Metabolomics for a Millenniums-Old Crop: Tea Plant (*Camellia sinensis*)

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Abstract

Tea cultivation and utilization dates back to antiquity. Today it is the most widely consumed beverage on earth due to its pleasant taste and several beneficial health properties attributed to specific metabolites. Metabolomics has a tremendous potential to correlate tea metabolites with taste and health properties in humans. Our review on the current application of metabolomics in the science of tea suggests that metabolomics is a promising frontier in the evaluation of tea quality, identification of functional genes responsible for key metabolites, investigation of their metabolic regulation, and pathway analysis in the tea plant. Furthermore, the challenges, possible solutions, and the prospects of metabolomics in tea science are reviewed.

KEYWORDS: metabolomics, technique, tea plant, metabolic mechanism

Introduction

The tea plant (*Camellia sinensis* (L.) O. Kuntze) is cultivated in more than 50 countries, as an essential cash crop in many developing countries.(1) Tea is consumed by approximately 70% of the human population.(2) The metabolites in the fresh leaves contribute to the resistance of tea plant to abiotic and biotic stresses, tea flavor/quality, and human health.(3−6) Metabolomics may help with the identification and quantification of metabolites, elucidation of metabolic pathways, and the regulation of these pathways.(7,8) This information is beneficial to genetic improvement, including screening and breeding of elite tea cultivars with excellent quality, high yield, and strong resistance.

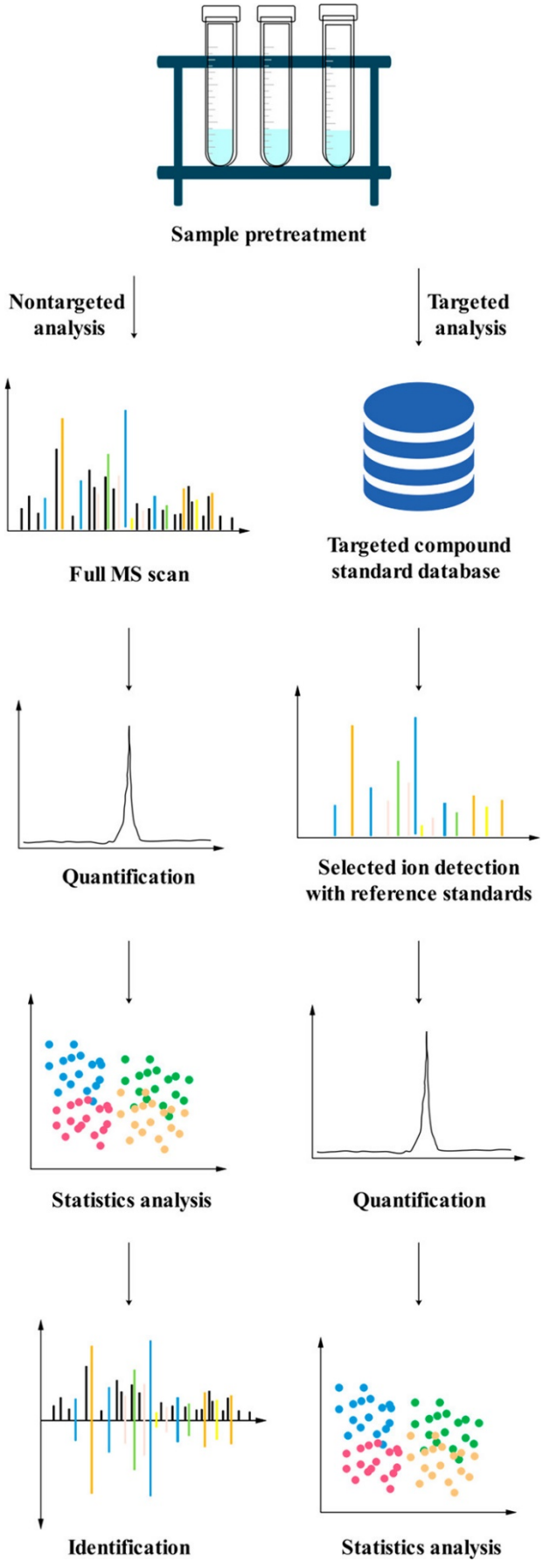

Figure 1. Workflow of metabolomics.

More recently, omics has been increasingly popular to decipher the complex biological processes in organisms, with the rapid development of bioinformatics. Genomics is an interdisciplinary field of biology focusing on the structure, function, evolution, mapping, and editing of genomes.(9) Transcriptomics is a set of all RNA molecules that can vary with external environmental conditions.(10,11) Proteomics is a comprehensive, quantitative description of protein expression.(12) Metabolomics is the study of the small-molecule intermediates and products of metabolism in the specific cellular processes from biological samples. It could be basically divided into nontargeted and targeted metabolomics based on two different strategies as presented in Figure 1. Metabolomics is one of the postgenomics techniques, exploring the relationship between gene sequences and metabolic networks, through a comprehensive investigation of many metabolites in a plant. Metabolomics is more capable to either reveal the relationship between genes, phenotype, and the environment or reflect on the nutritional level and physiological status than other omics. This capability is for the reason that the metabolites are the final stage that determines the phenotype after genomics, transcriptomics, and proteomics. It is time-consuming to demonstrate the expression level of genes based on visible phenotypes such as plant morphology. In addition, visible phenotypes sometimes fail to reflect the alternation of genotypes.(13) However, variations of some metabolites can be used for deduction of functional genes. Consequently, metabolites are the direct performer controlled by a complex series of biochemical reactions with the medium linking with the internal regulation of the genetic basis (genotype) and the external presence of the plant morphology (phenotype).(14)

Flavonoids, alkaloids, theanine, and terpenes are the subjects of intense research for the genetic improvement of the tea plant since they account for the majority of the unique compounds in tea. Accordingly, it is necessary to improve metabolomics for accurate analysis of a broad spectrum of metabolites in the tea plant. Specifically, it needs to improve the selectivity, sensitivity, economy, and convenience of the current metabolic detection platforms. Nonvolatile and volatile compounds of tea are usually determined through liquid chromatography–mass spectrometry (LC–MS) or gas chromatography–mass spectrometry (GC–MS), respectively. The genetic mechanisms in the tea plant underlying the metabolic pathways of the principle metabolites are still unclear due to the extremely complex genetic background. In particular, there have been only a few publications on biosynthesis, degradation, and regulation of metabolites in the tea plant at the whole-genome level to date.(15) Hence, we summarize the history, detection techniques, trends, perspectives, and limitations of current metabolomics in the tea plant. We also provide an insight into the exploration of metabolomics combined with other omics techniques that may eventually lead to the genetic improvement of the tea plant in this review.

Development of Metabolomics

The first study of metabolomics was the metabolite profiling analysis of steroids in human urine in the 1970s.(16) The conception of “metabolic control analysis” was put forward after obtaining the metabolic snapshot through tandem mass spectrometry as a tool in the elucidation of the function of novel genes in 1998.(17) Nicholson et al.(18) proposed “metabonomics” in 1999, the quantitative determination of metabolic processes, namely, small molecules in an organism with multiple dynamic parameters varying with time. Fiehn

et al.(19) presented “metabolomics” in the next year. Compared to “metabonomics”, “metabolomics” focuses on a static process, more precisely, the whole quantitative determination of a designated biological sample under certain conditions. Therefore, both of them refer to the metabolites of the whole organism. In general, the metabonome or metabolome is all of the molecules whose molecular mass is less than 1500 Da from a specific cell, organ, or organism. Metabolomics has been widely applied in functional genomics, nutritional genomics, clinical toxicology, clinical medicine, drug discovery, biomarker discovery, and other fields.(20−22)Figure 2 shows the number of papers per year with “metabolomics” or “metabonomics” in their titles, abstracts, or keywords from 2001 to 2018 searching on https://ccc.glgoo.top/scholar. This figure shows a clear ascending trend.

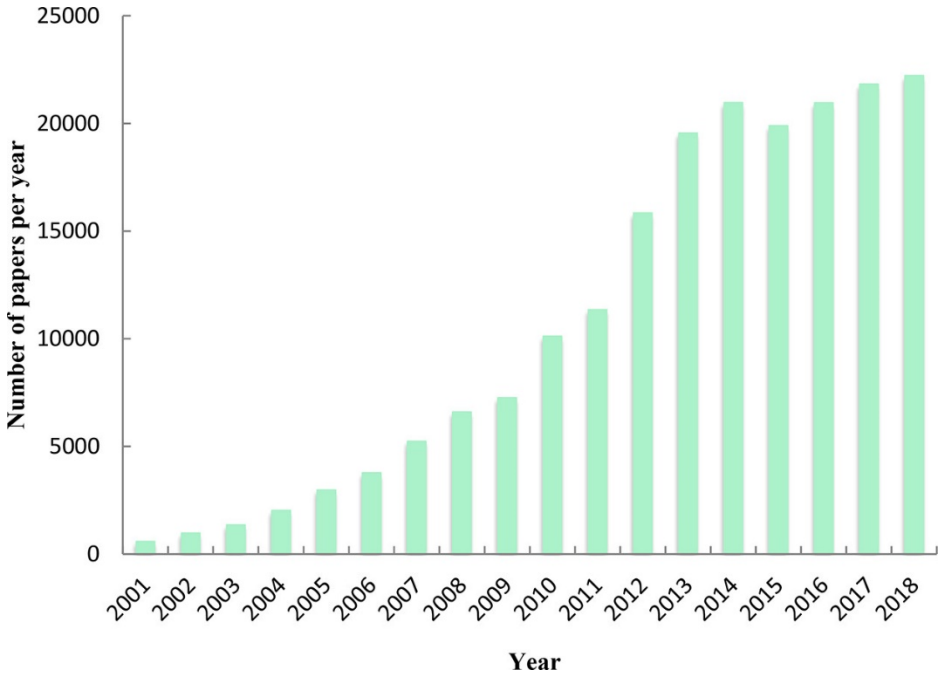

Figure 2. Summary of papers referring to “metabolomics” or “metabonomics” in their titles, abstracts, or keywords from 2001 to 2018 searching on https://ccc.glgoo.top/scholar.

Numerous high-abundance metabolites with distinct functions in plants could fall into the primary and secondary metabolites groups, e.g., glucosides, amino acids, flavones, terpenes, and alkaloids that are regulated by specific genes.(23−25) Metabolomics can promote the investigation of biosynthetic pathways in basic research. Metabolomics is also important for screening of crops possessing unique qualities, strong resistances, and high yields in agricultural practice. There are several advantages in metabolomics as follows: First, it is easier to detect the metabolites than the expression of genes or proteins, and it is well-known that the former is regulated by the latter. Second, it does not require the complete genomic sequence or a massive database. Third, it is time-saving owing to its lower diversity than genes or proteins. Fourth, metabolic biomarkers will be more universally applicable across species than transcriptomic or proteomic biomarkers.

Metabolic products could reflect growth, development, and disease of plants via genetic variation, epigenetic modification, and transcriptome and protein compositions.(26,27) Plant metabolomics mainly focus on the mechanism underlying disease and stress resistance, the biosynthetic pathway of secondary metabolites, the variation of quality-

related metabolites among diverse varieties, the characteristics of growth and development, and so on at present.(28−32) In a word, metabolomics is a powerful tool for the analysis of secondary metabolic networks and identification of rate-limiting steps, cellular activity, and the genetic relationship of plants based on the comprehensive study of complex metabolic processes and products when combined with other omics.

Technology of Metabolomics

Chromatography–Mass Spectrometry

Chromatographic techniques separate components depending on their affinity for the mobile and stationary phases. The mobile and stationary phase can be categorized into liquid chromatography (LC), gas chromatography (GC), and supercritical fluid chromatography (SFC) on the basis of the mobile phase.(33) Drift correction and time shift alignment are a critical first stage in chromatographic data analysis.(33) Mass spectrometry (MS) takes advantage of mass-to-charge ratios of fragment ions produced from a molecule for structure analysis and identification.(34) It also has excellent sensitivity, high specificity, and simplicity without elaborate preparatory procedures.(35) Thus, it can further improve the resolution of unknown metabolites, in particular for metabolites with isomers and poor separation with chromatography.(36) Matrix-assisted laser desorption ionization, secondary ion MS, and desorption electrospray ionization MS are the predominant types of MS in the current literature.(37) Matrix-assisted laser desorption ionization uses a laser energy absorbing matrix to create ions from large molecules with minimal fragmentation.(38) Secondary ion MS generates ions that are then transferred into a mass spectrometer across a high electrostatic potential and are referred to as secondary ions.(39) Desorption spectrometry observes desorbed molecules from a surface when the surface temperature is increased.(40) Most plant-based MS studies to date have addressed the temporal and spatial distribution of metabolites.(41,42)

Electrospray ionization (ESI) and atmospheric pressure ionization (API) are the two widely used ionization modes. ESI produces ions using an electrospray source in which a high voltage is applied to a liquid to create an aerosol.(43) API produces primary ions on a solvent spray.(44) GC–MS, pioneered in plant metabolomics, has both the high resolution ability of GC and the high specificity of MS.(11) GC coupled to electron-impact quadrupole MS, with remarkable accuracy and repeatability, is one of the most mature technologies capable of fulfilling the required criteria that is applied for evaluation of heat-stable and volatile small molecules. GC–MS is suitable for metabolic analysis of ultrasmall sample volumes of 10 nL to 2 μL. However, derivatization is required before analysis of high-boiling-point compounds. GC–MS allows the detection and quantification of more than 300 compounds from a single plant leaf extract.(19) LC–MS is more suitable for detection of molecules with a high-boiling-point, thermal instability, and strong polarity.(45,46) GC–MS and LC–MS achieve the measurement of a broad range of metabolites from low to high polarity, respectively.(47) Ultra performance liquid chromatography–quadrupole-time-of-flight mass spectrometry (UPLC–Q-TOF-MS) permits investigation of the fragmentation pathway of unknown compounds and predictions of their molecular formulas based on the recorded accurate masses. Ion trap-MS and TOF-MS have been used extensively in metabolomics, due to their high resolution, outstanding sensitivity, and strong structural

characterization.(48) An ion trap is a combination of electric or magnetic fields used to capture charged particles. As the main radio frequency voltage increases, ions of growingly greater mass-to-charge ratios eject through the slots in the linear trap. Ions are focused toward the ion detection system where they are detected. The linear ion trap provides MS^n capabilities, and the Orbitrap analyzer can provide a mass resolution of 100 000.(49) A variety of ions could also be successively separated after gaining the same initial kinetic energy produced from the mass-to-charge ratio by TOF-MS, as the arrival time in the receiver is influenced by their mass. In theory, there is no upper limit if the flight tube is long enough. It needs tandem mass spectrometry, involving quadrupole-time-of-flight-mass spectrometry (Q-TOF-MS), ion trap-time-of-flight-mass spectrometry (IT-TOF-MS), quadrupole mass spectrometry (QMS), and triple quadrupole tandem mass spectrometry (QqQ-MS), to identify the signal that is difficult for single TOF spectrometry. Q-TOF-MS further improves the resolution capability as a result of the integration of quadrupole and reflective time-of-flight spectrometry. IT-TOF-MS combines time-of-flight spectrometry and ion trap so that it allows obtaining secondary and higher order fragmentation spectrum data to help with structure identification or elucidation.

Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) is accessible to explore torsional angles, internuclear distances, bond orientations, and dynamics on a picoseconds to seconds time scale(50,51) based on the energy level transition of atoms after absorption of radio frequency radiation in the magnetic field outside the core.(52) Although NMR is less sensitive, it provides abundant structural information for its higher reproducibility and universality compared to MS.(53) NMR has been used in a number of particular cases to measure metabolic fluxes in dynamic metabolic networks in the past decade.(54) NMR spectroscopy transfers the time signal into a frequency signal for line intensity using Fourier transformation involving one-dimensional or two-dimensional NMR. Two-dimensional NMR spectroscopy is advantageous to acquire more detailed structural information on complicated compounds or the overlapping region of one-dimensional NMR spectroscopy. ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR are popular NMR approaches for plant metabolomics. ^{1}HNMR is predominantly used to gain an overview of compounds present in polar extracts.(55) However, the concentrations of numerous metabolic compounds in crude plant extracts are so low that they are easier to be captured by MS than NMR.

Other Technologies

Fourier transform-infrared spectroscopy (FT-IR) is a rapid, nondestructive, and high-resolution method for analyzing the molecular contents of complex samples.(56) Fourier-transform-ion cyclotron resonance (FT-ICR) is a type of mass analyzer of ions based on the cyclotron frequency of the ions in a fixed magnetic field.(57) Near-infrared spectrometry (NIR) observes the structure and content of the sample by virtue of its absorption property ranging from 780 to 2526 nm.(58,59) Capillary electrophoresis–mass spectrometry (CE–MS) could be utilized to separate positive, negative, and neutral ions for high-throughput nontargeted metabolomics, with the feature of small injection volume, separation with high efficiency, and short analysis time, despite its limitation of reproducibility and sensitivity.(59)

The appropriate analytical technique for metabolomics is the critical step that is intimately linked with the reliability of data obtained due to distinct features of different approaches. The common techniques and their features are listed in Table 1. Generally, an ideal analytical method should cater to the following requirements: universality, suitable for detecting a wide range of metabolites from various organisms, organs, or cells; accuracy, able to reflect the true value; reproducibility, stable for a long time for analyses of huge numbers of samples; sensitivity, a wide dynamic range; high resolution, able to distinguish slightly different components; high efficiency, easy sample pretreatment and high throughput. However, there is no single technique fulfilling all the above-mentioned requirements, and thus it is advisable to combine several techniques for the purpose of complementary advantages.

Table 1. Main Features of Most The Popular Techniques for Metabolomics

technique	CE–MS	GC–MS	LC–MS	NMR 1D	NMR 2D
universality	++	+	+/++	+++	+++
accuracy	–/++[a]	–/++[a]	–/++[a]	+++	+++
reproducibility	++/–[a]	++/–[a]	++/–[a]	+++	+++
sensitivity	+	++/+++[a]	+++	–	–
resolution	++	+++	+++	+	++
efficiency	++	–	+	++	++

[a] Accuracy, repeatability, and sensitivity mainly depend on the analyzer. GC–MS and LC–MS are usually performed either with a quadrupole or time-of-flight analyzer. “+” or “–” in front of and behind “/” represent quadrupole and time-of-flight analyzer, respectively.

Metabolic Characteristic of Principle Metabolites in Tea

The tea plant is an evergreen perennial woody C3 plant. Its utilization efficiency of solar radiation is far lower than C4 plants because of stronger photorespiration, higher light compensation point, and lower light saturation point.(60) The respiratory efficiency of vegetative organs of the tea plant from high to low is leaf, root, and stem. The primary metabolites, including sugars, amino acids, and fats for vital activity, are used to produce secondary metabolites such as flavonoids, carotenoids, alkaloids, etc. under specific conditions. The main secondary metabolic pathways in the tea plant are as follows: the shikimate pathway and the malonic acid pathway regarding erythrose-4-phosphate, phosphoenolpyruvic acid, and acetyl-CoA as the substrates for the synthesis of catechins, tannins, anthocyanins, lignins, and other polyphenols;(61,62) the methyl-amyl acid pathway and the pyruvate pathway regarding methyl-amyl acid and 3-phosphoglyceric acid as the substrates for the synthesis of gibberellins, carotenoids, triterpene alcohols, and other terpenes;(63) the amino acid pathways with aromatic and aliphatic amino acids as the substrates for the synthesis of quinine, caffeine, theobromine, and other alkaloids.(64,65)

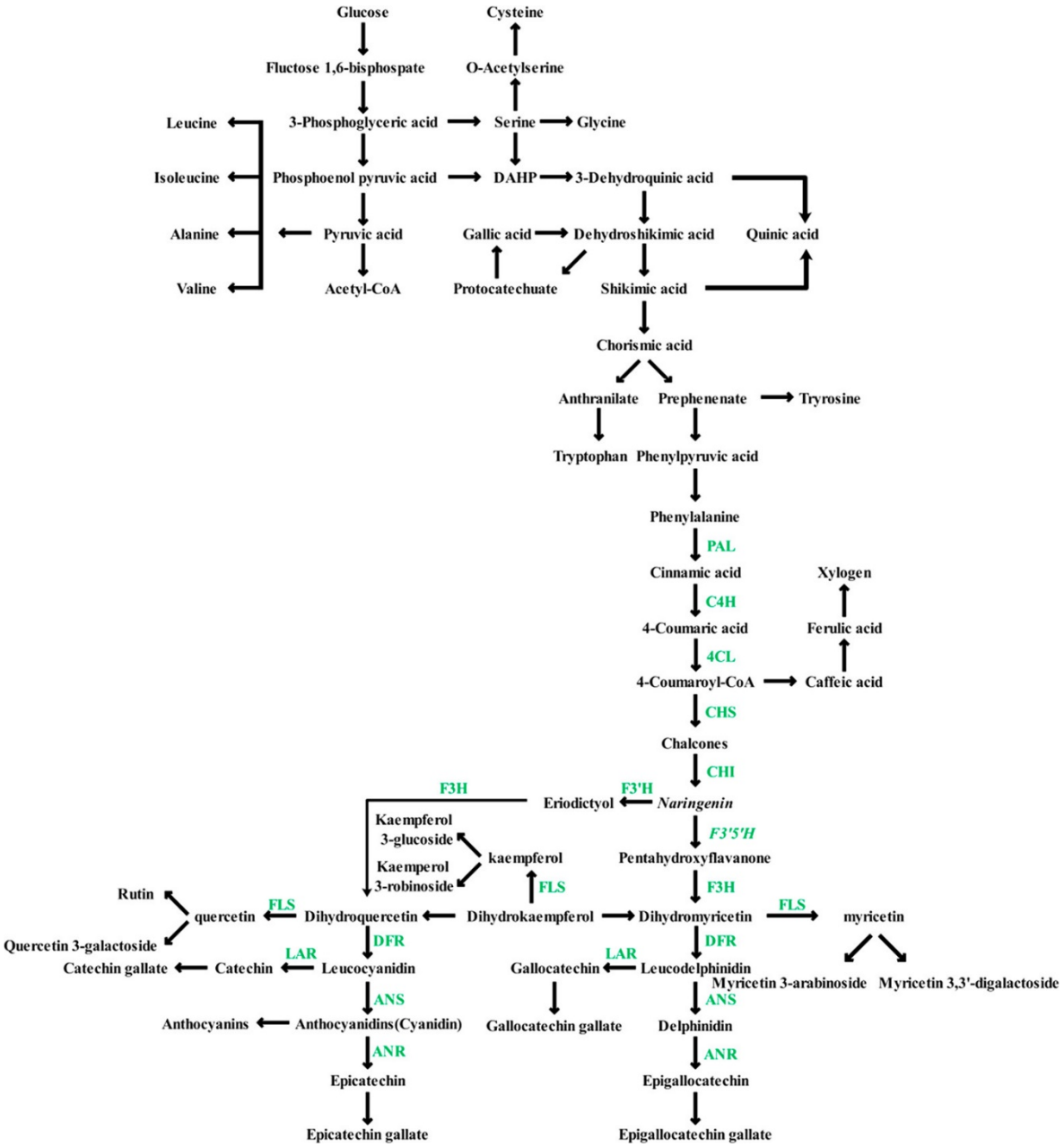

Figure 3. Metabolism of flavonoids in the tea plant. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate–CoA ligase; CHI, chalcone isomerase; CHS, chalcone synthase; F3’5’H, flavonoid 3’, 5’-hydroxylase; F3H, flavanone 3-hydroxylase; F3’H, flavonoid 3’-monooxygenase; FLS, flavonol synthase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase.

Metabolism of Flavonoids

Flavonoids are a large family ubiquitously distributed throughout the plant kingdom, classified as flavones, flavan-3-ols, isoflavones, flavanones, flavonols, anthocyanins.(66) The metabolism of flavonoids in the tea plant is shown in Figure 3. The tea polyphenols are a large category of flavonoids, more than half of which are catechins. The major catechins in tea are catechin (C), epicatechin (EC), gallocatechin (GC), epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), catechin gallate (CG), and gallocatechin

gallate (GCG).(67) Among them, EGCG accounts for the highest amount of catechins.(67) Furthermore, methylated catechins and catechins binding to digallic acid are also found in tea.(68,69) Some of the catechin derivatives have some special pharmacological effects. Tea catechins also bind DNA via hydrophilic and hydrophobic interactions.(67) For instance, O-methylated epigallocatechin-3-*O*-[3-*O*-methyl] gallate (O-methylated EGCG) could alleviate Japanese cedar pollinosis.(68) The amount of total catechins is usually high in the young and fast-growing leaves of tea. They are believed to provide a chemical defense to the young tender leaves from herbivores and pathogens.(69) The biosynthesis of catechins includes the shikimate pathway, the phenylpropanoid pathway, and the flavonoid pathway. The shikimate pathway connects the primary and secondary metabolism. Chorismic acid is its final product, as the precursor of aromatic amino acids, l-tryptophan, l-phenylalanine, and l-tyrosine, are further synthesized into pigments, phenols, alkaloids, hormones, and aromatic components.(70) The phenylpropanoid pathway is between the shikimate pathway and flavonoid pathway, producing salicylic acid lignin and isoflavonoid.(71) Flavonoids are formed into their derivatives such as flavonoid glycoside through methylation and galactosylation. The majority of polyphenols are located in the leaves of the tea plant; however, some of them distribute to the flower and fruit.(72)

Metabolism of Theanine

There are 26 amino acids identified in tea, including 20 protein amino acids and six nonprotein amino acids. l-glutamic acid is a precursor for theanine, catalyzed by theanine synthase in the roots. Theanine (*N*-ethyl-γ-l-glutamine) belongs to one of the nonprotein amino acids, accounting for more than 70% of the free amino acids in the dry weight of tea leaves. Theanine acts as a reservoir for nitrogen and as an initiator for carbon skeleton synthesis during germination.(73) The metabolism of theanine in the tea plant is presented in Figure 4. Theanine is hydrolyzed into glutamic acid and ethylamine in the presence of theanine hydrolase in the leaves with sunlight. Ethylamine is transformed into acetaldehyde induced by ammonia oxidase.(74,75) Acetaldehyde is a precursor of catechins.(75) Theanine is produced in the roots. It is also biosynthesized in the leaves as well, however, not as much as in the roots. Biosynthesis in the roots may be due to the absorption of nitrogen, as ammonia and nitrate by the roots. The content of theanine in leaves is reduced with leaf maturation,(73) and its content is lowest in the stem.(76)

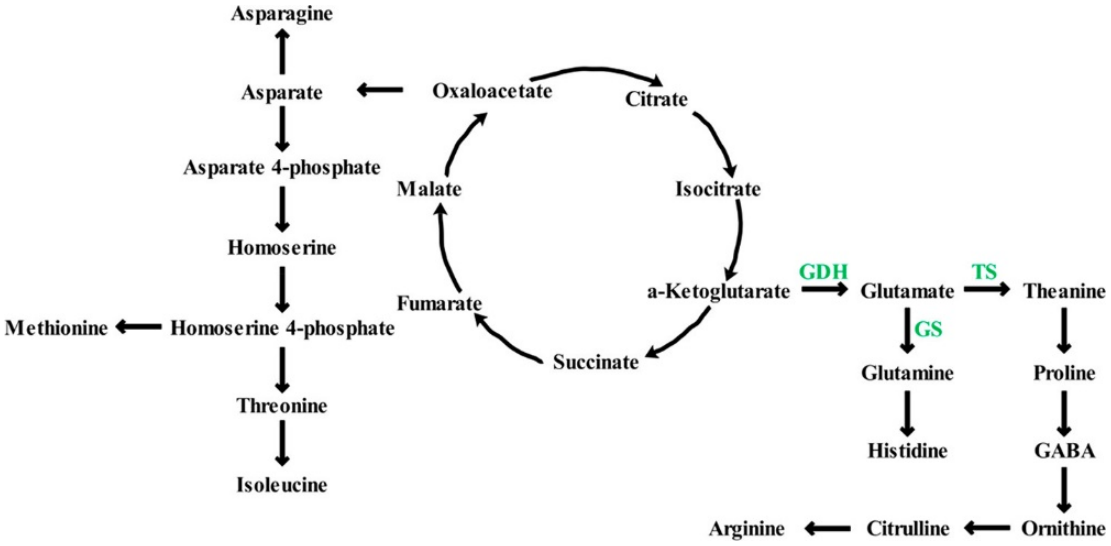

Figure 4. Metabolism of theanine in the tea plant. GDH, glutamate dehydrogenase; TS, theanine synthase; GS, glutamine synthase.

Metabolism of Caffeine

Caffeine (1,3,7-trimethyl xanthine), is produced from the de novo pathways and the salvage pathway.(77) The latter is the predominant way as displayed in Figure 5. The purine ring of caffeine comes from the de novo synthesis from glycine, glutamine, formate, and carbon dioxide or from the nucleotide pool, adenine serving as its main source. Theobromine is the precursor of caffeine. On the other hand, *S*-adenosyl methionine offers the methyl for caffeine catalyzed in the order of 7,3,1 by *N*-transmethylase.(78) Caffeine is mainly synthesized in the tender leaves. The rate of caffeine synthesis is significantly reduced with leaf maturity. Approximately 99% of caffeine is found in the leaves.(79,80) It is also produced in the roots, flowers, and fruit. Most caffeine located in the vacuole is binding to chlorogenic acid.(80)

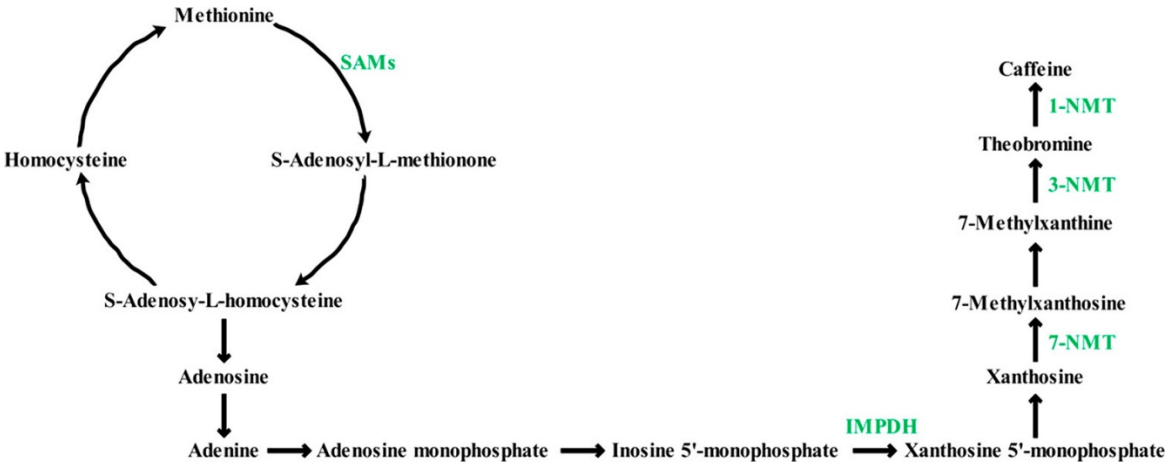

Figure 5. Metabolism of caffeine in the tea plant. SAMs, *S*-adenosyl-l-methionone synthase; IMPDH, inosine 5'monophosphate dehydrogenase; NMT, *N*-methyltransferase.

Metabolism of Terpenes

Terpenes exhibit a strong aroma. Terpenes are also the precursors of aromatic compounds in made tea and protect the tea plants from insects by releasing volatile deterrent compounds. The terpene index (TI) was used to evaluate the aroma quality of tea according to the formula:

$$\frac{\text{linalool and its oxidative}}{\text{linalool and its oxidative} + \text{geraniol} + \text{nerol} + \alpha\text{-terpinol}}$$

.(81) The TI of *Camellia sinensis* var. *assamica* is almost 1.0 with a relatively high content of linalool.(82) By comparison, the TI of *C. sinensis* var. *sinensis* ranges from 0.1 to 0.2 with a relatively high content of geraniol. The content and composition of monoterpene alcohols are highly dependent on genetic characteristics. Terpenes are derived from two common interconvertible five-carbon precursors, isopentenyl diphosphate and its allylic isomer, dimethylallyl diphosphate.(83) These precursors are synthesized via the following pathways: the cytoplasmic mevalonic acid (MVA) and the plastidial methylerythritol phosphate (MEP) pathways, as presented in Figure 6. The MVA pathway provides precursors to sterols, certain sesquiterpenes, and the side chain of ubiquinone,(84) while the MEP pathway synthesizes monoterpenes, diterpenes, carotenoids, and the side chains of chlorophylls.(85) The majority of monoterpenes and sesquiterpenes in tea present a floral or fruity aroma. Linalool and geraniol possess a woody

fragrance and a rose fragrance, respectively, while nerolidol is an applelike aroma. The terpenes mentioned above are the major volatile compounds of fresh tea leaves. The tea plant will synthesize specific volatile terpenes under some environmental conditions. For example, tea leaves infected by tea green leafhoppers would produce linalool derivatives.(86)

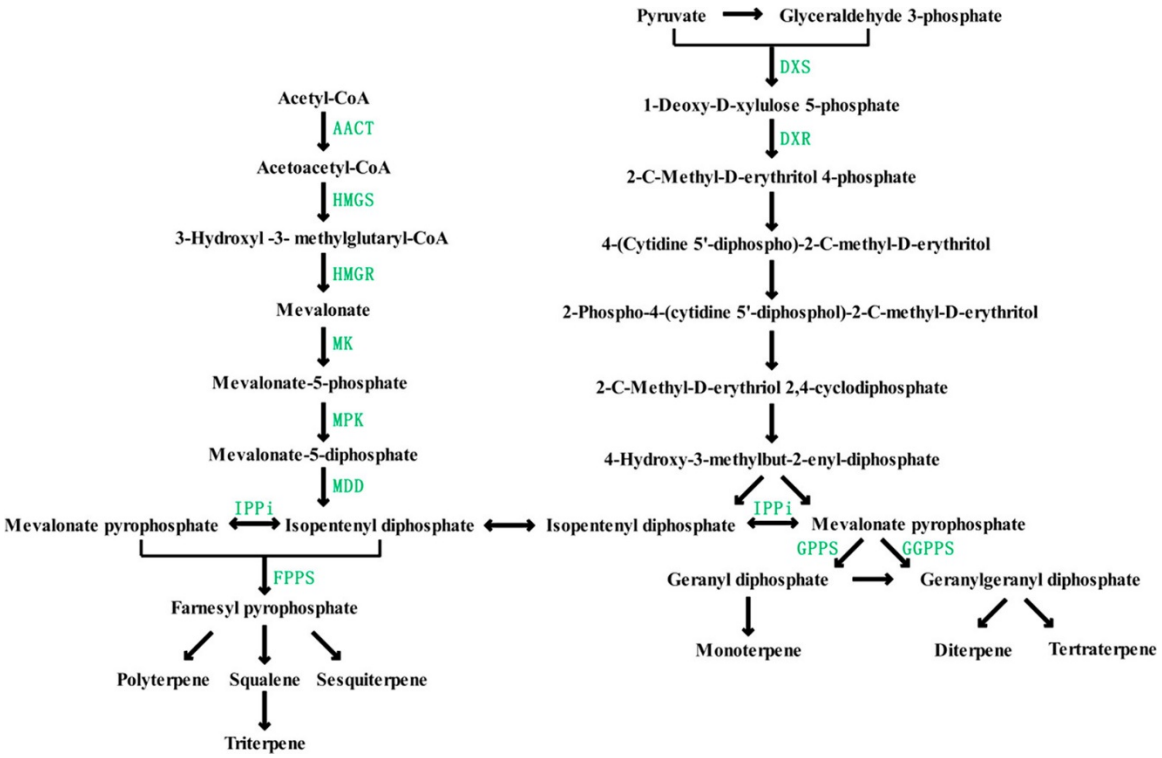

Figure 6. Metabolism of terpenes in the tea plant. AACT, acetoacety-CoA thiolase; HMGS, 3-hydroxyl-3-methylglutaryl-CoA synthase; HMGR, 3-hydroxyl-3-methylglutaryl-CoA reductase; MK, mevalonate kinase; MPK, mevalonate-5-phosphate kinase; MDD, diphosphomevalonate decarboxylase; IPPi, isopentenyl diphosphate isomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; GPPS, geranyl pyrophosphate synthase; GGPPS, geranylgeranyl diphosphate synthase.

Carotene and its oxidative degradation products are the classical terpenoids in the tea plant. Carotenoids, including β-carotene, lutein, zeaxanthin, neoxanthin, xanthophyll, and lycopene, have been identified as precursors for many tea flavors. Their biosynthetic pathway starts with the formation of isopentenyl diphosphate and dimethylallyl diphosphate. Isopentenyl diphosphate and dimethylallyl diphosphate are isomerized by isopentenyl diphosphate isomerase in the methylerythritol 4-phosphate pathway.(87) Besides free volatile compounds, glycosidically bound volatile compounds exist in tea. β-primeverosides and β-glucosides are the predominant aroma precursors, which can release free terpenes after being hydrolyzed by β-primeverosidases and β-glycosidases enzymes.(88)

In general, some genes regulating the metabolism of terpenes have been reported. It is known to us that the environmental elements, sunlight, temperature, water, and insects, play a role in the biosynthesis of terpenes in the tea plant. The basic mechanisms underlying

the biosynthesis of terpenes have been revealed.(83,84) In the future, it may be possible to improve the aroma of tea by regulating the cultivation conditions.

Application of Metabolomics in the Tea Plant

Metabolomics is broadly applied in tea sciences, including screening of elite tea, evaluation of tea quality, physiological changes in the tea plant induced by cultivation conditions, metabolic responses to abiotic and biotic stress, and construction of metabolic pathways.

Evaluation of Tea Quality

Variation of Metabolites Controlled by Genetic Basis

Tea metabolism is controlled by genetic materials and regulated by environments and agricultural practices. Thus, gene sequences are the basis of a considerable number of compounds in different tea accessions. Biochemical constitutes in fresh tea leaves are the precursors for tea manufacturing. Catechins, kaempferol, and quercetin derivatives were the key metabolites responsible for cultivar discrimination according to the metabolite profiling of 14 Wuyi Rock tea cultivars.(89) Flavan-3-ols, proanthocyanidins, flavonol glycosides, flavone glycosides, flavonone glycosides, phenolic acid derivatives, hydrolyzable tannins, alkaloids, and amino acids also revealed clear variations between tea cultivars.(89) The metabolomics approach with various tea cultivars indicated high levels of catechin derivatives in the EGCG- and EGCG3"Me-rich tea cultivars as well as the low levels of amino acids, in contrast to wild type tea cultivars.(90) Moreover, different tea accessions have unique regulatory mechanisms. The purple leaf color in 'Zijuan' was mainly caused by a decrease in flavonoids/anthocyanins. A decrease in flavonoids/anthocyanins, an enhancement of porphyrin, chlorophyll metabolism, carotenoid biosynthesis, and steroid, and a decrease in fatty acids synergistically gave rise to the leaf color change in 'Zixin'.(61)

Metabolites of tea plants varied with distinct verities would have a profound effect on tea quality and flavor. Nevertheless, less is known about the comprehensive understanding of characteristic components controlled by genetic backgrounds. One possible solution is comparing metabolites in a global tea accessions concerning cultivars, landraces, and wild types especially core germplasms.

Variation of Metabolites with Tea Category and Grade

Metabolomics may be useful to grade tea in an unbiased, scientific way based on the metabolite profile. Metabolomics may be used for the improvement of the tea processing conditions. The metabolites of 191 green teas from China, Japan, Vietnam, and other countries were determined through ^{1}H NMR metabolomics. The results indicated that theanine, gallic acid, caffeine, EGCG, and ECG in the highly valued Longjing tea were more abundant than other teas, while EGC was lower.(91) The levels of theogallin and gallocatechin were higher in the high-quality green tea, whereas neotheaflavin, neotheaflavin 3-*O*-gallate, trigalloyl-β-d-glucopyranose, catechin-(4α → 8)-epigallocatechin, kaempferol, and myricetin 3,3'-digalactoside were lower.(92) Furthermore, 2-

phenylacetaldehyde with a pungent and unpleasant odor presents an adverse effect on tea quality.(93)

Besides green tea, there are various studies on the flavor metabolites of other made teas. The umami taste of white tea was related to theanine, aspartic acid, asparagine, and adenosine. There are also correlations between bitterness and astringency with flavan-3-ols, theasinensins, procyanidin B3, and theobromine. Moreover, EGCG, ECG theogallin, γ-aminobutyric acid, and caffeine in white tea lead to astringency and bitterness.(5) The level of apigenin7-[galactosyl-(1 → 4)-mannoside], apigenin 6-C-glucoside 8-C-arabinoside, biochanin-A7-(6-methylmalonylglucoside), and garcimangosone D increased from high grade to low grade.(94) However, the authors have not discussed the reason for this result. Theaflavins and thearubigins significantly influenced the quality of tea as they impart desired color and taste to the tea liquor.(95) Theaflavins, phenolic acids, catechins, flavonoids in the form of aglycons and glycosides, and theasinensins are marker compounds to distinguish the grade of Keemun black tea through metabolomics.(96)
Some metabolites such as catechins, theanines, and caffeine are the common gradients in all kinds of made tea. Some are quality-related components in certain teas. As a result, tea metabolomics is an effective technique to seek for quality dependent compounds to promote evaluation of tea quality.

Variation of Metabolites with the Ambient Environment

It will be interesting to explore the variety and amount of metabolites under distinct environmental conditions, including temperature, rainfall, and duration of sunshine. Metabolomics combined with other omics techniques can identify genes that are affected by environmental conditions. The composition and content of components in fresh tea leaves differed in early and middle May in the Bulang Mountains.(97) The metabolites of tea were closely related to the growing conditions. White and oolong tea from China, Japan, and Korea could be clustered together based on 284 metabolites by targeted metabolomics. Meanwhile, there were differences among white teas concerning theanine and catechins from different countries and regions.(98) Likewise, metabolic components of tender shoot varied with growth stages in spring. As the season progressed from spring to summer, the content of amino acids reduced, whereas sugars, flavonoids, and their glucosides and the metabolites in the citric acid cycle and photorespiration pathway increased.(99) Most sesquiterpene contents were lowest in April, rose markedly in June, and declined in August, September, and October in Anhui, China.(100) While the majority of monoterpenes were on the contrary.(100) The content of caffeine, ECG, GC, and C reduced, while sweet, floral, honeylike compounds increased at high elevation, implying striking changes in tea metabolites due to elevation effects.(101) The biosynthesis of C, EC, EGC, GC, and EGCG3”Me decreased in tea leaves with shade treatments, while the biosynthesis of EGCG increased under shade treatment.(102) There were lower concentrations of amino acids in leaves with higher light intensity, especially leucine, isoleucine, valine, alanine, threonine, asparagine, and aspartate acid.(102)

The ambient elements, involving sunlight, temperature, and rainfall, influence the growth of tea plant and the quality of tea. Several variations of metabolites have been presented in

the previous papers.(98,103) However, the influence mechanism of complex climatic and cultivated factors and their interaction needs further investigation.

Variation of Metabolites with Developmental Period

Metabolomics has revealed increasing levels of theanine, caffeine, and gallic acid but decreasing levels of catechins, glucose, and sucrose with leaf age.(104) Flavonoids, caffeine, and carbohydrates decreased significantly, while isoleucine, tryptophan, serine, and allonic acid increased significantly in mature leaves from the early to the late spring. In addition, the biosynthesis of flavone and flavonol and the metabolism of glutathione, tryptophan, and linoleic acid were more active in the mature leaves than in the young shoots (two expanding leaves and a bud). These leaf-age-related differences suggest that the balance of carbon and nitrogen reserves is remobilized during different periods of the spring season.(99) C, EC, EGCG, total catechin, and monoterpene decreased, but EC, EGC, volatile fatty acid derivatives, and wall-bound phenolics increased with maturity.(105)

The tender shoot is the economically important part of the tea plant with different compounds at various developmental stages. Different plucking standards for different teas are recommended due to the different metabolites associated with young and mature leaves. These plucking standards can promote rational plucking and farming management of fresh tea processing.

Variation of Metabolites with the Manufacturing Process

The fresh tea leaves can be made into green, black, and oolong teas according to the degree of fermentation. The first step of tea processing is withering. Tea leaves are increasingly softened with moisture reduction. Meanwhile, their chemical compounds especially volatiles change to a certain proportion.(106) The most important process of green tea is fixation or de-enzymation. During this period, tea leaves are treated at a high temperature to deactivate the polyphenol oxidase enzyme localized within the cells of leaves, to stop the fermentation process, and to maintain the original green color. On the other hand, when the fresh leaves begin withering and are not treated at a high temperature, the tea polyphenols are oxidized by the polyphenol oxidase enzyme, thus producing fermented tea (black tea). When the enzymes in the tea leaves are not completely deactivated and the tea polyphenols are not fully oxidized, this produces intermediate tea products semifermented tea (oolong tea).(1) The manufacturing process, therefore, produces the unique flavor of various kinds of tea after a series of biochemical transformations. Metabolomics will provide a comprehensive picture of the metabolite changes during the manufacturing process, which is helpful to improve tea quality and set quality control standards for each type of tea. In order to characterize the entire fermentation process of tea, Tan et al.(107) prolonged fermentation duration of 14 h, although appropriate fermentation duration is about 6 h for unshredded black tea and 30–120 min for shredded black tea. C, EC, EGC, EGCG, ECG, epicatechin 3-*O*-methyl gallate, and epigallocatechin methyl gallate decreased within the initial 6 h of fermentation and then remained steady.(107) Most of the flavone glycosides continuously increased during the entire fermentation process, while theanine decreased slightly.(107) Compared with pan-frying fixation, steam fixation reduced the concentrations

of catechins and geraniol and linalool and its derivatives. In contrast to this, the concentration of indole and nonanal were significantly increased during steam processing.(108) Biochemical transformations were most active during the manufacture of oolong tea due to fixation at high temperature, and free amino acids and hydroxyl jasmonic acid increased during fermentation.(109)

The precursors in fresh tea leaves are the basis for producing the unique taste and aroma of the made tea types due to the complex chemical and enzymatic transformations that occur during the manufacturing (withering and fermentation) processes. Adjustments of the manufacturing processes could change the metabolic alternations of fresh tea leaves for quality optimization. Further research is recommended to uncover the metabolic flux and thus improve our understanding of the biochemical transformations during the manufacturing processes.

Mining of Functional Genes Responsible for Metabolism

Metabolomics permits mining of the functional genes in metabolic pathways combined with genomics and transcriptomics. The detection of metabolite quantitative trait loci (mQTLs) provides information about the association between genotype and phenotype for mining and validation of candidate functional genes. By using this strategy, genes encoding caffeine synthase were successfully cloned in 2000, opening up the opportunity for the development of a naturally low-caffeine tea cultivar.(110) Some other genes controlling metabolic pathway were identified since 2000. As an illustration, the expression levels of phenylalanine ammonia-lyase, dihydroflavonol reductase, and chalcone synthase were high in the bud and the first and second leaves while low in the mature leaf.(111) Dehydroflavonol reductase is closely related to catechin metabolism. Seven SNPs were characterized for caffeine synthase 1 (TCS1), eight genes for inosine-5′-monophosphate dehydrogenase (TIDH), and seven genes for S-adenosyl-l-methionine synthetase (sAMS), respectively.(111) The caffeine content was significantly dependent on SNPs in *TCS1* A995C, *TIDH* T573A and *sAMS* C393T.(15) A total of 87 and 120 SNPs were found in *TCS1* and flavonoid *3′,5′ hydroxylase* (*F3′,5′H*), respectively.(111) Ten SNPs in *F3′,5′H* influenced the ratio of dihydroxycatechin and trihydroxy catechin to total catechin content.(15) The S-RNA enzyme putative gene (*KU852488*) encoding 238 amino acids was cloned, in which one SNP was mined.(112) Approximately 2 000 unigenes were obtained from the transcriptome data of leaves and roots from ‘Baojing Huangjincha’ and ‘Fuding Dabaicha’ under different nitrogen treatments. *AMT*, *AQP*, *GS*, and *GOGAT* were identified, which were tissue-specific and mutually restricted in the network of nitrogen absorption. Moreover, three new genes regulating nitrogen utilization were discovered from two tea cultivars, possibly playing roles in stress, binding to manganese, and the transcription factor regulating gibberellin. Their expression level dramatically influenced the amino acid content.(113)

These genes encode enzymes controlling caffeine, catechin, and other secondary metabolites that are of extreme importance for metabolic pathway construction in the tea plant. Unfortunately, most of them are candidate genes without validation, gained from the transcriptome rather than the genome. The lack of proven transgenosis and tissue culture restricts the transformation system in the tea plant. As a consequence, it is a possible way

to quickly dig and decipher other unknown metabolism-related functional genes through the combination of metabolomics and molecular markers.

Investigation into the Metabolic Mechanism of the Tea Plant

Metabolic products of tea examined using metabolomics may provide insights into biosynthetic and degradative pathways and transformative and regulative relationships among metabolites under various stresses. The polysaccharides were hydrolyzed to raffinose, maltose, glucose, and fructose under low temperature in winter, and there were negative correlations between shikimic acid and quinic acid based on metabolomics.(114) Compared to green leaves, purple leaves showed a higher content of anthocyanin, total polyphenols and total catechins, a higher carotenoid-to-chlorophyll ratio, a lower content of glucose, fructose and sucrose, and a lower photosynthetic efficiency, CO_2 assimilation, and carbohydrate accumulation rate. The lower CO_2 assimilation rate correlates to lower yields of purple-leaved clones compared to green leaved clones.(115) The expressional levels of flavonoid 3'-monooxygenase, anthocyanin 5-aromatic acyltransferase, and phenylalanine ammonia-lyase were up-regulated in purple tea leaves.(115) The leaf purple color was fading with the reduction of flavonoids and anthocyanins in 'Zijuan'.(61) A decrease in flavonoids and fatty acids and an increase in porphyrin, chlorophyll, carotenoid, and steroids were observed during the color shift from purple to green in 'Zixin'.(61) These results suggest that the mechanisms of leaf color changes differ between the two purple-leaf tea cultivars.(61) It is likely that the expression levels of genes concerning the synthesis of catechin are sensitive to light at the beginning of the dark period. Particularly, the conversion of theanine to catechin derivatives is weaker under shade treatment, in contrast to the high efficiency of generating catechin from theanine under strong light.(102) Shading is the basis for achieving the highly desired high theanine content of matcha tea. The shading is achieved by covering the tea plants with shade nets or rice straw in Japan.(102) The nitrogen consumption declined as the result of the weakness of carbon anabolism in albino leaves yet whose catabolism was reinforced to generate more carbon skeletons for energy production, indicating a potential mechanism for regulating the balance of carbon and nitrogen metabolism under conditions of carbon deficiency.(116)

The integration of metabolomics and other omics allows a better understanding of the relationship between gene expression levels and metabolites and the construction of regulatory pathways. Li et al.(117) revealed that a total of 11 719 unigenes are concerned with secondary metabolism. Then they identified the dynamic regulating network of flavonol, caffeine, and theanine and expression pattern of genes controlling metabolites through the sequence assembly and annotation of 347 827 unigenes after RNA sequencing of 13 tea tissues at different developmental stages. Wei et al.(118) reported that 1 143 and 1 050 out of 112 233 unigenes were, respectively, up and down regulated in purple shoots of an individual plant hybridized between 'Longjing 43' and 'Baihaozao', respectively. One out of 28 differential expression transcription factors called *CsMYB* is highly aligned with *AtPAP1* in *Arabidopsis*.(118) The pathway of phenylpropanoid biosynthesis is the typical one of 26 pathways in terms of the formation of purple shoots.(118) There were 11 unigenes potentially affecting epimerization of catechins by making a comparison of catechins and RNA sequences from 'Fuding Dabaicha' cultivars.(118) The genes of anthocyanin reductase (*CsANR1*, *CsANR2*) and synthetase (*CsANS*) were regarded as the key genes, as it was likely

that the expression ratio of *CsANR1* to *CsANR2* and the expression of *CsANS* influenced the formation of nonepicatechins.(119,120) With the foliar age, EC, EGC, gallic acid, *cis*-*p*-coumaric acid, and aliphatic and aromatic substances increased, while C, ECG, EGCG, and monoterpenes decreased as determined by metabolite concentrations and expression level of genes in the catechin metabolism pathways. This study indicated that flavan-3-ols might lead to a shifting of carbon flow in the direction of linked metabolic pathways associated with chlorophyll, wall-bound phenolics, benzenoids, and biosynthesis of fatty acids.(105) A cDNA sequence encodes the anthocyanin transcription factor belonging to *CsMYB4a* that negatively correlated with catechins and phenolic acids. *CsMYB4a* overexpressed in tobacco down-regulated genes in the shikimic acid and phenylpropanoid pathway. The contents of lignin, rutin, chlorogenic acid, and phenylpropanoids were reduced in *CsMYB4a* transgenic tobacco based on metabolomics. This reduction may be attributed to inhibition of CsC4H, Cs4CL, CsCHS, CsLAR, and CsANR2 as promoters in the phenylpropanoid pathway.(121) A crosstalk regulatory network was constructed, concerning 13 transcription factors genes with possible crucial roles in the regulation of terpene metabolism by integration of metabolomics and transcriptomics.(100)

Secondary metabolic pathways in the tea plant have been constructed based on function genes related to metabolism and bioinformatics. The recent release of the tea genome from *C. sinensis* var. *assamica* and *C. sinensis* var. *sinensis* will accelerate these tasks.(122) There is a need to reconstruct and improve metabolic pathways particularly dealing with essential quality-related metabolites and reveal the mechanisms underlying the regulation of these networks. Thus, it will be possible to breed elite tea cultivars with low caffeine, high theanine, and other outstanding traits by regulating key genes in the metabolic pathway. The metabolic traits of the tea plant are quantitative traits regulated by the environment and minor genes whose dominance or recessivity is indistinguishable. A limited number of metabolic QTLs responsible for large variations of the phenotype have been published.(123) While the mQTLs with intermediate or micro effects in the tea plant are commonly identified through linkage analysis under a single genetic background or environment. Moreover, a tea population for linkage analysis is generated from two parents with contrasting characteristics. In such populations, there are limited alleles and recombination. It does not allow the discovery of the QTLs if the alleles from both parents are identical. Hence, metabolite-based genome-wide association study of natural population may provide more information.

Summary and Outlook

There is a wide diversity of metabolites in the tea plant with various physical and chemical properties. Some metabolites have been comprehensively probed into, and achievements have been made through metabolomics. However, the study of the metabolism of natural products on the whole genome level in the tea plant is still in its infancy, for it is a bottleneck to accurately characterize various metabolites and detect the trace compounds of tea. Moreover, only a small number of genes involved in metabolism have been explored, and the regulation mechanisms for the majority of the quality-related metabolites are currently unclear.

It is difficult to quantify a large number of compounds using a single separation and detection technique due to their enormous variations in concentrations. There is a need to improve the analytical techniques that require minimum sample pretreatment, easy separation for the purpose to maintain the original metabolites, and further enhance the integrity, accuracy, efficiency, and reproducibility. Furthermore, it is necessary to establish a standardized metabolite library for the tea plant with a convenient inquiry, accurate annotation, comprehensive statistics, and information on the analytical conditions. The first tea metabolome database (TMDB), http://pcsb.ahau.edu.cn:8080/TCDB/) has been created mainly relying on NMR information. It requires more globally improved databases that will make great contributions to the identification of unknown metabolites, the exploration of their functions, and the identification of regulation mechanisms.

The raw metabolomics data of tea is so huge that it is neither possible nor rational to seek biomarkers without data filtering. Nevertheless, the inappropriate filtering and processing may lose the objective necessity and the authoritative feeling and ultimately lead to skewed results. Consequently, some software suitable for the specific conditions of tea metabolomics is needed to cater for unbiased dimensionality reduction of data sets, followed by data standardization, normalization, transformation, filtering, and statistical analysis. Simultaneously, consideration needs to be given to the calculation efficiency.

A systematic and comprehensive understanding is what the various omics techniques strive to achieve. For instance, it is attractive to seek the gene loci responsible for small molecules in development and responses to different environmental stresses in the entire genome through genome-wide association studies. Moreover, the metabolites, in turn, give rise to the expression level of relative genes, which stimulates to expand the metabolic pathway. Also, it is favorable to probe into the metabolic regulation at the transcriptional level with a combination of metabolomics and transcriptomics.

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