Metabolomic analysis of the pharmacological active substances in four ginger varieties

Zhexin Li
Chongqing university of arts and sciences https://orcid.org/0000-0002-5097-8571
Shao-Fei Dang
Chinese academy of forestry
Yan-Ru Fan
Chinese academy of forestry
Hong-Lei Li
Chongqing university of arts and sciences
Wen-Lin Zhang
Chongqing university of arts and sciences
Jian-Bin Lan
Chongqing university of arts and sciences
Li-Wang Qi
Chinese academy of forestry
Jianmin Tang (Tangjmjy@163.com)

Research article

Keywords: ginger varieties, bioactive compounds, metabolomic analysis

Posted Date: February 5th, 2020

DOI: https://doi.org/10.21203/rs.2.22667/v1

Abstract

Background: The bioactive compounds of ginger (*Zingiber officinale*), including gingerols, diarylheptanoids, and flavonoids, are important for human health because of their anticancer, anti-oxidant, and anti-inflammatory properties.

Results: The UPLC/Q-TOF-MS profiles of four ginger samples, Zingiber officinale 'Yujiang No.1' (YJ), Zingiber officinale 'Shandong dajiang' (SD), Zingiber officinale 'Shandong xiaojiang' (SX) and Zingiber officinale 'Luoping xiaohuangjiang' (LP) were compared to gain insight into the differences in their rhizome content. A total of 1,810 metabolites were detected across the four varieties with 111, 72, 57, and 92 metabolites shared between the LP/YJ, SX/YJ, SX/SD and LP/SD ginger samples, respectively, with a p value of < 0.05 and a fold change of ≥ 1. Among the four libraries, 186 differentially expressed metabolites were identified. The metabolic pathways associated with the production of the bioactive compounds of ginger included those for stilbenoid, diarylheptanoid, and gingerol biosynthesis, flavonoid biosynthesis, steroid hormone biosynthesis, and diterpenoid biosynthesis. Among the differentially accumulated metabolites, gingerols and α-zingiberene were found in higher amounts in the SD and LP than in the SX and YJ varieties. The steroid hormones and terpenoids were found in higher amounts in LP than the other ginger samples.

Conclusion: The LP and SD varieties could be utilized as a source of medicinal ginger, while SX and YJ are more suited to incorporation into food because of their lighter taste related to their lower content of gingerols. The differences in active ingredients combined with analysis of the KEGG pathway allowed prediction of the metabolite synthetic pathways involved in the ginger component biosynthesis and lays the foundation for the further development and use of specific ginger varieties as medicinal resources.

Background

Ginger has been used extensively for more than 2,500 years in China for conditions including headaches, nausea, and colds (Grant and Lutz), and has high economic value as a medicine and food source. Compounds identified in ginger can be classified into three categories, essential oils, gingerol and diphenylheptane, with the key constituents of essential oils being mainly terpenoid compounds (Jiang and Xie et al.). Plants synthesize a variety of terpenoid natural products. The precursors of steroids are biosynthesized by two separate pathways, the mevalonate pathway (MVA) and the methyl erythritol phosphate pathway (MEP) (Vascular and Zulak, 2010). The MVA pathway provides farnesyl pyrophosphate, a precursor that is incorporated into sesquiterpenoids, triterpenoids, and steroids (Newman, 1999), while the MEP pathway provides geranyl diphosphate, a precursor used to synthesize monoterpenoids, and also geranylgeranyl pyrophosphate that is used to synthesize diterpenoids, gibberellin, and carotenoids (Lichtenthaler, 1999). Gingerols are core medicinal ingredients and the primary spicy substance in ginger, and includes more than 10 types of similar compounds such as 6-gingerol, 8-gingerol, and 10-gingerol with a β-hydroxyketone structure (Dedov and Tran et al., 2009). Typically ginger contains over 75% 6-gingerol, making it potentially useful as an objective indicator for

evaluating ginger quality. In addition to these compounds, flavonoids are also reported to have antioxidant, anticancer and anti-inflammatory activities (Luiz and De et al.; Ravishankar and Rajora et al.; Baldim and Domingos et al., 2017). Compounds that contain a phenylpropane structure are produced directly or indirectly through the phenylpropane metabolic pathway (Jaganath and Crozier, 2011; Zhao and Dixon, 2011). However, previous studies have revealed that metabolites with a phenylpropane structure like lignin and flavonoids are not accumulated in the rhizome of ginger. The main biochemical process in ginger is the conversion of sucrose into gingerols (Ramirez-Ahumada and Timmermann et al., 2006).

Metabolomics, developed in the middle and latter part of the last century, is the study of metabolites in a biological system involving the use of genomics, transcriptomics, and proteomics. The metabolite is closely associated with the phenotype of the organism and is considered to be a link between the geneotype and phenotype (Oliver, 2002). The use of metabolomic techniques to solve medicine, microbiology, and scientific issues in the fields of engineering, crop science and food nutrition has become central to the research of experts and scholars in many countries in recent years. In the present study, the UPLC/Q-TOF-MS profiles of four ginger samples were compared to gain insight into the differences in their rhizomes. The differences in active ingredients were determined and combined with analysis of the KEGG pathway, the metabolite synthetic pathways potentially involved in the building blocks of ginger were proposed. This work should help to lay the foundation for the further development and use of ginger as a medicinal resource.

Methods

Ginger material and sample preparation

The four ginger varieties that planted in our greenhouse base in Chong Qing, China (29°14' N, 105°52'E) were selected as the plant materials for this study. ‘Yujiang 1’ passed the field appraisal of experts in the Chongqing Crop Variety Approval Committee in 2014 (CVAC2015015). 'Shandong dajiang' was formally registered as a geographical indication certification mark by the State Administration for Industry and Commerce, 'Shandong xiaojiang' (Dense Seedling, Shandong Laiwu Traditional Local Variety) and 'Shandong dajiang' (Sparse Seedling Type, Shandong Laiwu Specialty Variety) were first provided by Laiwu academy of agricultural sciences. 'Luoping xiaohuangjiang' was also registered as geographical indication certification trademark product in Qujing, Yunnan province. Pro. Jian-Min Tang undertook the formal identification of the plant material used in our study. Underground rhizomes of the four well-developed varieties were rapidly frozen in liquid nitrogen and stored at -80 °C prior to analysis.

LC-MS analysis

LC-MS was performed on a Waters UPLC I-class system equipped with a binary solvent delivery manager and a sample manager coupled with a Waters VION IMS Q-TOF mass spectrometer equipped with an electrospray interface (Waters Corporation, Milford, USA).

Data analysis

The UPLC/Q-TOF-MS raw data were analyzed using Progenesis QI software (Waters Corporation) using the following parameters: retention time (RT) range of 0–14.0 min, mass range of 50–1,000 Da, and mass tolerance of 0.01 Da. Isotopic peaks were excluded for analysis, the noise elimination level was set at 10.00, the minimum intensity was set to 15% of the base peak intensity and the RT tolerance was set at 0.01 min. An excel file was obtained containing a three dimension data set including the m/z, peak RT, and peak intensities, and the RT–m/z pairs were used as the identifier for each ion. The resulting matrix was further reduced by removing any peaks with a missing value (ion intensity = 0) in more than 60% of the samples. The internal standard was used for data quality control and reproducibility. The positive and negative data were combined to obtain a combined data set and it was imported into the SIMCA-P + 14.0 software package (Umetrics, Umeå, Sweden). Principle component analysis (PCA) was carried out to visualize the metabolic alterations among the experimental groups. Pathway analysis was performed using online software found at http://www.genome.jp/kegg/tool/map_pathway2.html.

Results

Metabolomics analysis of the four ginger samples

Metabolomics is an important discipline within biology and can provide information critical for the determination of associations among genetic variants. Herein, LC–MS-based metabolomics detection was performed to identify differential metabolites among four ginger varieties, YJ, SD, SX, and LP (Fig. 1A). From the PCA plot, it was evident that there were metabolite differences in the components of the rhizomes of the four samples (Fig. 1B).

Differential metabolites identification of ginger samples

A total of 1,810 metabolites were detected across the four samples. Differentially expressed metabolites were identified between ginger varieties with the larger differences in their degrees of spicy taste. A total of 111, 72, 5,7 and 92 metabolites were shared between the LP/YJ, SX/YJ, SX/SD, and LP/SD ginger samples, respectively, with a p value < 0.05 and a fold change of ≥ 1 (Table 1). A comparison across the four libraries identified 186 differentially expressed metabolites (Fig. 1C). The detailed information of the differentially expressed metabolites is shown in Supplemental Files 1 and 2. A pathway analysis of the key bioactive compounds identified among the differentially accumulated metabolites, including gingerols, flavonoids, steroid hormones, and diterpenoids, is shown in Table 2.

Table 1
Identification of differentially expressed metabolites between ginger varieties with bigger differences in degrees of spicy taste.

Comparison group	Up-regulation	Down-regulation	Total number
LP/YJ	79	32	111
SX/YJ	44	28	72
SX/SD	35	22	57
LP/SD	62	30	92

Table 2
The significantly enriched KEGG pathways for differentially expressed metabolites selected from different ginger varieties ($p < 0.05$)

Pathway Name	metabolites	ID	LP/YJ	SX/YJ	SX/SD	LP/SD
Stilbenoid, diarylheptanoid and gingerol biosynthesis	6-gingerol	C10462	↑	↓	↓	↓
	8-gingerol	C17495	↑	↑	↓	↓
	10-gingerol	C17496	↓	↓	↓	↑
Flavonoid biosynthesis	Epicatechin	C09727	↓	↓	-	-
-	α-Zingiberene	C09750	↑	↑	↓	↓
Steroid hormone biosynthesis	Corticosterone	C02140	↑	-	-	-
	Tetrahydrocortisone	C05470	↑	-	-	-
	2-Methoxy-estradiol-17β-3-glucuronide	C11131	↑	↑	↑	↑
		C11132	↑	-	↑	↑
	2-Methoxyestrone-3-glucuronide	C11133	↑	↑	↑	↑
	Estrone glucuronide					
Diterpenoid biosynthesis	Gibberellin A14	C11858	↑	↑	-	↑
	Gibberellin A51-catabolite	C11854	-	-	-	↑

Bioactive compounds analysis

Gingerols like 6-Gingerol was found in higher amounts in the SD rhizomes, with the order of D > LP > YJ > SX, (Figs. 2 and 3). Other gingerols like 8-gingerol and 10-gingerol in the four rhizome varieties were found in the order of SD > LP > SX > YJ and YJ > LP > SD > SX, respectively (Table 2, Supplemental File 2). The concentration of α-zingiberene was found in the order of SD > LP > SX > YJ (Table 2, Supplemental File 2). The amounts of the steroid hormones corticosterone and tetrahydrocortisone were 2.4 and 6.27 times higher in LP compared with YJ, while 2-methoxy-estradiol-17β-3-glucuronide, 2-methoxyestrone-3-glucuronide, and estrone glucuronide revealed a similar accumulation pattern (LP > SX > YJ > SD) (Figs. 2 and 4). The concentration of gibberellin A14 was found in the order of LP > SX and SD > YJ and the

concentration of gibberellin A51-catabolite was significantly higher at 1.90-fold in LP compared with SD (Table 2, Supplemental File 2).

Discussion

Gingerols and Flavonoid biosynthesis

Gingerols are reported to have a variety of pharmacological properties like anti-inflammatory, anticancer, and anti-oxidant activities (Lee and Seo et al., 2008; Dugasani and Pichika et al., 2010; Liao and Leu et al., 2012; Tiwari and Mishra, 2016; Liang and Sang et al., 2018). Three types of gingerols, 6-gingerol, 8-gingerol, and 10-gingerol, were detected in the rhizomes of the four ginger varieties according to the LC–MS-based metabolomics results (Supplemental File 2). 6-Gingerol was primarily enriched in the stilbenoid, diarylheptanoid, and gingerol biosynthesis pathway (map 00945) and was found in higher amounts in the SD rhizomes, and in decreasing amounts in the LP, YJ and SX varieties (SD > LP > YJ > SX, Figs. 2 and 3). Since the concentrations of 8-gingerol and 10-gingerol were much lower compared with that of 6-gingerol (Jiang and Huang et al., 2018), 6-gingerol could be used as an indicator to evaluate the quality of ginger drugs. The amounts of 8-gingerol and 10-gingerol in the four rhizome varieties were found in the order of SD > LP > SX > YJ and YJ > LP > SD > SX, respectively (Table 2, Supplemental File 2). According to our results, the concentrations of gingerols are generally proportional to the spicy taste of ginger. This is consistent with the results from a previous study that described that the pungent components of ginger, which mainly consists of gingerols, are the main contributors to the taste of ginger (Ramirez-Ahumada and Timmermann et al., 2006).

Other bioactive compounds were also identified among the four ginger varieties. Epicatechin is a flavonoid that is a polyphenolic antioxidant plant secondary metabolite. The concentration of epicatechin in YJ was found to be 21.27 and 47.61 times higher than that of LP and SX (Figs. 2 and 3). In fact, the stilbenoid, diarylheptanoid, gingerol, and flavonoid biosynthesis pathways are branches of the general phenylpropanoid biosynthesis pathway (Fig. 3). α-Zingiberene is a sesquiterpene that constitutes the main component of the volatile oil of ginger. The concentration of α-zingiberene was found in the order of SD > LP > SX > YJ, with SD having 8.06 times more α-zingiberene than YJ (Table 2, Supplemental File 2). According to the above results, gingerols and α-zingiberene were found in higher amounts in SD and LP than in SX and YJ, indicating that SD and LP could be used as medicinal source of ginger while SX and YJ could be used in food because of their anticipated lighter taste as a result of their lower amount of gingerols.

Biosynthesis Pathway of Bioactive Steroids

Steroid hormones are a class of widely relevant, biologically active compounds originating from cholesterol that are altered in a stepwise fashion while maintaining a basic 17-carbon, 4-ring structure (Payne and Freishtat, 2012). They were reported to act as signaling molecules important for normal growth, development and differentiation of plants (Friedrichsen and Chory, 2010), and were also revealed as bioactive compounds that help control metabolism, inflammation, immune functions, salt and water

balance, the development of sexual characteristics, and withstand illness and injury in animals(Payne and Freishtat, 2012; Yadav and Yadav et al., 2014). In this study, the concentrations of five metabolites significantly enriched in the steroid hormone biosynthesis pathway were found to vary among the four ginger varieties (Table 2, Fig. 4). The amounts of the steroid hormones corticosterone and tetrahydrocortisone were 2.4 and 6.27 times higher in LP compared with YJ, while 2-methoxy-estradiol-17β-3-glucuronide, 2-methoxyestrone-3-glucuronide, and estrone glucuronide revealed a similar accumulation pattern (LP > SX > YJ > SD), with amounts 4.85, 6.19, and 6.07 times higher in LP than SD, respectively (Figs. 2 and 4). Thus, LP might be a valuable ginger variety for producing larger quantities of bioactive compounds for medical applications.

Gibberellin is an important diterpenoid phytohormone with extensive and complex biological functions that regulates the growth and development of plants throughout their life cycle (Richards and King et al., 2001; Ludmila and Thomas et al., 2004; Colebrook and Thomas et al., 2014; Tang and Liu et al., 2018). Two types of gibberellins, gibberellin A14 and gibberellin A51-catabolite, were detected among the four ginger varieties (Fig. 5). The concentration of gibberellin A14 was found in the order of LP > SX and SD > YJ, while no significant difference in gibberellin A14 was detected between SX and SD. The concentration of gibberellin A51-catabolite was significantly higher at 1.90-fold in LP compared with SD (Table 2, Supplemental File 2). Thus, terpenoids accumulated to a higher extent in the LP ginger compared with the other varieties.

Conclusions

LC/MS-based method was used and non-target metabolomics studies were performed on four ginger varieties, and the data were subjected to both PCA multidimensional and single dimensional statistical analyses. The differential metabolites between the groups were identified and KEGG analysis of these metabolites was performed. Our results provide the basis for the use of a specific ginger variety depending on which secondary metabolites are required to provide the desired medicinal or taste benefits.

Abbreviations

YJ: Zingiber officinale 'Yujiang No.1'; SD: Zingiber officinale 'Shandong dajiang; SX: Zingiber officinale 'Shandong xiaojiang'; LP: Zingiber officinale 'Luoping xiaohuangjiang'; MVA: the mevalonate pathway; MEP: the methyl erythritol phosphate pathway; RT: retention time.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no conflict of interest.

Funding

The authors are grateful for the financial support provided by The Natural Science Foundation of Chongqing (cstc2019jcyj-msxmX0697 and cstc2019jcyj-msxmX0300), The Scientific and Technological Research Program of Chongqing Municipal Education Commission (KJQN201901302) and Chongqing University of Arts and Sciences Talent Introduction Project (2017RTZ22 and P2018TZ05). These funding bodies had no role in the design of the study, collection, analysis, and interpretation of data, or in writing the manuscript.

Authors' Contributions

ZL conceived, designed and carried out the study and wrote the manuscript. JT, LQ and SD provided important suggestions on the experimental design and analyses. YF and HL offered varying degrees of help during the experimental operation. WZ and JL helped to modify the manuscript. All authors read and approved the manuscript.

Acknowledgements

Not applicable

References

1. Baldim, J. L. and O. D. S. Domingos, et al. (2017). "The Correlation between Chemical Structures and Antioxidant, Prooxidant, and Antitrypanosomatid Properties of Flavonoids." Oxidative Medicine and Cellular Longevity,2017,(2017-7-2) **2017** (4): 1-12.
2. Colebrook, E. H. and S. G. Thomas, et al. (2014). "The role of gibberellin signalling in plant responses to abiotic stress." Journal of Experimental Biology **217** (1): 67-75.
3. Dedov, V. N. and V. H. Tran, et al. (2009). "Gingerols: a novel class of vanilloid receptor (VR1) agonists ." British Journal of Pharmacology **137** (6): 793-798.
4. Dugasani, S. and M. R. Pichika, et al. (2010). "Comparative antioxidant and anti-inflammatory effects of [6]-gingerol, [8]-gingerol, [10]-gingerol and [6]-shogaol." J Ethnopharmacol **127** (2): 515-20.

5. Friedrichsen, D. and J. Chory (2010). "Steroid signaling in plants: from the cell surface to the nucleus." Bioessays **23** (11): 1028-1036.
6. Grant, K. L. and R. B. Lutz "Ginger." Am J Health Syst Pharm **57** (10): 945-947.
7. Jaganath, I. B. and A. Crozier (2011). Flavonoid Biosynthesis.
8. Jiang, H. and Z. Xie, et al. "Metabolic profiling and phylogenetic analysis of medicinal Zingiber species: Tools for authentication of ginger (Zingiber officinale Rosc.)." Phytochemistry **67** (15): 1673-1685.
9. Jiang, Y. and M. Huang, et al. (2018). "Transcriptome Analysis Provides Insights into Gingerol Biosynthesis in Ginger (Zingiber officinale)." Plant Genome **11** (3).
10. Lee, H. S. and E. Y. Seo, et al. (2008). "[6]-Gingerol inhibits metastasis of MDA-MB-231 human breast cancer cells." J Nutr Biochem **19** (5): 313-9.
11. Liang, N. and Y. Sang, et al. (2018). "Anti-Inflammatory Effects of Gingerol on Lipopolysaccharide-Stimulated RAW 264.7 Cells by Inhibiting NF-kappaB Signaling Pathway." Inflammation **41** (3): 835-845.
12. Liao, Y. R. and Y. L. Leu, et al. (2012). "Anti-platelet aggregation and vasorelaxing effects of the constituents of the rhizomes of Zingiber officinale." Molecules **17** (8): 8928-37.
13. Lichtenthaler, H. (1999). "The 1 deoxy D xylulose 5 phosphate pathway of isoprenoid biosynthesis in plants." Annual Review of Plant Physiology & Plant Molecular Biology **50** (50): 47-65.
14. Ludmila, T. and S. G. Thomas, et al. (2004). "Della proteins and gibberellin-regulated seed germination and floral development in Arabidopsis." Plant Physiology **135** (2): 1008-1019.
15. Luiz, B. J. O. and A. B. G. A. De, et al. "The Correlation between Chemical Structures and Antioxidant, Prooxidant, and Antitrypanosomatid Properties of Flavonoids." Oxidative Medicine and Cellular Longevity **2017**: 1-12.
16. Newman, J. J. (1999). "Isoprenoid biosynthesis in plants: carbon partitioning within the cytoplasmic pathway." Crc Critical Reviews in Biochemistry **34** (2): 95-106.
17. Oliver, F. (2002). "Metabolomics--the link between genotypes and phenotypes." Plant Molecular Biology **48** (1-2): 155-171.
18. Payne, A. S. and R. J. Freishtat (2012). "Conserved steroid hormone homology converges on nuclear factor κB to modulate inflammation in asthma." Journal of Investigative Medicine the Official Publication of the American Federation for Clinical Research **60** (1): 13-7.
19. Ramirez-Ahumada, M. D. C. and B. N. Timmermann, et al. (2006). "Biosynthesis of curcuminoids and gingerols in turmeric (Curcuma longa) and ginger (Zingiber officinale): identification of curcuminoid synthase and hydroxycinnamoyl-CoA thioesterases." Phytochemistry **67** (18): 2017-2029.
20. Ramirez-Ahumada, M. D. C. and B. N. Timmermann, et al. (2006). "Biosynthesis of curcuminoids and gingerols in turmeric (Curcuma longa) and ginger (Zingiber officinale): identification of curcuminoid synthase and hydroxycinnamoyl-CoA thioesterases." Phytochemistry **67** (18): 2017-2029.

21. Ravishankar, D. and A. K. Rajora, et al. "Flavonoids as prospective compounds for anti-cancer therapy." International Journal of Biochemistry & Cell Biology **45** (12): 2821-2831.
22. Richards, D. E. and K. E. King, et al. (2001). "HOW GIBBERELLIN REGULATES PLANT GROWTH AND DEVELOPMENT: A Molecular Genetic Analysis of Gibberellin Signaling." Annu Rev Plant Physiol Plant Mol Biol **52** (52): 67-88.
23. Tang, Y. and H. Liu, et al. (2018). "OsmiR396d Affects Gibberellin and Brassinosteroid Signaling to Regulate Plant Architecture in Rice." Plant Physiology **176** (1): 946.
24. Tiwari, M. K. and P. C. Mishra (2016). "Anti-oxidant activity of 6-gingerol as a hydroxyl radical scavenger by hydrogen atom transfer, radical addition and electron transfer mechanisms." Journal of Chemical Sciences **128** (8): 1199-1210.
25. Vascular, S. and K. G. Zulak (2010). "Cells of Conifer Defense." **52** (1).
26. Yadav, R. and N. Yadav, et al. (2014). "Steroid Chemistry and Steroid Hormone Action: A Review." Asian Journal of Research in Chemistry.
27. Zhao, Q. and R. A. Dixon (2011). "Transcriptional networks for lignin biosynthesis: more complex than we thought?" Trends in Plant Science **16** (4): 227-233.

Figures

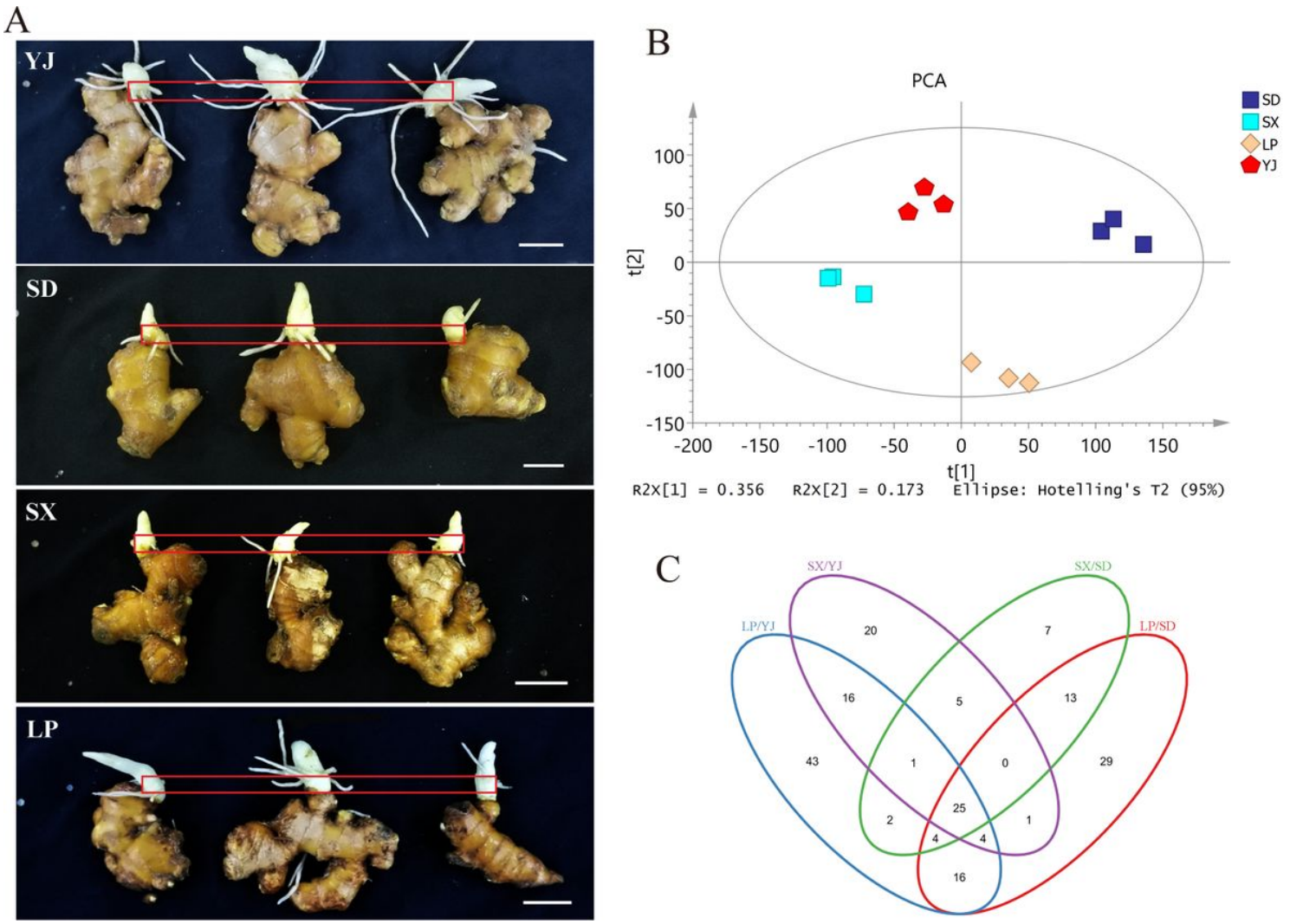

Figure 2

A:The four ginger materials selected in the present study. Scale bar represent 2 centimeter; B: PCA analysis of the four samples; C: Differentially expressed metabolites shown in Venn figue.

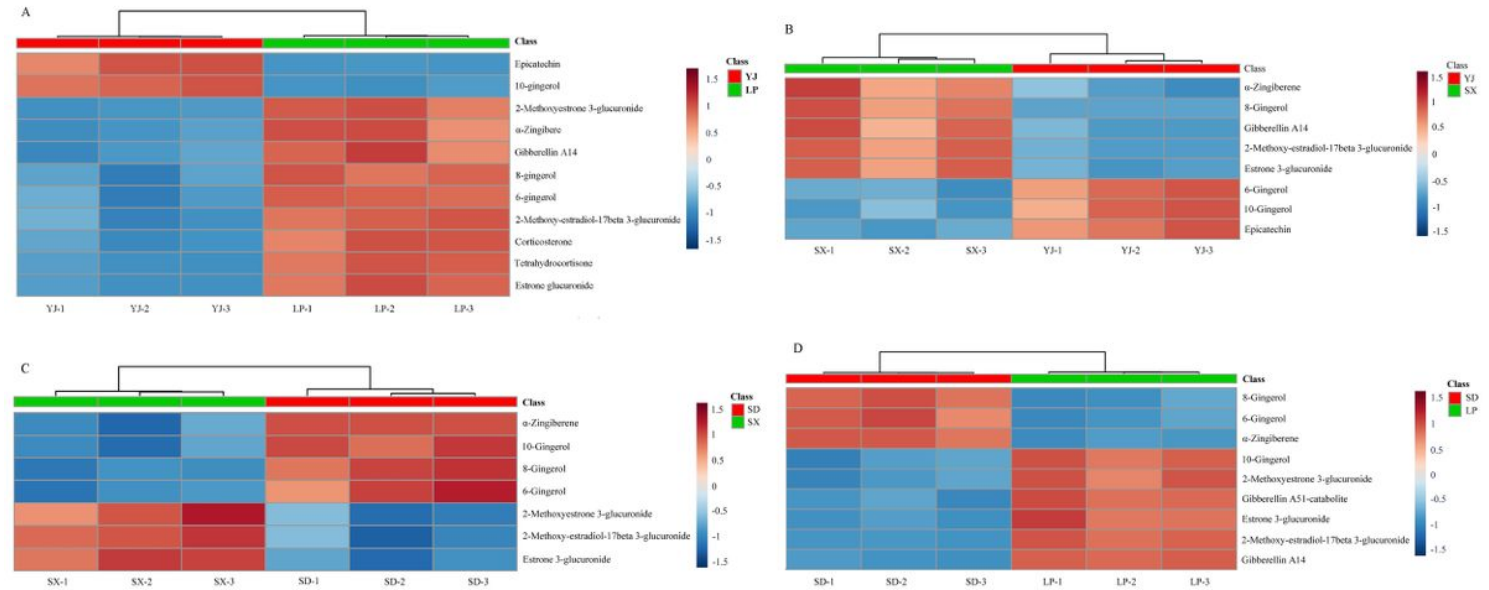

Figure 4

Heatmap visualization of metabolomics data for the ginger rhizome. The heatmap was plotted based on levels of the differential metabolites. The data were normalized by rescaling between -1.5 and 1.5. Rows: metabolites; Columns: samples. Color key indicates expression value of the metabolites: red, highest; blue, lowest.

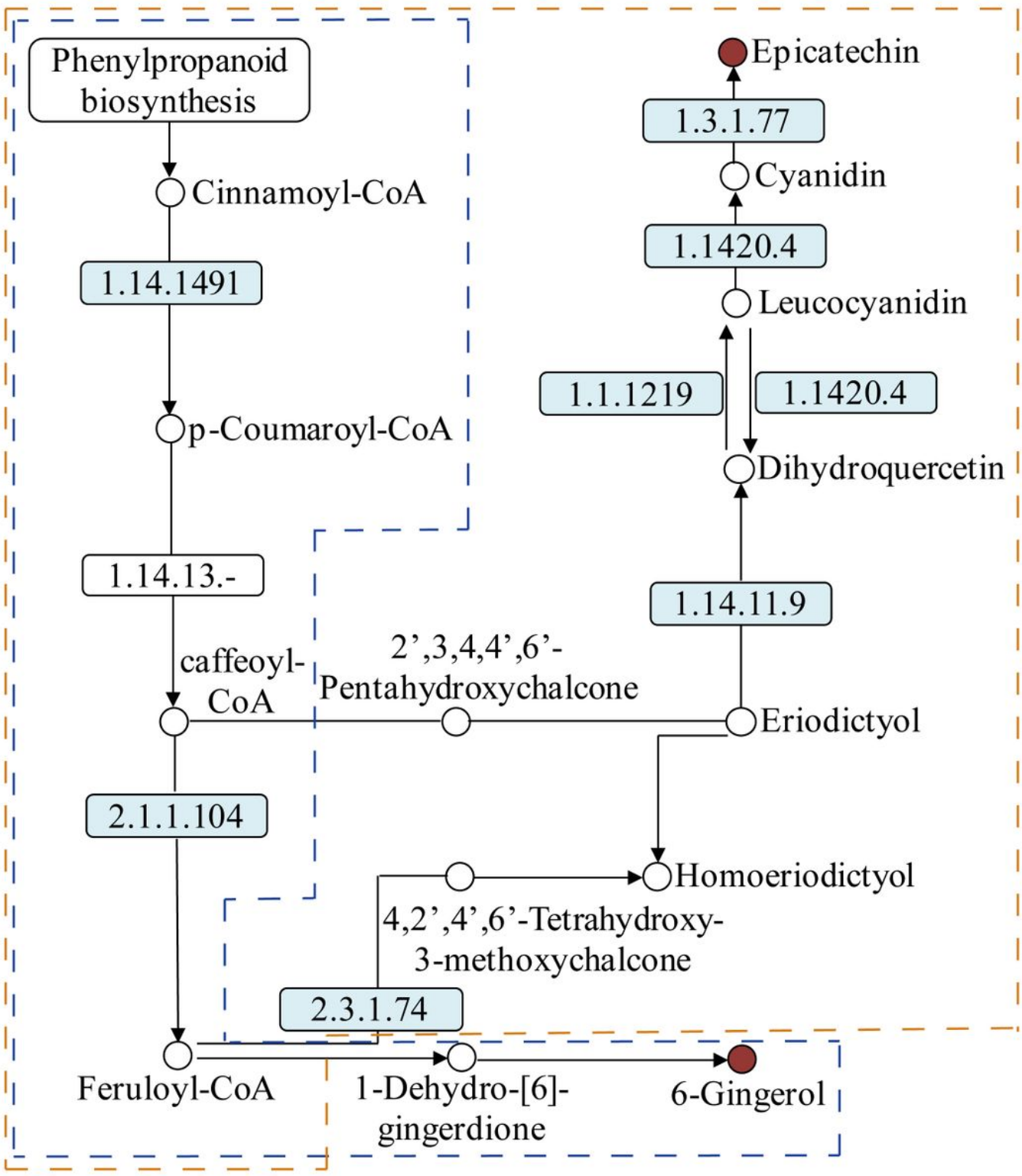

Figure 6

Diferentially accumulated metabolites involved in the "stilbenoid, diarylheptanoid and gingerol biosynthesis" and "flavonoid biosynthesis" pathway of ginger. The red columns indicate metabolites expressed at a signifcantly high level in different rhizome samples. This map of DEGs corresponds to map00941 (in blue box) and map00945 (in orange box) in the KEGG database.

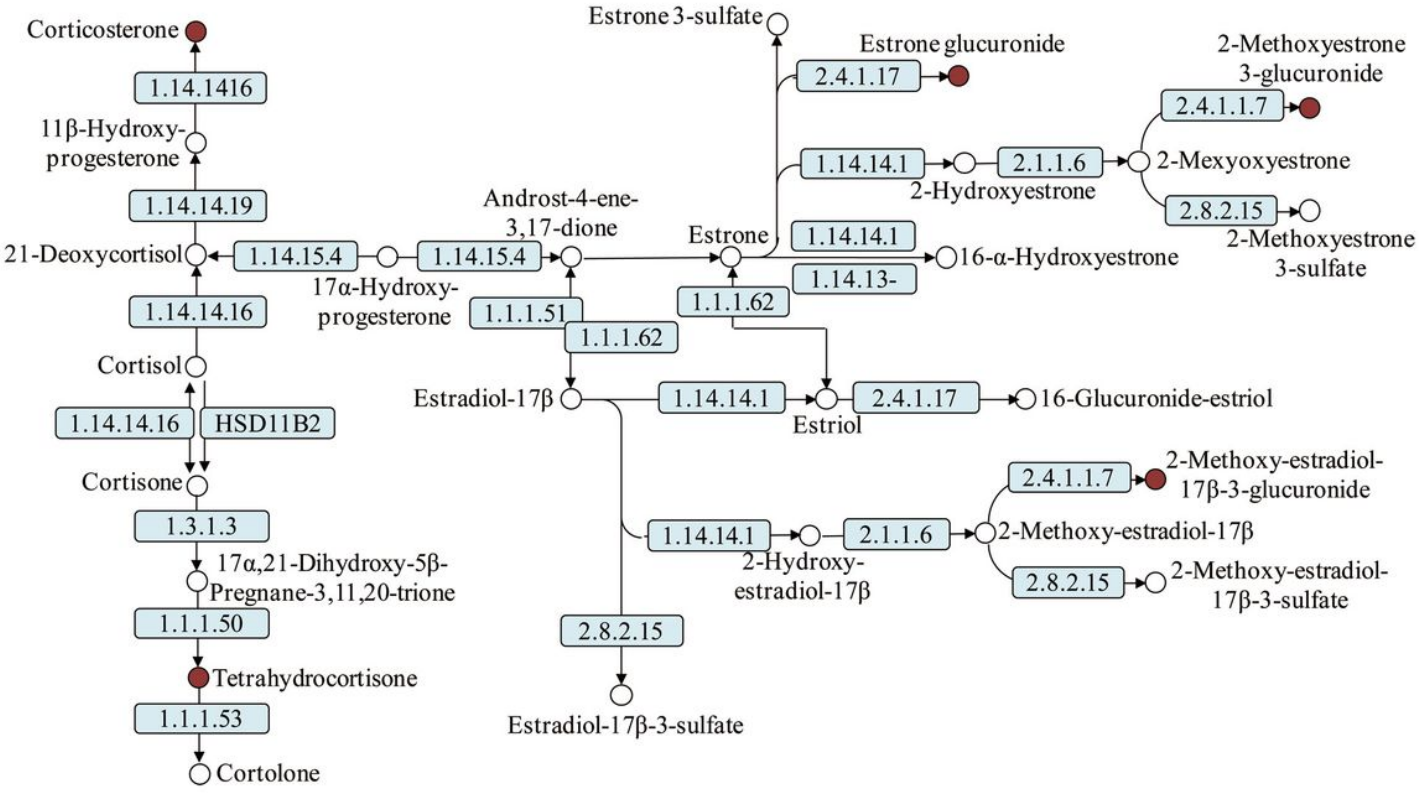

Figure 8

Diferentially accumulated metabolites involved in the "Steroid hormone biosynthesis" pathway of ginger. The red columns indicate metabolites expressed at a signifcantly high level in different rhizome samples. This map of DEGs corresponds to map00140 in the KEGG database.

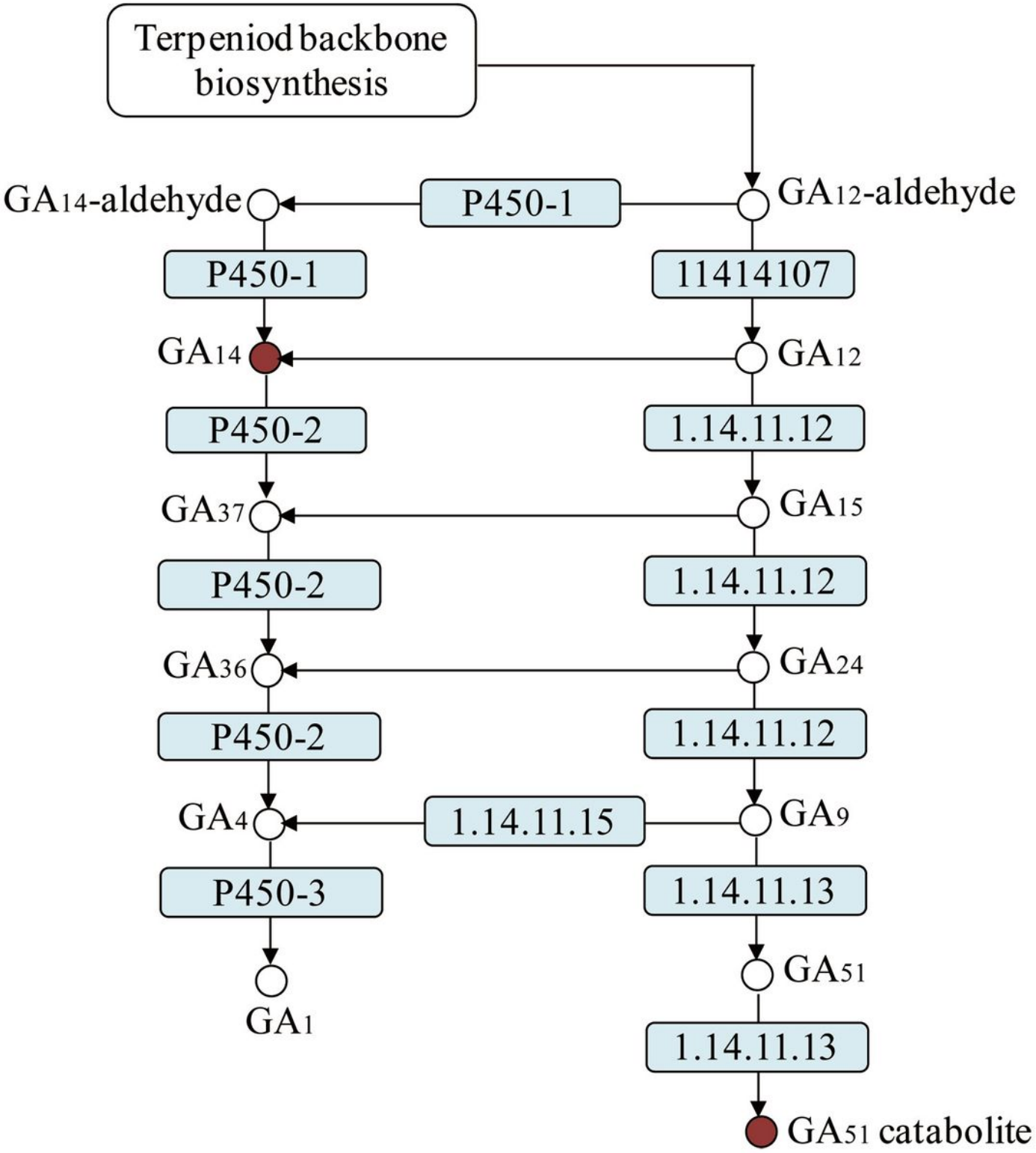

Figure 10

Diferentially accumulated metabolites involved in the "Diterpenoid biosynthesis" pathway of ginger. The red columns indicate metabolites expressed at a signifcantly high level in different rhizome samples. This map of DEGs corresponds to map00904 in the KEGG database.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.xlsx
- Additionalfile2.xlsx
- Additionalfile2.xlsx
- Additionalfile1.xlsx