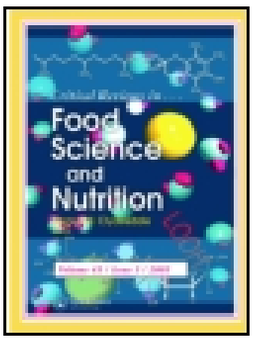

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REVIEW

Strategies for studying *in vivo* biochemical formation pathways and multilevel distributions of quality or function-related specialized metabolites in tea (*Camellia sinensis*)

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ABSTRACT

Tea (*Camellia sinensis*) contains bioactive metabolites such as catechins, amino acids, caffeine, and aroma compounds that contribute to characteristic tea function and flavor. Therefore, studies on biochemical formation pathways and occurrences of these characteristic specialized metabolites in tea plants are important, providing essential information for the regulation and improvement of tea quality and function. Owing to the lack of a stable genetic transformation system, obtaining direct in vivo evidence of the formation of characteristic tea specialized metabolites is difficult. Herein, we review potential strategies for studying in vivo biochemical formation pathways and multilevel distributions of specialized metabolites in tea. At the individual plant level, stable isotope-labeled precursor tracing is an approach to discovering the pathways of some specialized metabolites specifically occurring in tea and elucidating the formation of tea specialized metabolites in response to stresses. At the within-tissue level, imaging mass spectrometry can be used to investigate the in situ localization of characteristic specialized metabolites within tea tissue without sample destruction. At the cellular or subcellular level, nonaqueous fractionation is a feasible method for characterizing the distributions of nonvolatile metabolites in subcellular organs. These approaches will help explain the characteristic scientific problems in tea secondary metabolism and provide more precise information to improve tea quality or function.

KEYWORDS

Aroma; amino acid; biosynthesis; *Camellia sinensis*; in vivo evidence; regulation; tea; volatile

HIGHLIGHTS

- Multilevel distributions of metabolites in tea are important for tea quality improvement.
- Stable isotope-labeled precursor tracing method can be used to study formations of tea metabolites at individual plant level.
- Imaging mass spectrometry can be used to investigate the *in situ* localization of metabolites within tea tissue.
- Nonaqueous fractionation is a feasible method for characterizing the distributions of metabolites in subcellular organs.

Introduction

Tea (*Camellia sinensis*) leaves are generally selected to make tea, which is the second most popular beverage in the world. The occurrence of specialized metabolites in tea leaves makes a major contribution to the health benefits of tea beverages, as well as their unique taste and fragrance. In addition to common metabolites distributed in most plants, tea leaves are rich in some characteristic specialized metabolites, including catechins, caffeine, specialized amino acids (such as L-theanine), and aroma compounds (Wan 2003; Wan and Xia 2015; Yang, Baldermann, and Watanabe 2013; Yu and Yang 2020; Zeng, Watanabe, and Yang 2019). Therefore, studies on the biosynthesis and regulation of characteristic specialized metabolites in tea plants have attracted increasing interest in plant biology. These studies provide essential information for the regulation and improvement of tea quality and function. Knowledge regarding the biosynthesis and regulation of tea characteristic specialized metabolites consists of biochemical pathways, involved genes/enzymes and cellular or subcellular localization. Research targets include metabolites, proteins (enzymes), and genes, with studies involving qualitative analysis, quantitative analysis, and localization monitoring of these targets (Figure 1). With the development of modern analytical techniques and molecular biological methods, these techniques (as shown in Figure 1) can be applied to

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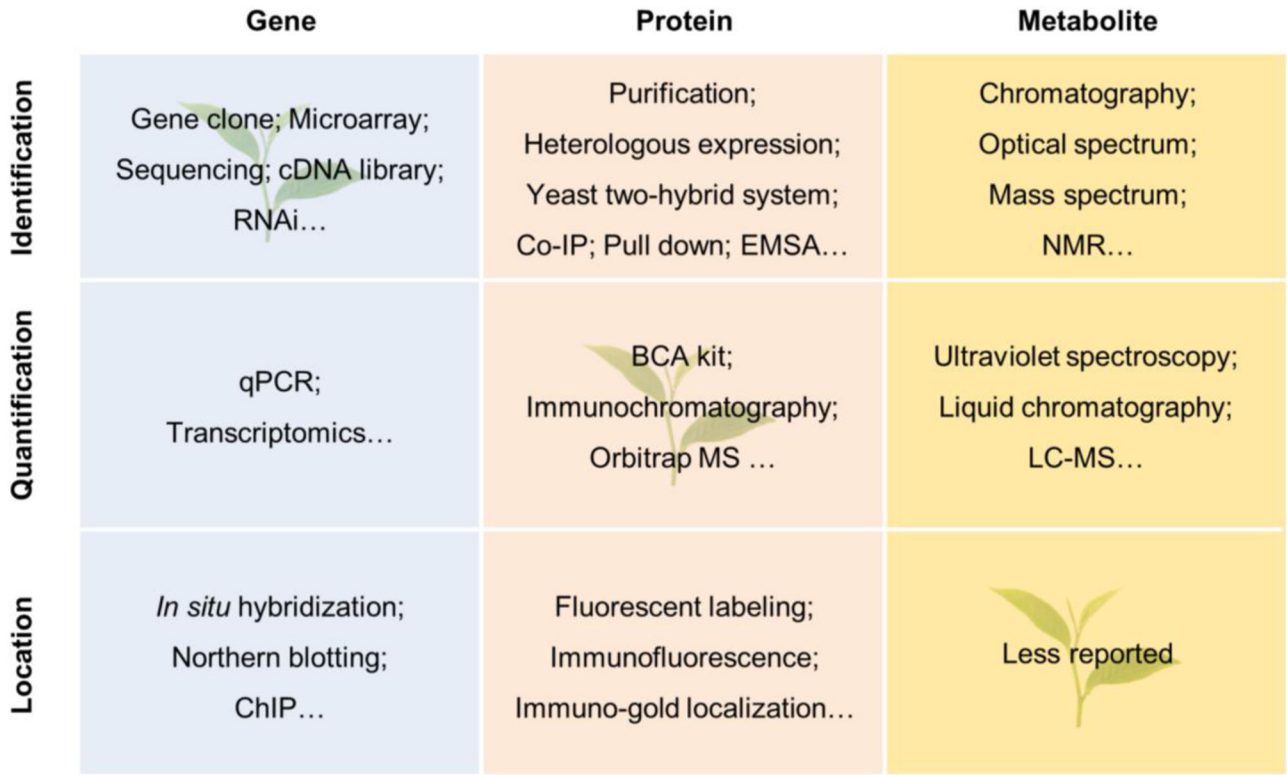

Figure 1. Potential approaches to studying the genes, proteins (enzymes), and metabolites involved in biosyntheses and regulation of tea specialized metabolites. BCA: bicinchoninic acid; Co-IP: co-immunoprecipitation; ChIP: chromatin immunoprecipitation; EMSA: electrophoretic mobility shift assay; LC-MS: liquid chromatography mass spectrometer; NMR: nuclear magnetic resonance; qPCR: Quantitative real-time polymerase chain reaction; RNAi: RNA interference.

study the biosynthesis and regulation of tea specialized metabolites.

Currently, researchers in the field of tea specialized metabolites are mostly focused on studying enzymes/genes related to their biosynthesis and regulation. In particular, the decoding of tea genome information has recently benefited the study of enzymes/genes involved in the biosynthesis and regulation of tea specialized metabolites (Xia et al. 2017; Wei et al. 2018). However, compared with model plants and other crops, there is insufficient in vivo evidence from tea plants to support the study of enzymes/genes involved in the biosynthesis and regulation of tea specialized metabolites. Very few of these enzymes have been isolated and purified directly from tea plants for functional verification, including β-primeverosidase (responsible for the hydrolysis of β-primeveroside-bound alcoholic volatile compounds) (Mizutani et al. 2002), glycosyltransferases (responsible for the formation of glycosidically bound volatile compounds) (Ohgami et al. 2015), galloyltransferase (involved in catechin galloylation) (Liu et al. 2012), and tannase (involved in the hydrolysis of tannin compounds, depsides, and phenolic glycosides) (Dai, Liu, et al. 2020). Most studies have focused on identifying target enzymes/genes through homology analysis, with the recombinant proteins obtained by heterologous expression systems (prokaryote, *Escherichia coli*; eukaryote, yeast; insect cell, *Arabidopsis* or *Nicotiana*) and their functions verified. Owing to the low in vitro propagation ability and immature genetic transformation system of tea plants, obtaining transgenic plants and conducting further genetic analysis is difficult. Therefore, only these heterologous expression systems are employed for functional verification. In addition to enzyme/gene function identification, localization of the enzymes involved is generally conducted through transient expression in model plants, such as *Arabidopsis* or *Nicotiana*. Although these heterologous systems provide some characteristics of tea specialized metabolic enzymes or genes, the results obtained might not represent the true situation in tea plants owing to differences between the heterologous systems used and tea plants. To deeply elucidate the molecular mechanisms underlying biosynthesis and metabolism of tea specialized metabolites in the tea plants, the difficulty of gene transformation into tea plants needs to be overcome. Therefore, precise information for improving tea quality or function would not be provided at present.

To date, studies on the biosynthesis and regulation of characteristic specialized metabolites in tea plants have mainly focused on the enzymes and genes involved. Qualitative approach to identify tea genes include gene clone, microarray, sequencing, cDNA library, RNAi, etc. Purification, heterologous expression, yeast two-hybrid system, Co-IP, pull down, EMSA, etc. are available for studying tea proteins (enzymes) (Figure 1). For example, 33,932 highly reliable tea plant genes were annotated with application of second and third generation sequencing technology to conduct whole-genome sequencing for tea plant (Wei et al. 2018). Most of the tea protein identification depends on heterologous expression due to the absence of a mature genetic transformation. For quantitative analysis, qPCR and transcriptomics are routinely used for genes, while for proteins (enzymes), BCA kit, immunochromatography, orbitrap

MS, etc. are preferred in tea (Figure 1). There are also plenty of alternatives available for localization monitoring of genes and proteins (enzymes) in tea plant. In situ hybridization, northern blotting and ChIP enable the in situ analysis of genes. The methods for protein localization include fluorescent labeling, immunoflurescence, and immuno-gold localization (Figure 1). However, the tea specialized metabolites are also important research targets. The study of tea specialized metabolites mostly involves the analysis of their contents and changes therein. Qualitative and quantitative analysis for tea characteristic metabolites are same with those of other plant species, with chromatography, optical spectrum, mass spectrum, and NMR employed for qualification; and ultraviolet spectroscopy, liquid chromatography, and LC-MS for quantification. However, few studies on the locations of tea metabolites are available (Figure 1). These information on their in vivo biochemical formation pathways and multilevel distributions (such as within-tissue distribution and cellular or subcellular localization) can provide direct in vivo evidence concerning the biosynthesis and regulation of tea specialized metabolites. In this review, we have mostly focused on strategies for studying in vivo biochemical formation pathways and multilevel distributions of tea specialized metabolites related to quality or function. First, we have summarized investigations into the elucidation of biochemical formation pathways of these specialized metabolites and their responses to environmental stresses using stable isotope-labeled precursor tracing techniques. Furthermore, we describe the use of imaging mass spectrometry to investigate the in situ localization of characteristic specialized metabolites in tea without sample destruction. Finally, nonaqueous fractionation, a feasible method for characterizing the distribution of nonvolatile metabolites in subcellular organs, is discussed. These approaches will help solve current scientific problems in tea secondary metabolism and provide more precise information to improve tea quality or function.

Stable isotope-labeled precursor tracing method for studying in vivo biochemical formations of tea specialized metabolites at individual plant level

Isotope-labeled precursor tracing is a classical method for elucidating unknown biochemical formation pathways of metabolites in plants. In the last century, the isotope-labeled precursors used for tracing in plants were mainly radioactive owing to detection instruments at the time having relatively low sensitivity and radioactive isotope-labeled metabolites being relatively easy to detect with less background interference. With the rapid development of analytical instruments and increasing focus on the safety of experimental operations, stable isotope-labeled precursors are gradually replacing radioactive isotope-labeled precursors for tracing in plants and elucidating the biochemical formations of target metabolites. In tea plants, the stable isotope tracing method using a labeled precursor can be applied to three aspects of research (Figure 2), as follows: (i) Discovering unknown biochemical formation pathways; (ii) explaining the presence of some specialized metabolites, such as the specific accumulation of L-theanine in tea plants; and (iii) investigating the formation of specialized metabolites in tea plants in response to external factors, such as stresses.

Characteristic specialized metabolites present in tea can be subdivided into nonvolatile and volatile metabolites. Characteristic nonvolatile metabolites include L-theanine, caffeine, and catechins, etc. In the last century, biochemical formation pathways of some representative specialized metabolites, such as L-theanine, caffeine, and catechins, in tea plants have been elucidated using radioactive isotope-labeled precursor tracing methods (reviewed by Wan 2003; Wan and Xia 2015). Therefore, current research on the biosynthesis and regulation of these specialized metabolites has mostly focused on the key enzymes or genes involved. In recent years, the biosynthesis and regulation of tea volatile metabolites have attracted increasing interest (Yang, Baldermann, and Watanabe 2013; Zeng, Watanabe, and Yang 2019). Volatile metabolites in tea plants include volatile phenylpropanoids/benzenoids (namely aromatic compounds), volatile fatty acid derivates, and volatile terpenes, according to different biosynthetic pathways (Yang, Baldermann, and Watanabe 2013). In addition to volatile fatty acid derivates and volatile terpenes, aromatic compounds play important, but relatively underappreciated, roles in tea aroma quality and the ecological functions of tea plants. Therefore, the biochemical formation pathways of aromatic compounds in tea plants are attracting increasing attention. Most aromatic compounds are derived from L-phenylalanine (Yang, Baldermann, and Watanabe 2013), with stable isotope-labeled L-phenylalanine being available commercially. Using [$^2H_8$]L-phenylalanine, aromatic compounds such as 1-phenylethanol and 2-phenylethanol were found to occur in multiple biochemical formation pathways in tea plants, with several 1-phenylethanol or 2-phenylethanol products containing different numbers of ^2H atoms detected. After supplementing flowers of tea plants with [$^2H_8$]L-phenylalanine, [$^2H_5$] and [$^2H_6$]-1-phenylethanol products were detected. Based on [$^2H_5$] or [$^2H_6$]-1-phenylethanol products being derived from [$^2H_8$]L-phenylalanine, and the determination of deuterium-labeled intermediate metabolites, two pathways leading from L-phenylalanine to 1-phenylethanol were proposed in tea plants, namely, pathway I *via trans*-cinnamic acid, 3-hydroxy-3-phenylpropionic acid, 3-oxo-3-phenylpropionic acid, and acetophenone, and pathway II *via* phenylpyruvic acid, phenyllactic acid, *trans*-cinnamic acid, 3-hydroxy-3-phenylpropionic acid, 3-oxo-3-phenylpropionic acid, and acetophenone (Dong et al. 2012). After supplementing leaves of tea plants with [$^2H_8$]L-phenylalanine, [$^2H_7$] and [$^2H_8$]-2-phenylethanol products were detected. Based on derivation of [$^2H_7$] or [$^2H_8$]-2-phenylethanol products from [$^2H_8$]L-phenylalanine, and the determination of deuterium-labeled intermediate metabolites, three pathways leading from L-phenylalanine to 2-phenylethanol in tea plants were proposed, namely, pathway I *via* phenylacetaldehyde, pathway II *via* phenylpyruvic acid and phenylacetaldehyde, and pathway III *via* (*E*/*Z*)-phenylacetaldoxime and phenylacetaldehyde (Zeng, Tan, et al. 2019).

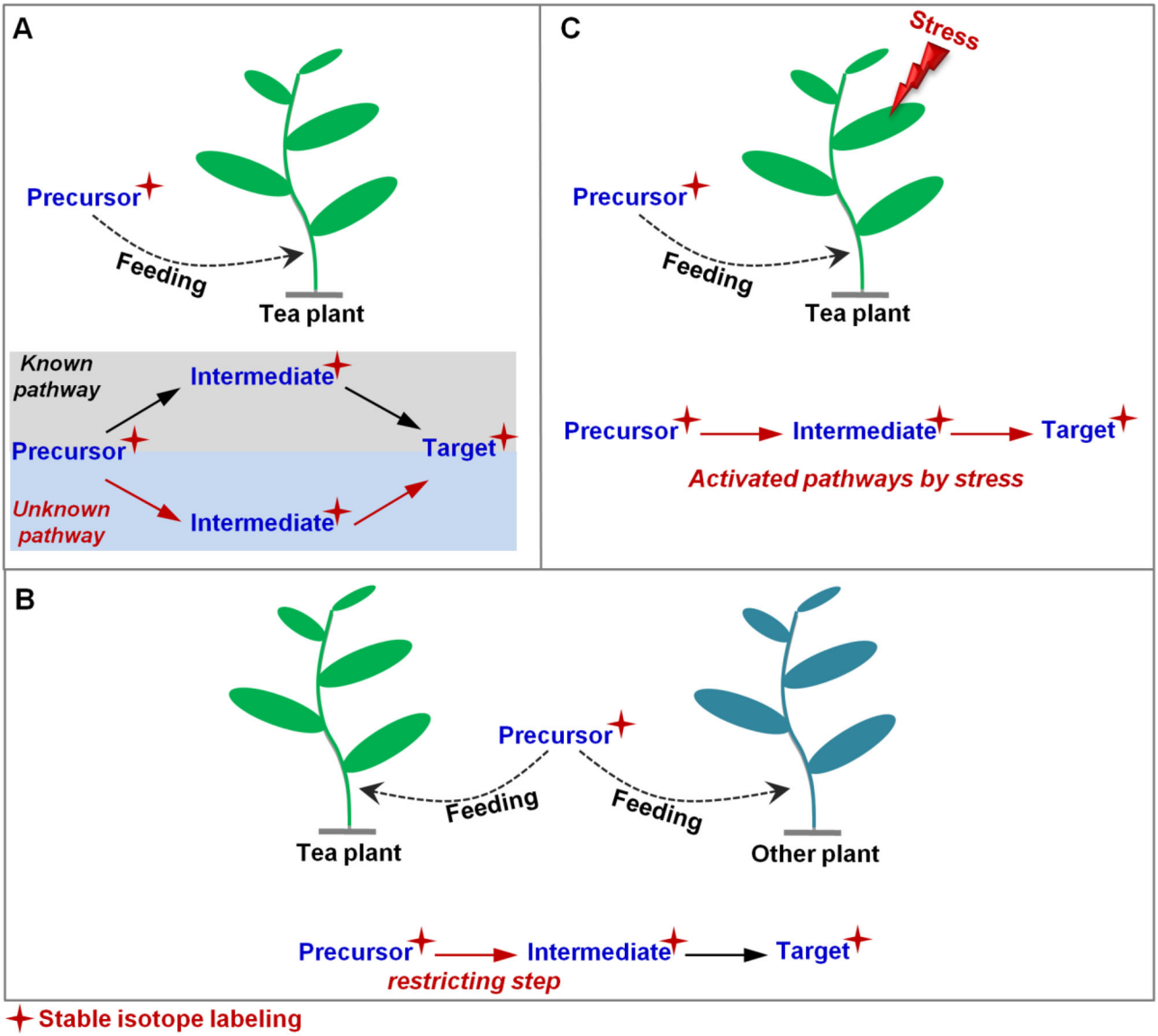

Figure 2. Application of stable isotope labeling precursor tracing method in studying in vivo biochemical formations of tea specialized metabolites at the plant individual level.
(A) Discovery of unknown biochemical formation pathways. (B) Elucidation of reasons for some specialized metabolites such as L-theanine specifically accumulated in tea plants. (C) Investigation on formations of specialized metabolites in tea plants in response to external factors such as stresses.

These multiple biochemical formation pathways for 2-phenylethanol have also been demonstrated in other plants containing 2-phenylethanol, such as tomato fruit, petunia flower, and rose flower, using [$^2H_8$]L-phenylalanine tracing (Hirata et al. 2012, 2016; Zeng, Tan, et al. 2019; Zeng, Wang, et al. 2019). Importantly, an alternative pathway leading from L-phenylalanine to *trans*-cinnamic acid, which is an important precursor of several downstream metabolites and plays crucial roles in the growth, development, reproduction, and environmental responses of plants (Huang et al. 2010), was discovered. The deamination of L-phenylalanine is generally considered to be the only pathway to *trans*-cinnamic acid formation in plants. After supplementing leaves of tea plants with [$^2H_8$]L-phenylalanine, [$^2H_6$] and [$^2H_7$]*trans*-cinnamic acid products were detected. Based on the derivation of [$^2H_6$] or [$^2H_7$]*trans*-cinnamic acid products from [$^2H_8$]L-phenylalanine, and the determination of deuterium-labeled intermediate metabolites, an alternative pathway to direct deamination, leading from L-phenylalanine to *trans*-cinnamic acid *via* phenylpyruvic acid and phenyllactic acid, was proposed (Zeng et al. 2020). Furthermore, this alternative pathway was also identified in other plants, such as *Arabidopsis thaliana* leaves, rose flowers, and tomato fruits, using [$^2H_8$]L-phenylalanine tracing. These observations suggest that the stable isotope-labeled precursor tracing method can be used to discover multiple and unknown biochemical formation pathways of metabolites in tea plants, representing in vivo evidence from tea plants (Figure 2A).

Some specialized metabolites are known to specifically accumulate in tea plants. L-Theanine is a representative specialized metabolite in tea plants that plays important roles in tea taste and function (Wan and Xia 2015). L-Theanine mainly occurs in *Camellia* species, and is particularly abundant in *C. sinensis* (Nagata and Sakai 1984; Deng et al. 2010). Although the biochemical formation pathway of L-theanine in tea plants has been elucidated, the reason for its

abundance remains unclear, with little or no L-theanine occurring in other plant species. After supplementing some plants that contain no L-theanine, such as *Zea mays*, *Arabidopsis thaliana*, and *Solanum lycopersicum*, with [$^2H_5$]ethylamine (a precursor of L-theanine), [$^2H_5$]L-theanine was detected in all cases. This suggested that ethylamine availability accounts for the difference in L-theanine content between tea plants and other species (Cheng et al. 2017). The stable isotope-labeled precursor tracing method has also been employed to elucidate the differential accumulation of L-theanine in albino-induced yellow and green tea leaves. In tea plants, albino-induced yellow leaves usually contain more L-theanine than normal green leaves, but the reason for this was unclear. After feeding with [$^2H_5$]L-theanine, yellow leaves showed less [$^2H_5$]L-theanine catabolization compared with green leaves, suggesting that the higher level of L-theanine in yellow tea leaves was due to weak L-theanine catabolism (Cheng et al. 2019). These observations suggested that stable isotope-labeled precursor tracing provided in vivo evidence of the mechanisms involved in differential L-theanine accumulation both intra- and inter-species. In addition to L-theanine as an example of a nonvolatile metabolite, stable isotope-labeled precursor tracing has also been used to explain the specific accumulation of some volatile specialized metabolites in tea plants. 1-Phenylethanol is a main aromatic volatile in tea flowers, with a much lower level of distribution in tea leaves and almost no occurrence in many other plants. [$^2H_8$]L-Phenylalanine and [$^2H_5$]acetophenone tracing experiments demonstrated that most plants contain the enzyme/gene being responsible for the catabolism of acetophenone to form 1-phenylethanol, with the availability of acetophenone from L-phenylalanine accounting for the difference in 1-phenylethanol occurrence between tea flowers and tea leaves or other plant species (Dong et al. 2016; Zhou et al. 2018). These observations suggest that the stable isotope-labeled precursor tracing method can be used to explain the specific accumulation of nonvolatile and volatile specialized metabolites in tea plants (Figure 2B).

Many studies have validated that external factors, especially stresses, significantly affect the biosynthesis of specialized metabolites in tea plants, especially volatile metabolites. Therefore, understanding of the specialized relationships between stresses and characteristic aroma compounds has been proposed as essential for the safe and effective improvement of tea quality (Zeng, Watanabe, and Yang 2019). When studying the formation of tea specialized metabolites in response to stresses, only investigating changes in the contents of metabolites involved might not provide clear information. As the precursor or intermediate metabolite might also be affected by the stress affecting the target metabolite, it is difficult to monitor the pathways affected by stresses. By feeding tea plants with the same amount of exogenous stable isotope-labeled precursor before stress treatment, the biochemical formation pathways leading to the stable isotope-labeled target metabolite in response to stress can be more accurately monitored. The formation of volatile metabolites in response to postharvest manufacturing stress has several applications. The effect of leaf cell disruption during black tea processing on the formation and transformation of aroma compound indole has been studied using [^{15}N]anthranilic acid tracing experiments. The results suggested that tea leaf cell disruption due to intense rolling during black tea manufacture terminated indole synthesis, rather than enhanced its catabolism. This explained the much lower indole content in black tea compared with that in oolong tea (Zeng et al. 2016). The effect of continuous wounding stress at the turn-over stage of oolong tea manufacture on the formation of volatile compounds jasmine lactone and benzyl nitrile was also elucidated through stable isotope-labeled precursor tracing experiments. After supplementing with [$^{13}C_{18}$]linoleic acid or [$^{13}C_{18}$]-α-linolenic acid, followed by stress treatment, the content of [$^{13}C_{10}$]jasmine lactone was significantly increased, suggesting that continuous wounding, and dual stresses of low temperature (15 °C) and wounding, activated jasmine lactone biosynthesis in tea leaves (Zeng et al. 2018). Similarly, based on [$^2H_8$]L-phenylalanine tracing experiments, the pathway leading from L-phenylalanine to benzyl nitrile *via* (*E*/*Z*)-phenylacetaldoxime was found to be activated by continuous wounding stress (Liao et al. 2020). In addition to investigating the formation of volatile metabolites in response to abiotic stresses, stable isotope-labeled precursor tracing can also be applied to study the effect of biotic stresses on volatile metabolite formation in tea plants. After prior supplementation of tea flowers with [$^2H_5$]acetophenone, the emission of (*R*)-[$^2H_5$]-1-phenylethanol was found to be enhanced by *T. hawaiiensis* attack, suggesting that this attack mostly affected the pathway from acetophenone to 1-phenylethanol, resulting in a variation in the emitted ratio of (*S*)/(*R*)-1-phenylethanol (Zhou et al. 2017). These observations suggested that the stable isotope-labeled precursor tracing method can be used to investigate the formation of specialized metabolites in tea plants in response to abiotic or biotic stresses (2C).

The general procedure for stable isotope-labeled precursor tracing experiments includes the following steps: (i) Selection of potential precursor metabolites for use in tracing experiments based on previous reports on plants or microorganisms, and chemical structural similarity with target metabolites; (ii) structural elucidation of the stable isotope-labeled target metabolite and potential intermediate metabolites based on the isotope number and position of the isotope-labeled precursor metabolites, and the inferred pathway; and (iii) identification of the stable isotope-labeled target metabolite and potential intermediate metabolites using mass spectrometry. Although the procedure is not complicated, several factors must be considered. First, stable isotope-labeled precursor metabolites are generally not commercially available and must be chemically synthesized, which requires professional chemistry knowledge. Furthermore, in some cases, during chemical synthesis, an unstable stable isotope-labeled precursor metabolite might be transformed into the stable isotope-labeled target metabolite and potential intermediate metabolites, even in small or trace amounts, which would interfere with the tracing

results (Liao et al. 2020; Zeng et al. 2020). Second, exchange reactions of some isotopes, such as deuterium, might occur during tracing experiments. This would incorrectly imply that the detected stable isotope-labeled target metabolites were derived from the biotransformation of stable isotope-labeled precursor metabolites, resulting in an incorrect pathway. For example, in the feeding experiment of [$^2H_8$]L-phenylalanine into tea flowers, [$^2H_5$]acetophenone and [$^2H_5$]-1-phenylethanol were formed. However, their formation was mainly due to deuterium exchange reactions with the two intermediate metabolites during feeding experiments (3-hydroxy-3-phenylpropionic acid labeling changed from [$^2H_7$] to [$^2H_6$], while 3-oxo-3-phenylpropionic acid labeling changed from [$^2H_6$] to [$^2H_5$]) (Dong et al. 2012). Third, exogenous stable isotope-labeled precursor metabolites might not precisely enter the in situ cellular location of the precursor metabolites in tea plants. As much research has focused on detecting stable isotope-labeled target metabolites after stable isotope-labeled precursor tracing experiments, this phenomenon has attracted less attention. When the target metabolites have different configurations, this phenomenon is clear. For example, 1-phenylethanol naturally occurs in tea flowers with a higher ratio of the (*R*)-enantiomer (more than 80%) (Dong et al. 2012). However, tea flowers supplemented with [$^2H_5$]acetophenone produced more [$^2H_5$]-(*S*)-1-phenylethanol than [$^2H_5$]-(*R*)-1-phenylethanol (Zhou et al. 2019). This might be due to exogenous [$^2H_5$]acetophenone entering tea subcellular locations and contacting (*S*)-1-phenylethanol and (*R*)-1-phenylethanol synthases, in contrast to its natural *in-situ* location and interaction with related synthases. A similar phenomenon was observed in another experiment regarding (*E*/*Z*)-phenylacetaldoxime. In nature, tea leaves contain more (*Z*)-phenylacetaldoxime than (*E*)-phenylacetaldoxime. However, after supplementing tea leaves with [$^2H_8$]L-phenylalanine, equal amounts of [$^2H_8$]-(*E*)-phenylacetaldoxime and [$^2H_8$]-(*Z*)-phenylacetaldoxime were detected (Liao et al. 2020). This suggested that the cellular location entered, and the reaction with enzymes of the exogenous stable isotope-labeled precursor metabolite, might differ from those that naturally occur in tea plants.

Use of imaging mass spectrometry to visualize and analyze within-tissue spatial distributions of tea specialized metabolites

Mass spectroscopy plays an incomparable role in the qualitative and quantitative analysis of plant metabolites owing to its high resolution and high sensitivity (Kueger et al. 2012). Quantity might not be sufficient to gain insight into the synthesis, transportation, stress response mechanism, and biological function of metabolites in organisms. As mentioned earlier, exogenous stable isotope-labeled precursor metabolites entering different locations in tea plants might result in distinct products. Consequently, spatial location information for metabolites within tissues, even single cells, is urgently needed to achieve precise physiological processes. Mass spectrometry imaging (MSI), derived from MS technology, is becoming a promising in situ analytical tool for obtaining structural information on metabolites (such as small-molecule compounds, peptides, and proteins) via MS, while also providing their spatial distribution and relative contents via in situ ionization (Buchberger et al. 2018). The earliest application of MSI was proposed by Caprioli, who mapped specific molecules on mammalian tissue sections using matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) (Caprioli, Farmer, and Gile 1997). After two decades of research, various novel MSI technologies have been reported (such as secondary ion mass spectrometry (SIMS), desorption electrospray ionization mass spectrometry (DESI-MS), and laser ablation electrospray ionization mass spectrometry (LAESI-MS) (Buchberger et al. 2018). MSI has become an important modality for metabolism analysis of various biological samples owing to its unique advantages, providing broad application prospects in clinical medicine, drug research, pathology research, food research, plant physiology, and other fields. MSI is primarily composed of MS and in situ ionization, with the latter providing visualization of the distribution of molecules inside living materials. MSI can usually be divided into four major categories depending on the different ionization principles of the ion source (Figure 3). The MALDI source requires the sample to be embedded in a specific matrix that can absorb energy from an ultraviolet/infrared laser and sublimate, protonate, and carry the sample analytes to the mass spectrometer for detection (Figure 3; Lin 2014). The secondary ion (SI) ionization source uses a high-energy primary ion beam to bombard the sample surface, with the atoms or compounds inside taking up energy and sputter from the surface to generate secondary ions (Figure 3; Lin 2014). The DESI source sprays charged high-speed droplets onto the sample surface, microextracts compounds from the sample, and ionizes them, with charged particles then desolvated by N_2 gas and transferred into the mass spectrometer (Figure 3; Lin 2014). The LAESI ionization source combines the microsampling capabilities of mid-infrared lasers with the high-ion yield of electrospray (Figure 3; Lin 2014). The mid-infrared laser bombards the sample surface with water molecules, evaporating the compounds, which are then captured and ionized by the charged spray. MALDI-MS is more widely applicable than other ion sources owing to its high spatial resolution (as low as 5 μm), high ionization efficiency, high sensitivity (fmol order-of-magnitude), and wide molecular weight range (Lin 2014; Neumann et al. 2019). This technique has become predominant in the imaging of high-molecular-weight molecules, such as proteins. The main drawbacks are complicated sample preparation (embedding, cryosection, matrix application), a vacuum working atmosphere (which might cause sample deformation, volatile metabolite evaporation, and render the imaging of live cells impossible), and severe matrix interference (especially in small-molecule detection) (Bjarnholt et al. 2014). The newly introduced AP-MALDI technology employs a high-frequency laser (up to 10 kHz) for ionization, allowing samples to be imaged at atmospheric pressure, which perfectly addresses the second shortcoming

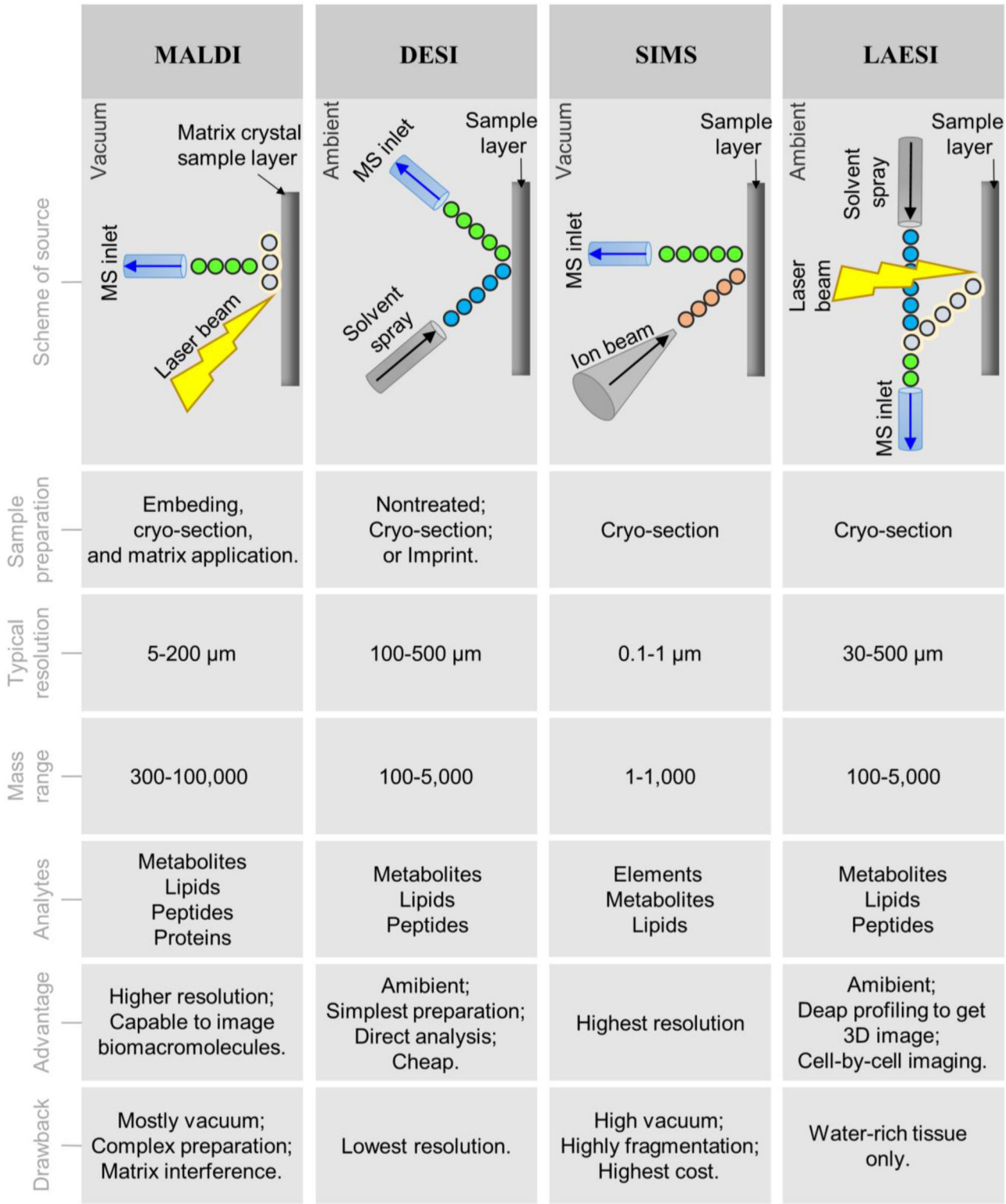

Figure 3. Comparison of common mass spectrometry imaging (MSI) technologies.

mentioned above (Guenther et al. 2011). The major advantage of DESI-MS should be real-time imaging (Ifa et al. 2007). As no processing and atmosphere analysis are required, maximum distribution accuracy is ensured. Furthermore, the time period from sample settling to results output can be as fast as 5 min (Fresnais et al. 2020). Meanwhile, DESI is also a "soft" ionization technique, producing the lowest amount of debris, making it ideal for obtaining snapshots of low-weight regional compounds. The DESI source can be seamlessly mounted on all types of mass spectrometer, improves mass spectrometer utilization, and makes the technique more affordable than other ion sources (Guenther et al. 2011). However, DESI-MS technology limits in low spatial resolution, which makes obtaining a precise distribution map difficult. SIMS is characterized by its extreme spatial resolution, reaching subcellular level

(100 nm) (Bjarnholt et al. 2014). The scanning depth can be resolved to a single atomic layer on the sample surface, and a three-dimensional image of the tissue can be produced through superposition of multiple depth scans (Hansen and Lee 2018). SIMS can analyze all elements and even distinguish isotopes (Guenther et al. 2011). Furthermore, SIMS also possesses an ultrafast scan speed (10,000 spectra s^{-1}) and high detectability (ppm magnitude) (Lin 2014). The disadvantages of SIMS include operation under a vacuum environment, the high-energy ion beam, which can cause sample destruction, a high fragmentation rate, which results in the absence of a complete molecular ion peak, and high costs (Bjarnholt et al. 2014). LAESI combines the advantages of both MALDI and DESI techniques to improve spatial imaging resolution and achieve ionization at atmospheric pressure. The highlight of this technique is that it allows 3 D imaging analysis of living tissue for the first time, with a resolution sufficient to enable imaging of a single onion epidermal cell (Shrestha, Patt, and Vertes 2011). However, specific requirements for the sample moisture content are a disadvantage. In conclusion, all of these commonly used MSI technologies have unique skills and shortcomings, with each meeting different application criteria.

Presently, the application of MSI in clinical disease research, drug metabolism, and strain identification is relatively mature, and has been gradually extended to plant physiology and biochemical research in recent decades. The techniques commonly used for in situ analysis of macromolecules in plant tissues include fluorescent labeling and immunofluorescence, which require the preparation of specific antibodies or probes (Figure 1). MSI is advantageous due to being comparatively high-throughput, high-resolution, high-sensitivity, and label-free. However, other methods for the in situ analysis of small-molecule metabolites in plant tissues have seldom been reported. Dai, Hu, et al. (2020) cut tea leaves into small fragments ($1.7 \times 1.7\ mm^2$), performed LC-MS analysis on each small slice, and finally spliced the results into a complete distribution image. This method guaranteed accurate quantification, isomer separation, and low costs, but was accompanied by complicated preparation and low spatial resolution. Furthermore, the mechanical damage caused by cutting might induce changes in the tea leaf metabolic profile that do not reflect its original distribution. Despite its unsolved problems, MSI is currently the preferred method for in situ analysis of plant metabolites, ranging from model plants (*Arabidopsis thaliana*, tobacco) to varieties of economic plants (trees, fruits, vegetables, flowers, Chinese herbs) (Bjarnholt et al. 2014). MSI detection methods for root, stem, leaf, flower, fruit, seed, and other plant organs have been established gradually (Horn et al. 2013; Cha et al. 2008; Cabral et al. 2013). The imaging precision has also been upgraded from within-tissue level to single-cell or even suborganelle level in situ analysis (Hansen and Lee 2018). Growing interest in this technology will continue to drive developments and improvements in this field. Next, we discuss the key steps of MSI in plant applications.

In a classical MSI procedure, plant samples are pretreated and fixed on the worktable. The ion source energy beam then scans the sample pixel by pixel, with the corresponding independent mass spectrum obtained for every pixel. Using the targeted *m/z* distributions from each mass spectrum, a 2 D/3D molecular distribution map is generated. Therefore, well-designed sample preparation and the optimization of ion source parameters are essential for obtaining a successful image (Bjarnholt et al. 2014). For sample treatment, frozen sections of root, stem, fruit, and seed tissues, which represent the histological characteristics, can be easily accessed. However, the leaf and petal are usually thin and fragile, making slices difficult to obtain. DESI-MS appears to be an excellent choice for the detection of plant leaves and petals owing to its direct imaging. Tea leaf is the most valuable part of the plant, and is rich in characteristic metabolites beneficial to human health (Wan and Xia 2015; Zeng, Watanabe and Yang 2019). Studying the distribution of metabolites in tea leaf is important for elucidating the biosynthesis regulation and biological functions. However, tea leaves have a thick waxy layer over the surface (Liao et al. 2019). This physiological structure leads to poor signals when tea leaves are scanned directly. Imprinting is also an effective preparation method for plant leaves, where metabolites in the sample are transferred in situ to the carrier by exerting pressure. Liao et al. (2019) found that PTFE imprinting was suitable for transferring metabolites on tea leaves. When the slice is ready, optimization of the ion source begins, except for MALDI, which requires an additional matrix application process. Common matrixes include α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (2,5-DHB). There are also specific matrixes, including 3-aminoquinoline, which is well-suited to glucosinolates, and lithium 2,5-dihydroxybenzoate, which specializes in nonpolar metabolites, such as lipids (Shroff et al. 2008; Vrkoslav et al. 2010). In the ion source debugging step, optimization must be performed in accordance with the features of the chosen ion source. For DESI-MS, the spray solvents, spray velocity, atomization pressure, voltage, nozzle angle, distance among sprayer, nozzle, and MS inlet, and pixel size, among others, need to be coordinated (Lin 2014). The key parameters of the MALDI source are the number of laser shots, extraction delay, laser intensity, and pixel size (Lin 2014). Factors affecting LAESI-MS are the same as the above two ion sources. To keep the molecular positions on the sample surface relatively stable, the ion stream velocity from the SI source is limited to 1×10^{13} ions/cm^2, meaning that the desired spatial resolution of imaging is determined by the primary ion flow intensity, which affects the metabolite detection limit (Lin 2014). When DESI-MS was first applied to tea, two patterns were observed for catechins on the tea leaf, namely, epigallocatechin gallate/gallocatechin gallate distributed on two sides of the leaf, and epigallocatechin/gallocatechin distributed near the leaf vein (Figure 4; Liao et al. 2019). This suggested that catechins might be synthesized at different sites, despite sharing the same pathway. L-Theanine was mainly distributed around the outer root cross-section, which indicated

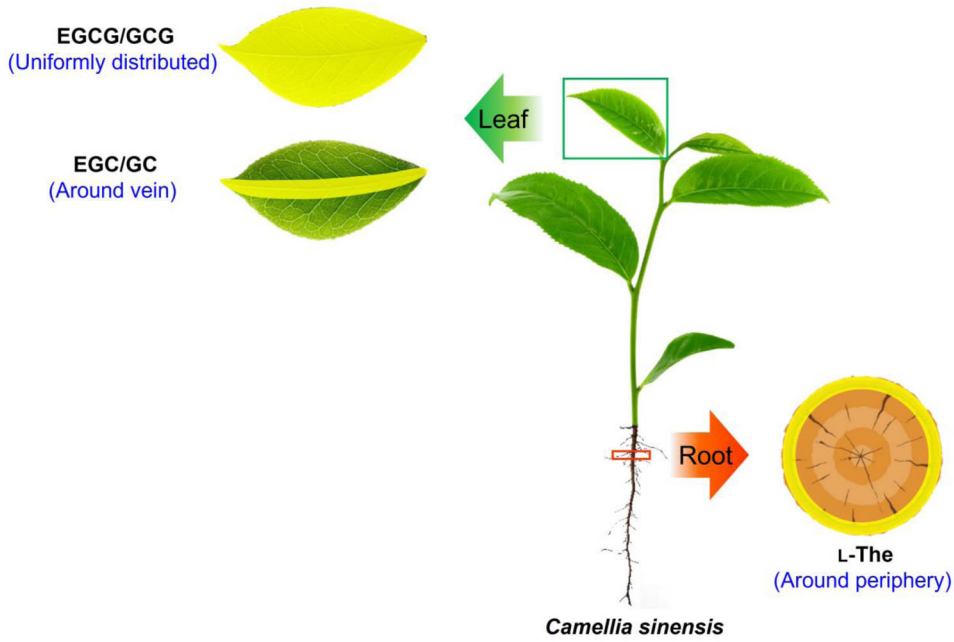

Figure 4. Schematic diagram of small molecules distributed in tea tissue.
EGC: epigallocatechin; EGCG: epigallocatechin gallate; GC: gallocatechin; GCG: gallocatechin gallate; L-The: L-Theanine.

that it might possess antipathogen functions (Figure 4; Liao et al. 2019). These visualized results offer valuable insight for future investigations on tea plants. The application of MSI to tea remains in its infancy. Many scientific problems regarding tea remain to be solved by the development of advanced MSI methods. For example, a method should be established to allow more metabolites (especially volatile metabolites) to be detected in response to stress. AP-MALDI-MS or SI-MS method have been employed to analyze differences in the distributions of characteristic metabolites in tea epidermal cells and mesophyll cells. The precise 3 D distribution of metabolites in living tea tissue could be captured using LAESI-MS technology, revealing the physiological phenomena involved in their synthesis, accumulation, and transport in tea.

Use of nonaqueous fractionation method to investigate distributions of nonvolatile specialized metabolites in subcellular organs of tea

Generally, numerous metabolites occur in plant species. Around 100,000 secondary metabolites have been discovered in plants, although this might only account for 10% of the actual total in nature (Wink 1988). As many metabolites in the cell are water-soluble, with fast turnover and transport between different compartments, it is necessary to avoid aqueous solutions during metabolite determination between subcellular compartments and organelles. Nonaqueous fractionation (NAF) might be the most extensive solution available today for determining subcellular distributions of metabolites in plant cells. NAF was developed by Behrens for the fractionation of animal nuclei in 1932 (as quoted in Gerhardt and Heldt 1984), then applied to isolate nuclei from wheat germ, and finally improved by Gerhardt and Heldt in 1984 to analyze subcellular metabolite levels in spinach leaves (Gerhardt and Heldt 1984). Along with omic technologies, the analysis of the distributions of large numbers of metabolites has become a selective method. The nonaqueous isolation method has been used to isolate chloroplasts from lyophilized leaves using a nonaqueous medium, but suffers from the loss of a large portion of chloroplasts, making subcellular metabolite levels difficult to determine (Stocking 1959). NAF is not based on the purification of intact organelles, but rather the enrichment of subcellular material in each part of a continuous nonaqueous density gradient. In this method, the frozen tissue is homogenized and lyophilized, the freeze-dried material is further sonicated in nonaqueous solvents and filtrated, and the resulting homogenate is fractionated by centrifugation. The frozen state before lyophilization and absence of water after drying prevent metabolite interconversion by enzymes, and water-soluble enzymes and metabolites are not leached during the isolation using nonpolar solvents. Subcellular metabolite distributions can be calculated by assaying compartment-specific marker enzyme abundance and metabolite levels in each of the gradient fractions (Gerhardt and Heldt 1984; Klie et al. 2011; Krueger et al. 2011).

The main steps in the nonaqueous fractionation procedure, according to Krueger et al. (2014), are as follows (Figure 5). The first step is tissue preparation. The tissues are first rapidly snap-frozen in liquid nitrogen after harvesting to prevent the enzymatic interconversion of metabolites. This is followed by homogenization of the frozen sample using a ball mill or pestle and mortar. Thawing of the plant materials during homogenization must be avoided, because it can lead to mixing of compartment-enriched particles and, therefore, false metabolite distributions within the NAF fractions. The frozen homogenized sample (−80 °C) is then lyophilized at 0.02 bar and around −30 to −80 °C for 48–60 h. The lyophilized material is then resuspended in a

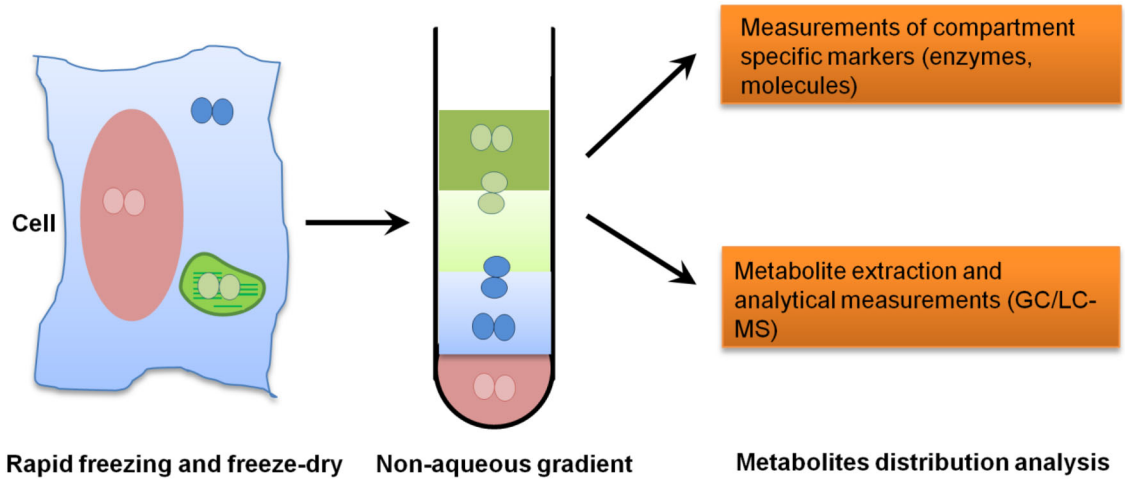

Figure 5. The main steps in the nonaqueous fractionation procedure.

nonaqueous mixture (C_2Cl_4/C_7H_{16}; 66:34, v/v) and sonicated to achieve sufficiently homogenization. Finally, to remove large particulates that could destroy the integrity of the NAF gradient in the centrifugation process, the sonicated suspension is filtered through a nylon net with a pore size of 20 µm before centrifugation. The second step is fractionation. The solvent density applied for nonaqueous fractionation varies dramatically among different species or tissues. For example, 1.28–1.50 g/mL with a cushion at 1.59 mg/mL for spinach leaves (Gerhardt and Heldt 1984), or 1.28–1.51 g/mL plus a cushion at 1.62 g/mL for spinach leaves (Riens et al. 1991), and 1.43–1.62 g/mL for *Arabidopsis* leaves (Krueger et al. 2011; Arrivault et al. 2014). In addition, the optimal density is different among tissues even from the same plant, for example, 1.30–1.50 g/mL for tea shoot and 1.30–1.55 g/mL for tea root (Fu, Liao, et al. 2020). Therefore, the optimal density range for the object needs to be screened and determined before preparing the NAF density gradient. After centrifugation, five or six fractions, or even up to 12 fractions, will be divided according to the special case. Aliquots of these gradient fractions are used to analyze compartment-specific marker enzymes (molecules) or a broad range of compounds using MS-based analytical approaches (such as LC/GC-MS). The third step is compartment-specific marker enzyme abundance analysis. Specific markers of a compartment are crucial for NAF analysis because they represent compartmental boundaries, and are used to calculate the compartmental distribution of the detected cellular metabolites. The NAF technique commonly recommends separating the three distinct compartments of plastids, cytosol, and vacuoles (Riens et al. 1991; Krueger et al. 2009). Dividing into more compartments can be performed as long as the number of fractions taken exceeds the number of compartments (Klie et al. 2011). For example, if four compartment-specific markers are to be analyzed, then at least five fractions will need to be divided. In addition, multiple markers specific to the same compartment are recommended for use to prevent using nonspecific markers or their measurement errors. In addition to marker enzymes, the abundance of different marker molecules can be analyzed. For example, glyceraldehyde-3-phosphatedehydrogenase (GAPDH), starch, and digalactosyldiacylglycerols (DGDGs) can be used to represent plastids, uridine diphosphate glucose pyrophosphorylase (UGPase), glyceroceramides (GlcCer), and triacylglycerides (TAGs) can represent cytosol, and nitrate, glucosinolates, and flavonoids can be designated as vacuoles. Furthermore, the protein content of compartment-specific enzymes detected by Western or ELISA assays is an appropriate alternative strategy, as long as the antibody to the protein is compartment-specific (Krueger et al. 2009). The final step is metabolic subcellular distribution analysis. Krueger et al. (2011) proved that two different computational strategies, the non-negative least squares (NNLS) algorithm (Lawson and Hanson 1974) and the best fit algorithm (BFA) (Riens et al. 1991), led to similar determinations of three-compartmental distribution. Subsequently, Klie et al. (2011) developed the *BestFit* command line tool (v1.2), which calculates and estimates subcellular metabolite distributions using two important data sets (the NAF distributions of compartment-specific markers and target or nontarget metabolites).

NAF method was first applied to investigating the subcellular contents of malate and sucrose in spinach leaves by Gerhardt and Heldt (1984), and has gradually been used to investigate metabolite compartmentalization in different tissues of various plant species, including bean, maize, barley, *Arabidopsis*, apple, and rose (Riens et al. 1991; Gerhardt et al. 1987; Winter et al. 1993; Fettke et al. 2005; Shannon et al. 1998; Farré et al. 2001; Yamada et al. 2009; Krueger et al. 2009). For example, Farré et al. (2001) first applied this NAF technique to elucidating metabolite compartmentation in potato tubers. A recommended technique is combined metabolite–protein analysis on single tissue samples (Arrivault et al. 2014). Recently, Ashykhmina et al. (2019) showed the contribution of $PAPST_2$ to organellar PAP uptake and metabolism by analyzing PAP levels in the chloroplast and cytosol fractions of respective mutant *Arabidopsis* plants using the NAF method. Interestingly, NAF can potentially determine suborganelle compartments, such as thylakoids, from the stroma in chloroplasts, by separating fragments of organelles, as long as specific markers

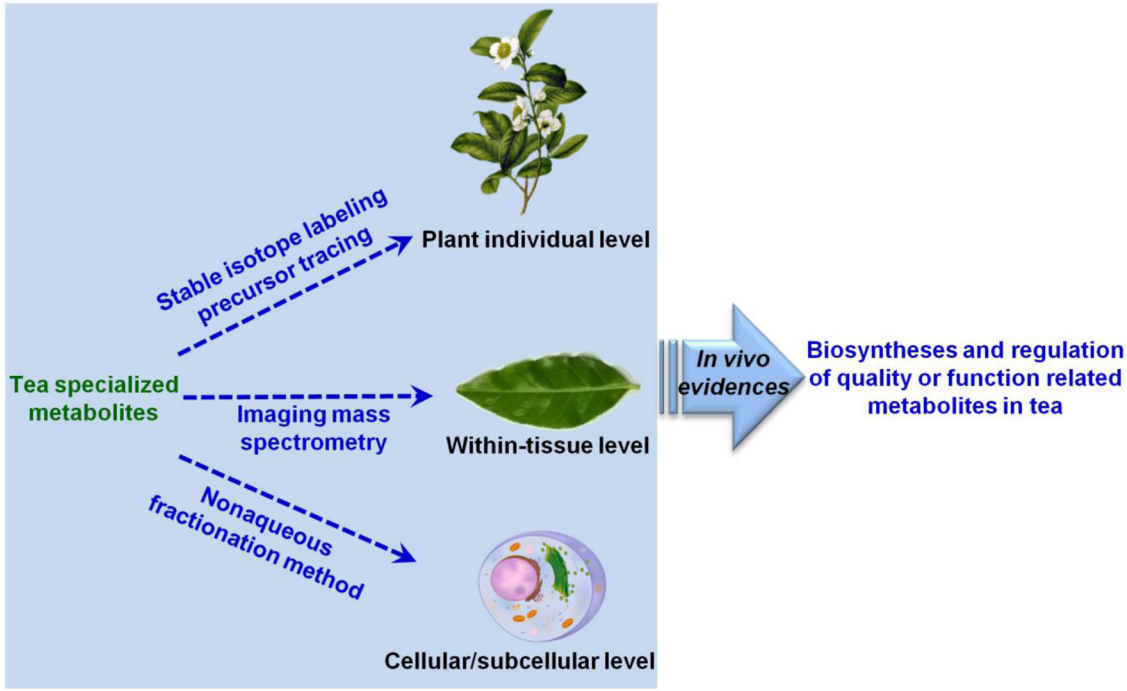

Figure 6. Summary on the feasible methods to study tea specialized metabolites at individual level, within-tissue level, and cellular or subcellular level.

represent these compartments. Recently, nonaqueous fractionation method has also been successfully applied to analyze the subcellular distribution of characteristic metabolites in tea plants (Fu, Liao, et al. 2020; Fu, Cheng, et al. 2020; Zhou et al. 2020). Based on this technology, Fu, Liao, et al. (2020) found that the subcellular distribution of specific metabolite L-theanine in tea is affected by different tissues and light environment conditions. Polyphenols such as gallic acid and methyl gallate were mainly accumulated in peroxisome of tea leaf cells (Zhou et al. 2020).

Every method has advantages and disadvantages. The main advantage of NAF is that a large number of polar, semipolar, and lipophilic subcellular metabolite levels, as well as proteins, can be measured in whole leaves or other tissues by combining comprehensive omic technologies and modern computational biology approaches. Meanwhile, metabolism is stopped during NAF isolation by rapid freezing and the nonaqueous medium. Therefore, unlike protoplast fraction and intracellular perfusion approaches (Stitt et al. 1989; Takeshige and Tazawa 1989), NAF avoids the conversion and translocation of metabolites during isolation. However, NAF is a time-consuming (generally two weeks) and labor-intensive process, and must produce consistent gradients. Meanwhile, the dividing line of each fraction is not very clear and requires estimation. It would be hard for the colorless tissue to separate these fractions. Furthermore, NAF analysis is fully dependent on compartment-specific markers. Therefore, inaccurate conclusions would be obtained if a nonspecific marker was applied. Currently, suitable markers for mitochondria, especially other unconsidered compartments, such as the endoplasmic network and peroxisome, have yet to be identified. Finally, NAF cannot be used to monitor dynamic transport processes and count metabolite exchange rates. It is known that some tea specialized metabolites dynamically transport between cells and subcellular organelles such as L-theanine (Dong et al., 2020). Therefore, these methods could not monitor these metabolites in vivo at real time in the tea plants. Bioluminescent proteins and fluorescent proteins have been explored to as a powerful tool for monitoring biological processes in real time such as gene expression in living tissues (Saito et al., 2010). Therefore, to develop a technology that can observe metabolites in plants in vivo at real time in plants is essential for further biological study at the metabolite level.

Concluding remarks and perspectives

Knowledge of the biosynthesis and regulation of specialized metabolites in tea plants are crucial for improving tea quality and function. In this review, we have summarized feasible methods for studying tea specialized metabolites at the individual level, within-tissue level, and cellular or subcellular level, including the stable isotope-labeled precursor tracing, imaging mass spectrometry, and nonaqueous fractionation. These methods will provide better in vivo evidence of the biosynthesis and regulation of specialized metabolites in tea plants (Figure 6). This review also summarizes the advantages and drawbacks of each method when applied to tea plants. Based on this information, we will select appropriate methods to solve corresponding scientific problems in a more targeted fashion.

In future, when these methods are used, several important issues should be considered, improved, or solved. First, precise introduction of the stable isotope-labeled precursor metabolite into the original site of the tea plant is key to obtaining more accurate in vivo evidence. Furthermore, metabolic flux in pathways leading from stable isotope-

labeled precursor metabolite to target metabolite needs be precisely calculated to determine key regulated steps in these pathways. Second, the detection resolution of mass spectrometry imaging must be significantly improved to obtain clearer profiles of within-tissue distributions of tea specialized metabolites. Furthermore, the scanning speed needs to be increased to avoid changes in tea specialized metabolites during analysis. Finally, the subcellular distribution of tea specialized metabolites obtained by nonaqueous fractionation needs to be combined with the subcellular distribution of the related proteins/genes to analyze the biosynthesis sites of tea specialized metabolites and their dynamic changes and transport. Besides, the biosynthesis and transport of tea specialized metabolites are generally happened at same time. Hence, if we want to precisely monitor the biochemical formation and multilevel distribution of tea specialized metabolites, whole tea plant level studies need to be considered except for certain tissue of tea plants.

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No potential conflict of interest was reported by the author(s).

Author contributions

Yang Z. constructed the manuscript outline. Yang Z., Liao Y., and Fu X. wrote the manuscript. Zeng L. helped editing the manuscript. All authors reviewed the manuscript.

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