the critical role of N-derived metabolites in the development of ginseng roots. In addition, the reallocation of N that has been stored as protein reserves is important for the spring growth of perennials (Rentsch et al. 2007). In ginseng, the high level of Gln was shown to be related to the low-branching and slow-growing hairy root in vitro (Jung et al. 2006). Therefore, this N assimilation could be an important factor in regulating ginseng root morphology during long time cultivation.

C/N ratio has been reported to be a key signal in plant growth and development (Nunes-Nesi et al. 2010; Balazadeh et al. 2014). Our observation of the predominance of N-transporting amino acids and derivates in aged leaves and roots, in contrast to the decreased level of the C status at the Y6 stage, suggests a general imbalance of C/N in roots that point toward a C deficit. C supply is largely connected to the photosynthetic activity in source leaf (Kooke and Keurentjes 2011). Ginseng has less number of stomata (Lee 1988) and thinner leaves which contain mostly sponge-type mesophyll cells without clearly distinguished compact palisade cells (Park 1980), which may cause reduced photosynthetic rate. These accumulated C/N sources might compensate the reduced levels of photosynthesis and photorespiration metabolites after 4 years of growth.

From an evolutionary point of view, we propose that ginseng has become a slow-growing plant after adaptation to the reduced energy requirement under limited photosynthesis and osmotic stress exposure, as slow-growing plants are generally found in low nutrient and stressful environments (Atkinson et al. 2012). Wild ginseng that has been grown in nature for hundreds of years develops smaller root and a slow growth rate compared with field-grown ginseng. Cultivated ginseng increases the number of leaves each year, whereas wild ginseng increases leaf numbers every 3–5 years and eventually produces 4–6 compound leaves. Therefore, we hypothesize that the difficulty of cultivating ginseng for more than 6 years in the field may be caused by a limited C supply from leaves. Supporting our hypothesis is the finding that compared with ginseng grown in the field, hydroponically cultured ginseng grows more quickly as it receives sufficient light and N supply (Kim et al. 2010). Taken together, C metabolism and N allocation are important for ginseng root development and C deficiency may be the major limitation for ginseng growth in the field.

Ginseng accumulates defense metabolites during growth

Our results suggested that ginseng plants may have evolved strategies to avoid oxidative damages, such as producing endogenous antioxidants including ascorbate, glutathione, and vitamins, and generating secondary metabolites including triterpenes, phenylpropanoids, and flavonoids (Fig. 6). In addition, ascorbate, glutathione and tocopherol pools act as redox buffers in plant cells (Foyer and Noctor 2005). Met sulfoxide is an oxidized metabolite generated by a variety of reactive molecules and is implicated in plant oxidative stress (Bechtold et al. 2004). The relative ratio of Met sulfoxide

to Met increased in Y6L, suggesting that Y6 plants have undergone oxidative stress. The reduced level of ascorbate and increased level of dehydroascorbate in ginseng leaves as plants grow older (Fig. 5) is consistent with the age-dependent oxidative stress imposed on the plant (Supplemental Fig. 1). Thus, increased oxidative stress and loss of membrane integrity are key characteristics of aging in ginseng. However, ginseng plants contain higher levels of antioxidants against ROS production in Y6 than Y4 plants, suggesting stress adaption in aged plants by balancing the production and quenching of ROS.

Given that aged ginseng leaves contain more osomoprotectants (Fig. 8), and accumulated salts in the field with 4- to 6-years' cultivated ginseng, we postulate that these plants are mainly suffering from osmotic stress. It is striking that compounds with strong osmotic properties, such as the sugar alcohol mannitol, quaternary ammonium compounds, and GPC, are highly accumulated in Y6L (Tables 1; S5). Notably, we found that in ginseng plants, amino acid-derived metabolites including GABA and polyamines accumulated in aging leaves. The accumulated stachydrine in Y4L is known as an osmoprotectant that is especially related to salinity stress (Trinchant et al. 2004). Therefore, our metabolomic analyses illustrate that ginseng has developed a whole range of strategies to adapt its metabolism to unfavorable growth conditions and that enhanced stress resistance is not due to the action of a single compound.

Accumulation and long-distance transport of ginsenosides

Although ginseng root is used for medicinal purpose, ginsenosides are distributed in all tissues. Ginsenoside concentrations fluctuate dramatically in response to environmental stimuli such as salt, light, JA, and other signaling molecules (Oh et al. 2014; Rahimi et al. 2015). In plants, different environmental circumstances evoke different effects on the balance between primary and secondary metabolisms. Under nutrient-limiting or stressful environments, plants grow slower because they allocate more resources to constitutive defense (Endara and Coley 2011). In support of these views, low nitrate concentrations could induce ginsenoside production in cell culture (Liu and Zhong 1997), whereas ginseng roots cultured by high-N hydroponics display faster growth but lower ginsenoside contents (Kim et al. 2010). As osmotic stress could cause growth inhibition but stimulate the accumulation of secondary metabolites including ginsenosides (Wu et al. 2005), we propose that many secondary metabolites in ginseng might be produced for defense response. Little is known about the physiological role of ginsenosides in plants beside the major role as defense against pathogens (Nicol et al. 2002; Kochan et al. 2013). Recent studies, however, revealed its association with plant growth (Zhang et al. 2011) and ginsenosides' different localizations at cortex, xylem and periderm in different ages of root (Lee et al. 2017). In addition, contents of ginsenosides and total ginsenosides are not positively correlated with 5- to 18-year-old ginseng root (He et al. 2016), contents of ginsenoside Rb1, Rc, and Rd varied widely with ages in samples from different cultivation regions (Xiao et al. 2015), and changes in the ratio of Re and Rg1 are dependent on the harvest season (Liu et al. 2017a), suggesting that ginsenosides accumulation varies to physiological status. Different change patterns of individual ginsenosides in leaves and roots along plant growth (Fig. 7) may be associated with their different biological activities in plant growth (Kim et al. 2015), which remains to be further comprehensively elucidated.

The level of squalene, the precursor of ginsenosides, was increased in leaves and decreased in roots over growth years (Fig. 7). It suggests the active biosynthesis of ginsenosides or their precursors in leaves and their dynamic transport into the root. In agreement with this hypothesis, Schramek et al. (2014) proposed that derivatives of photosynthetic metabolites such as glucose and fructose contribute to the synthesis for ginsenosides glycosylation or its movement into roots. More recently, Liu et al. (2017b) proposed that higher rates of photosynthesis and higher energy consumption in aboveground parts (leaves, petioles, and stems) regulate the bioactive compound biosynthesis of underground (lateral root and root) tissues. Although investigations have been done to understand the mechanism of ginsenoside synthesis and distribution, the long-distance allocation of ginsenosides remains largely unknown.

Our non-targeted metabolic profiling data revealed a high degree of metabolic dynamics in both leaves and roots of ginseng plants in different growth years. Notably ginseng metabolomes are closely associated with plant growth and development, and C metabolism and N allocation are critical for ginseng's root formation. The metabolomic data identified in this study suggest that during evolution ginseng plants developed a wide range of metabolic strategies to adapt to unfavorable conditions. Ginsenosides synthesized in leaves can be transported and accumulated in roots. These findings provide insight into the metabolic adjustments of ginseng plants to unfavorable growth conditions as they develop, in particular, the dynamics of metabolites in the source (leaves) and sink (roots) tissues of ginseng.

Materials and methods

Plant material

Panax ginseng cv. K-1 were grown at a ginseng field in Kyung Hee University (provided by Ginseng Bank), Suwon, Korea (GPS: E 127°08′25″N 37°24′55″). Primary leaves and

main roots were harvested from three plants at 1-, 4-, or 6-year-old on 1 August 2013 (temperature 23–33 °C, humidity 72.8%). To avoid the effects of diurnal variations in the metabolic profiles, samples were collected at approximately 15:00 p.m. and immediately frozen in liquid nitrogen. The fine-ground sample powders prepared using a ceramic mortar and pestle under liquid nitrogen were lyophilized and delivered to Shanghai Jiaotong University, Shanghai, China on dry ice, and stored in a − 80 °C freezer prior to metabolite extraction. Each metabolite profiling has three biological replicates.

Metabolite profiling

Forty mg of lyophilized powder of each ginseng sample was extracted with 400 µl methanol under vigorous shaking. Four chemical standards were added in the methanol prior to extraction to monitor the extraction and recovery efficiency. After centrifugation, the resulting supernatant was collected and divided into four aliquots: one for analysis by UPLC/MS/MS with negative ion mode electrospray ionization optimized for basic species; one for analysis by UPLC/MS/MS with positive ion mode electrospray ionization optimized for acidic species; one for analysis by GC/MS, and the last was reserved for backup. The detailed information on these platforms, including instruments, data acquisition and processing, and compound identification and quantitation, had been published previously (Evans et al. 2009; Oliver et al. 2011; Clarke 2013; Hu et al. 2014, 2016; Lin et al. 2014; Qu et al. 2014). Briefly, the UPLC–MS–MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer that consists of an electrospray ionization (ESI) source and a linear ion-trap (LIT) mass analyzer. The MS analysis alternated between MS and data-dependent MS/MS scans using dynamic exclusion to record retention time, mass-to-charge ratio, and MS/MS of all detectable ions. For GC platform, samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization (EI) and operated at unit mass resolving power. The samples were derivatized with bis trimethyl-silyl trifluoroacetamide prior to injection, and the retention time and mass-to-charge ratio for all detectable ions were measured. Metabolite identification is achieved by automatic matching of detected ion features of the samples to chemical standard entries in metabolom's proprietary reference metabolite library. The library includes retention time, molecular weight (M/Z), preferred adducts, and in-source fragments as well as associated MS/MS spectra of each chemical standard. This library allows rapid identification of metabolites in the experimental samples with high-confidence (Evans et al. 2009). Experimental samples were randomized with quality control samples across the platform and run days for quality control of each analysis.

Metabolomic data analysis

Relative abundance of a metabolite in each sample was calculated. For data normalization, missing values for a given metabolite in specific samples (below the instrument's detection limit) were first filled with the observed minimum detection threshold, and then all of the raw data values were normalized against the median. PCA was done with SIMCA-P 12.0 software. Significantly changed metabolites were further determined by partial least squares-discriminant analysis (PLS-DA). Heat maps of metabolites were visualized using Multi Experiment Viewer (MeV) version 4.8 (Saeed et al. 2003). Metabolic differences between ages and tissues of ginseng were determined using one-way ANOVA in the R package, and metabolic pathways were composed with Cytoscape version 2.8.3. The corresponding P values of statistical significance ($P \leq 0.05$), as well as those close to be statistically significant ($0.05 < P < 0.10$) were calculated by the T scores.

Author contribution statement YJK, DZ, and DCY designed the experiments. JS and SQ designed and performed metabolomic analysis. YJK, SCJ, and CH analyzed the data. JS conducted DAB staining. YJK, DZ, JS, JH, and PM co-wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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