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REVIEW

Understanding the biosyntheses and stress response mechanisms of aroma compounds in tea (*Camellia sinensis*) to safely and effectively improve tea aroma

Lanting Zeng[a,b], Naoharu Watanabe[c], and Ziyin Yang[a,b]

[a]Key Laboratory of South China Agricultural Plant Molecular Analysis and Genetic Improvement & Guangdong Provincial Key Laboratory of Applied Botany, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China; [b]College of Advanced Agricultural Sciences, University of Chinese Academy of Sciences, Beijing, China; [c]Graduate School of Science and Technology, Shizuoka University, Naka-ku, Hamamatsu, Japan

ABSTRACT

Metabolite formation is a biochemical and physiological feature of plants developed as an environmental response during the evolutionary process. These metabolites help defend plants against environmental stresses, but are also important quality components in crops. Utilizing the stress response to improve natural quality components in plants has attracted increasing research interest. Tea, which is processed by the tender shoots or leaves of tea plant (*Camellia sinensis* (L.) O. Kuntze), is the second most popular beverage worldwide after water. Aroma is an important factor affecting tea character and quality. The defense responses of tea leaves against various stresses during preharvest (tea growth process) and postharvest (tea manufacturing) processing can result in aroma formation. Herein, we summarize recent investigations into the biosyntheses of several characteristic aroma compounds prevalent in teas and derived from volatile fatty acid derivatives, terpenes, and phenylpropanoids/benzenoids. Several key aroma synthetic genes from tea leaves have been isolated, cloned, sequenced, and functionally characterized. Biotic stress (such as tea green leafhopper attack) and abiotic stress (such as light, temperature, and wounding) could enhance the expression of aroma synthetic genes, resulting in the abundant accumulation of characteristic aroma compounds in tea leaves. Understanding the specific relationships between characteristic aroma compounds and stresses is key to improving tea quality safely and effectively.

Introduction

Tea, which is processed by the tender shoots or leaves of tea plant (*Camellia sinensis* (L.) O. Kuntze), is the second most popular beverage worldwide after water (Rietveld and Wiseman 2003). The popularity of tea as a beverage is dependent on its unique quality and function. In contrast to many other plants, tea plants are rich in a wide variety of secondary metabolites that contribute to the tea quality, such as tea color, flavor, and taste. These metabolites can mostly be divided into three classes, phenolic compounds, amino acids, and aroma compounds (volatiles). Phenolic compounds and amino acids affect the tea taste, while phenolic compounds also contribute to tea color (Yang, Baldermann, and Watanabe 2013). Meanwhile, aroma compounds are responsible for tea flavor (Yang, Baldermann, and Watanabe 2013). Consequently, aroma is an important factor affecting the character and quality of tea. In general, phenolic compounds and amino acids account for 18%–36% and 1%–4% of tea content, respectively, while aroma compounds account for less than 0.03% (Wan 2003). Gas chromatography (GC), GC–mass spectrometry (GC–MS), nuclear magnetic resonance (NMR) spectroscopy, and other

techniques have been used to isolate and identify more than 700 tea aroma compounds. Although numerous aroma compounds have been identified in tea, only a few components with concentrations exceeding their odor thresholds contribute to the tea aroma profiles. Different aromas possess different flavor threshold concentrations because the human olfactory system detection limits for aromas are not consistent. In some cases, there are thousand-fold differences in threshold concentrations between two different aroma compounds. For example, rose-like aroma compound, 2-phenylethanol, can be detected by human beings at 1000 μg/L in water, while another aroma compound, damascenone, with a honey-fruity fragrance, can be perceived only at 4 ng/L in water (Schuh and Schieberle 2006). This evidence suggests that some abundant aroma compounds may make less of a contribution to tea aroma profiles than those with low or even trace amounts. Tea aromas, and their biosyntheses, are various and complex. Insight into tea aroma formation is of significant interest and has attracted increasing research attention recently.

According to the different reaction pathways, tea aroma formation can be classified into three types (Fig. 1), namely, enzymatic reactions when the leaf cell is alive (mainly occurring in preharvest tea plant development and partial postharvest manufacturing stages, and involving the activation of tea aroma-related genes and reactions between the substrate and enzymes), enzymatic reactions when the leaf cell is disrupted (mainly occurring in partial postharvest manufacturing stages and involving reactions between the substrate and enzymes), and thermophysical and chemical reactions (mainly occurring in partial postharvest manufacturing and deep processing stages). In this review, we have mostly focused on tea aroma formation from enzymatic reactions when the leaf cell is alive or disrupted. Firstly, we have summarized recent investigations into the biosyntheses of several characteristic aroma compounds prevalent in teas, which are derived from volatile fatty acid derivatives (VFADs), volatile terpenes (VTs), and volatile phenylpropanoids/benzenoids (VPBs). Several key aroma synthetic genes from tea leaves were isolated, cloned, sequenced, and functionally characterized. Furthermore, biotic stress (such as tea green leafhopper attack) and abiotic stress (such as light, temperature, and mechanical damage) can enhance the expression levels of aroma synthetic genes, resulting in abundant accumulation of the characteristic aroma compounds in tea leaves. Understanding the specific relationships between characteristic aroma compounds and stresses is key to achieving improved tea quality safely and effectively.

Direct investigation of volatile synthesis in tea plants

Similar to volatiles in other plants, tea volatiles can be classified into four major classes according to their metabolic origin: VTs, VPBs, VFADs, and volatiles derived from carotenoids (Yang, Baldermann, and Watanabe 2013). Some plant volatile formation pathways are shared among different plant species, while some differ owing to the complex networks of plant volatile biosynthesis. Therefore, direct evidence of volatile biosynthesis in tea leaves is required. As a genetic transformation system for tea has yet to be established, it is difficult to study the biosynthetic pathways of specialized metabolites in tea leaves directly. In many model plants, countless biosynthetic enzymes have been identified using a classical purification approach (O'Connor 2009). In this strategy, the desired enzyme is purified by traditional biochemical chromatography and protein sequence information is used to isolate the desired gene from the plant cDNA (O'Connor 2009). Nevertheless, in tea plants, current knowledge of volatile biosynthesis has been mostly obtained from studies on other plant species. The biochemistry is typically elucidated by feeding isotopically labeled precursors into the
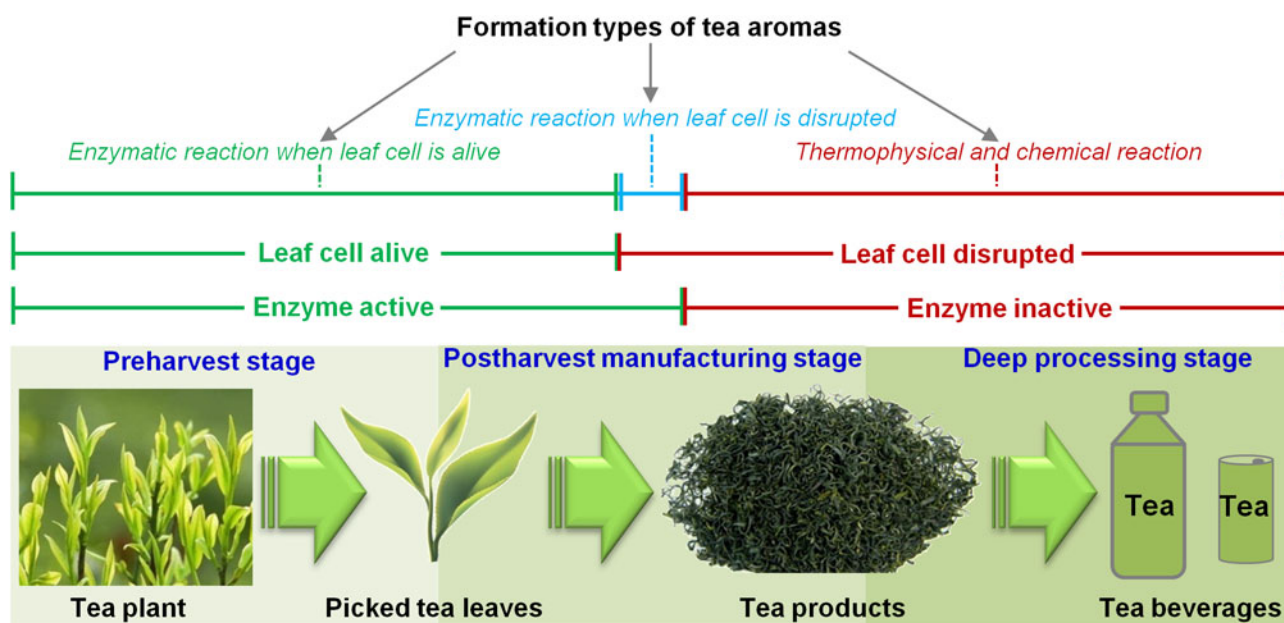

Figure 1. Types of tea aroma formation during the preharvest stage, postharvest manufacturing stage, and deep processing stage.

plant or plant culture and mapping the placement of isotopes within the final natural product (O'Connor 2009). Therefore, stable isotope labeling experiments are usually used to confirm whether biosynthetic pathways elucidated in other plant species also exist in tea plants (Zeng et al. 2018; Zhou et al. 2017b, 2018). After confirmation, or sometimes without confirmation, the homogenous genes are obtained using BLAST, and expressed in a heterologous expression system. The recombinant proteins are then typically monitored in an *in-vitro* enzymatic assay to confirm that the genes encode enzymes with the expected function. Furthermore, the homogenous genes are functionally characterized in a model plant transgenic system, providing *in vivo* evidence to some extent.

To date, major progress has been achieved using molecular and biochemical techniques, with many genes encoding enzymes of tea aroma biosynthesis having recently been reported (Table 1 and Fig. 2). In the last decade, most identified genes have been expressed in *Escherichia coli* (*E. coli*) or yeast expression systems and functionally characterized *in vitro* (Table 1). A few genes, such as (*E*)-*nerolidol synthase* (*CsNES*) and *linalool synthase* (*CsLIS*), have been further shown to catalyze the expected biochemical activity *in vivo*, namely, the formations of (*E*)-nerolidol and linalool from farnesyl diphosphate (FPP) and geranyl diphosphate (GPP) (Liu et al. 2018). In short, the functionally identification systems used to investigate synthetic genes involved in tea aroma formation are mostly conducted *in vitro*. The foreseeable shortage of a functional identification system for synthetic genes has prompted great urgency in establishing appropriate and precise research methods for tea. Therefore, the lack of direct evidence for functional identification obtained from a tea genetic transformation system is among the biggest bottlenecks restricting the development of basic tea research.

Both free volatile compounds and glycosidically bound volatile compounds (GBVs) occur in tea leaves. As the latter are more water-soluble and less reactive than their free aglycone counterparts, aroma compounds in tea leaves typically accumulate as glycoside forms (Winterhalter and Skouroumounis 1997). Many GBVs, including β-primeverosidases and β-glucosidases, can be hydrolyzed by β-glycosidases to liberate various free tea aromas (Ma et al. 2001). Therefore, determining the biosynthesis of GBVs catalyzed by β-glycosidases in tea plants is of particular interest. To date, one β-primeverosidase and three β-glucosidases have been isolated from tea, identified, and functionally characterized (Table 1) (Mizutani et al. 2002; Zhou et al. 2014; Zhou et al. 2017a). Subcellular localization analysis showed that β-primeverosidase targets the cell wall (Gui et al. 2015), while β-glucosidases are multi-located, such as in the cytosol and vacuole, other than cell wall (Zhou et al. 2017a). Glycosyl transferases (GTs), which are involved in the transformation of glycoside-bound aromas into free aromas in tea leaves, have also been intensively studied and functionally characterized (Ohgami et al. 2015). These results revealed the mechanism of GBV formation and aroma β-primeveroside biosynthesis in tea, providing not only molecular insight into volatile metabolism, but also crucial molecular tools for controlling tea aroma quality.

Other than glycosidic hydrolysis, many aroma compounds are formed by *de novo* synthesis pathways. Recently, several enzymes and genes involved in the final steps of the biosyntheses of tea aroma compounds, including (*S*)-linalool, (*E*)-nerolidol, β-ocimene, methyl salicylate, indole, and 3-(*Z*)-hexenal, have been isolated, identified, and functionally characterized (Deng et al. 2016, 2017; Fu et al. 2015; Liu et al. 2018; Mei et al. 2017; Xu et al. 2018; Zeng et al. 2016; Zhou et al. 2017c). In some cases, more than one enzyme are involved in the last step of tea aroma formation. For instance, in an investigation into indole formation, only the combination of tryptophan synthase α-subunit (CsTSA) and tryptophan synthase β-subunit 2 (CsTSB2) recombinant protein produced in *E. coli* exhibited the ability to transform indole-3-glycerol phosphate (IGP) into indole (Fig. 2) (Zeng et al. 2016), because TSA activity is largely dependent on its interaction with TSB, which is consistent with findings in other plant (Schullehner et al. 2008). Related information is also summarized in Table 1. Notably, while many

Table 1. Aroma compounds and their related biosynthetic genes.

Aroma compound	Gene name	Designation[a]	Accession number	Expression system[b]	Subcellular localization	References
β-Primeveroside-bound aroma	β-Primeverosidase	CsPD	BAC78656	*E. coli*	Cell wall	Mizutani et al. (2002) and Gui et al. (2015)
β-Glucoside-bound aroma	β-Glucosidase	CsGH1BG1	KY379513	Yeast	Cytosol	Zhou et al. (2017a)
		CsGH3BG1	KY379523	Yeast	Vacuole	
		CsGH5BG1	KY379530	Yeast	Cell wall	
(*S*)-Linalool	Linalool synthase	CsLIS1	KF006849	*E. coli*	–	Fu et al. (2015) and
		CsLIS2	KY033151	*E. coli*	–	Mei et al. (2017)
		CsLIIS/NES-1	KF006849	*E. coli*, tobacco	Chloroplast	Liu et al. (2018)
(*E*)-Nerolidol	Nerolidol synthase	CsNES	KY033151	*E. coli*	Cytosol	Zhou et al. (2017c)
		CsLIS/NES-2	–	*E. coli*, tobacco	Cytosol	Liu et al. (2018)
β-Ocimene	β-Ocimene synthase	CsOS2	TEA004606.1	*E. coli*	–	Xu et al. (2018)
Indole	Tryptophan synthase α-subunit	CsTSA	KX022968	*E. coli*	–	Zeng et al. (2016)
	Tryptophan synthase β-subunit	CsTSB2	KX022970	*E. coli*	–	
Methyl salicylate	Salicylic acid carboxyl methyltransferase	CsSAMT	MG459470	*E. coli*	Chloroplast	Deng et al. (2017)
3-(*Z*)-Hexenal	Hydroperoxide lyase	CsHPL	ADO51747	*E. coli*	–	Deng et al. (2016)

[a]The designation refers to the name in the original publication.
[b]Expression system used for the functionally identification of the related gene. *E. coli*, *Escherichia coli* expression system; Yeast, yeast expression system; Tobacco, tobacco transgenic system.
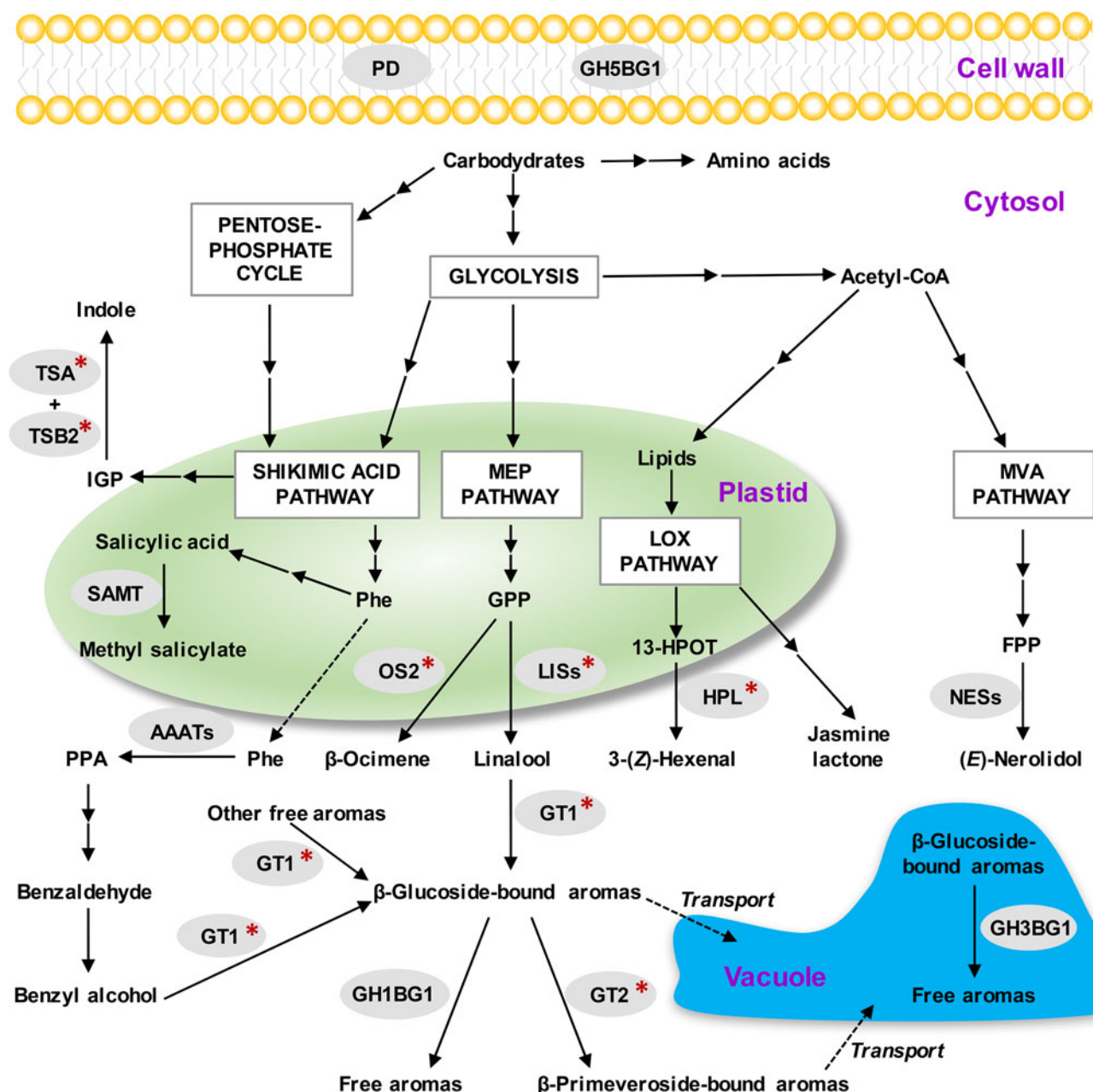'-'The accession number and subcellular localization of these proteins are unknown.

Figure 2. Known biosynthetic genes involved in the aroma formation in *Camellia sinensis*. Abbreviations: AAATs, aromatic amino acid aminotransferases; CoA, coenzyme A; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; GT, glycosyltransferase; HPL, hydroperoxide lyase; 13-HPOT, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)octadecatrienoic acid; IGP, indole-3-glycerol phosphate; LISs, linalool synthases; LOX, lipoxygenase; MEP, methylerythritol phosphate; MVA, mevalonic acid; NESs, (*E*)-nerolidol synthases; OS2, *β*-ocimene synthase 2; PD, *β*-Primeverosidase; Phe, phenylalanine; PPA, phenylpyruvic acid; SAMT, salicylic acid carboxyl methyltransferase; TSA, tryptophan synthase *α*-subunit; TSB2, tryptophan synthase *β*-subunit 2. *The subcellular localization of these proteins is unknown. One solid arrow represents one step involved in the conversion, and two solid arrows represent more than one step. Dotted arrows represent transportation between two different organelles. Most related references are shown in the Table 1, and the information on *CsAAATs* was referred from Wang et al. (2019).

studies have reported the biosynthesis of VTs and VPBs, few have investigated VFADs in tea plants (Fig. 2). VFADs, such as alcohols, aldehydes, and lactones, are derived from both saturated and unsaturated fatty acids (Yang, Baldermann, and Watanabe 2013). Some studies have identified the lipoxygenase (LOX) enzyme family, which catalyzes the first step of the biosynthetic pathway of VFADs (Zeng et al. 2018; Zhu et al. 2018). However, few genes involved in the final steps of the biosynthesis of VFADs have been functionally identified and reported, except for a *hydroperoxide lyase* (*CsHPL*) gene closely related to 3-(*Z*)-hexenal formation (Fig. 2) (Deng et al. 2016).

Owing to the existence of homogenous genes, *in vitro* evidence indicates that more than one gene can play a role in the biosynthesis of an aroma compound. For instance, four *E. coli*-expressed 1-phenylethanol (1PE) synthases (CsPESs) showed enzymatic activity in the transformation of acetophenone (AP) into 1PE (Zhou et al. 2018). Although CsPES1 has the highest K_{cat}/K_m value, showing that is the most catalytically active toward AP as a substrate *in vitro* (Zhou et al. 2018), it is still insufficient to vindicate that CsPES1 has a unique role in 1PE biosynthesis in tea flowers because, in some cases, there is a significant difference

between *in vitro* and *in vivo* evidence. Liu et al. (2018) proved that CsLIS/NES-1 and CsLIS/NES-2 can both produce (*E*)-nerolidol and linalool *in vitro*. However, transgenic tobacco studies and gene-specific antisense oligodeoxynucleotide suppression have further indicated that CsLIS/NES-1 acts as linalool synthase, while CsLIS/NES-2 functions as a potential (*E*)-nerolidol synthase, not a linalool synthase (Liu et al. 2018). This is attributed to alternative splicing resulting in the different subcellular localizations of these two enzymes, with CsLIS/NES-1 localized in the chloroplasts and CsLIS/NES-2 localized in the cytosol (Fig. 2).

Formation of tea aromas from enzymatic reactions where a live tea leaf is exposed to biotic stress

Under normal and non-stress conditions, most volatile compounds (including aroma compounds) occur as liquids in plants (Pichersky and Gershenzon 2002). Plants, especially vegetative parts, synthesize and emit some volatile compounds when exposed to environmental stresses. These stress-induced volatiles have functions that reduce the negative effects of stresses on plants (Dong et al. 2016; Pichersky and Gershenzon 2002). In contrast to abiotic stress, the effect of biotic stress, especially herbivore attack, on plant volatiles has been intensively studied. Similar to other plants, during tea plant growth, many herbivores, such as piercing-sucking insects, including tea green leafhoppers and tea aphids, and chewing insects, including tea geometrid, tea tortrix, and spider mites, attack tea leaves and influence their yield, appearance, and quality (Dong et al. 2011). These herbivore attacks can induce tea leaves to produce and emit numerous volatile compounds, such as VFADs (including (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, (*E*)-2-hexenal, and (*E*)-2-hexenoic acid), VPBs (including benzyl nitrile, benzaldehyde, and indole), and VTs (including geraniol, farnesene, ocimenes, linalool, and nerolidol). These herbivore-induced volatiles may have tea-plant-protecting functions (Han and Chen 2002; Ishiwari, Suzuki, and Maeda 2007). Most reports are focused on the ecological functions of herbivore-induced plant volatiles. However, herbivore-induced plant volatiles may influence the tea aroma properties. In tea practical applications, there is the classical example that tea leaves infested by tea green leafhoppers (*Empoasca* (*Matsumurasca*) *onukii* Matsuda) are used to make the famous oolong tea (Oriental Beauty), which has a unique aroma reminiscent of ripe fruit and honey (Cho et al. 2007). This unique aroma is attributed to characteristic volatile monoterpenes, such as 2,6-dimethyl-3,7-octadiene-2,6-diol (diendiol I), induced by *E.* (*M.*) *onukii* attack, and hotrienol, derived from diendiol I under heating conditions during the oolong tea manufacturing process (Fig. 3A) (Cho et al. 2007; Kinoshita 2011, Liu 2018, Mei et al. 2017). Interestingly, wounding and attack from other herbivores, such as piercing-sucking insects including tea aphids, and chewing insects including tea geometrid, tea tortrix, and spider mites, did not induce the formation of diendiol I in tea leaves, suggesting an inducement mechanism characteristic of tea green leafhoppers (Fig. 3B) (Kinoshita 2011; Liu 2018).

Currently, although the biosynthesis of diendiol I in plants is unknown, several reports have suggested that diendiol I is derived from linalool (Etoh, Ina, and Iguchi 1980; Luan et al. 2006, Williams, Strauss, and Wilson 1980). *E.* (*M.*) *onukii* attack has been reported to significantly enhance the emission of (*S*)-linalool from tea leaves due to the upregulation of *LISs* (*CsLIS1* and *CsLIS2*). Furthermore, continuous wounding was found to be a key factor in the *E.* (*M.*) *onukii* attack-induced formation and emission of (*S*)-linalool from tea leaves (Fig. 3C) (Mei et al. 2017). In addition to (*S*)-linalool, geraniol, another volatile monoterpene, was also significantly enhanced in tea leaves exposed to *E.* (*M.*) *onukii* attack. However, *E.* (*M.*) *onukii* attack did not affect the enzyme activity of geraniol synthase in tea leaves. Interestingly, an enzyme extract of *E.* (*M.*) *onukii* specifically produced geraniol from GPP, suggesting that *E.* (*M.*) *onukii* has its own geraniol synthase that contributes to the formation and emission of geraniol from tea leaves (Fig. 3C) (Liu 2018). These studies also indicated that the mechanisms of formation and emission of (*S*)-linalool and geraniol from tea leaves exposed to *E.* (*M.*) *onukii* attack are different, although both volatile monoterpenes are derived from the same precursor GPP.

Diendiol I is a proposed marker of *E.* (*M.*) *onukii*-infested tea leaves because it is only detected in those tea leaves (Fig. 3B) (Liu 2018). Furthermore, it is well-known that phytohormones, especially jasmonic acid, are involved in the formation and emission of plant volatile compounds exposed to herbivore attacks (Maffei, Mithöfer, and Boland 2007). As wounding and other herbivore attacks can induce increased jasmonic acid levels in tea leaves, the formation of diendiol I in *E.* (*M.*) *onukii*-infested tea leaves may be independent of jasmonic acid signals. Our preliminary experiment showed that exogenous jasmonic acid treatment of tea leaves did not produce diendiol I (unpublished data), which partially validated the occurrence of an inducement mechanism characteristic of tea green leafhoppers (Fig. 3B). When herbivores attack plants, in addition to wounding, herbivore-derived elicitors are involved in inducing the formation and emission of plant volatiles. The already-reported herbivore-derived elicitors that induce the formation and emission of plant volatiles include fatty acid-amino acid conjugates (Alborn et al. 1997; Bonaventure, van Doorn, and Baldwin 2011), β-glucosidase (Mattiacci, Dicke, and Posthumus 1995), inceptin (Schmelz et al. 2006), caeliferins (Alborn et al. 2007), and an unidentified heat-labile constituent (Allmann and Baldwin 2010). Some of these reported herbivore-derived elicitors are conservatively present in many herbivores. As herbivores that cannot induce diendiol I formation in tea leaves (Fig. 3B) might contain the reported herbivore-derived elicitors, *E.* (*M.*) *onukii* may contain unique elicitors able to induce diendiol I formation in tea leaves (Fig. 3A). The major challenge is that the amount of elicitors produced by small amounts of *E.* (*M.*) *onukii* is insufficient for analysis and difficult to obtain (Dong et al. 2016). Our group is now attempting to isolate and identify the *E.* (*M.*) *onukii*-derived elicitors able to induce diendiol I formation in tea leaves.
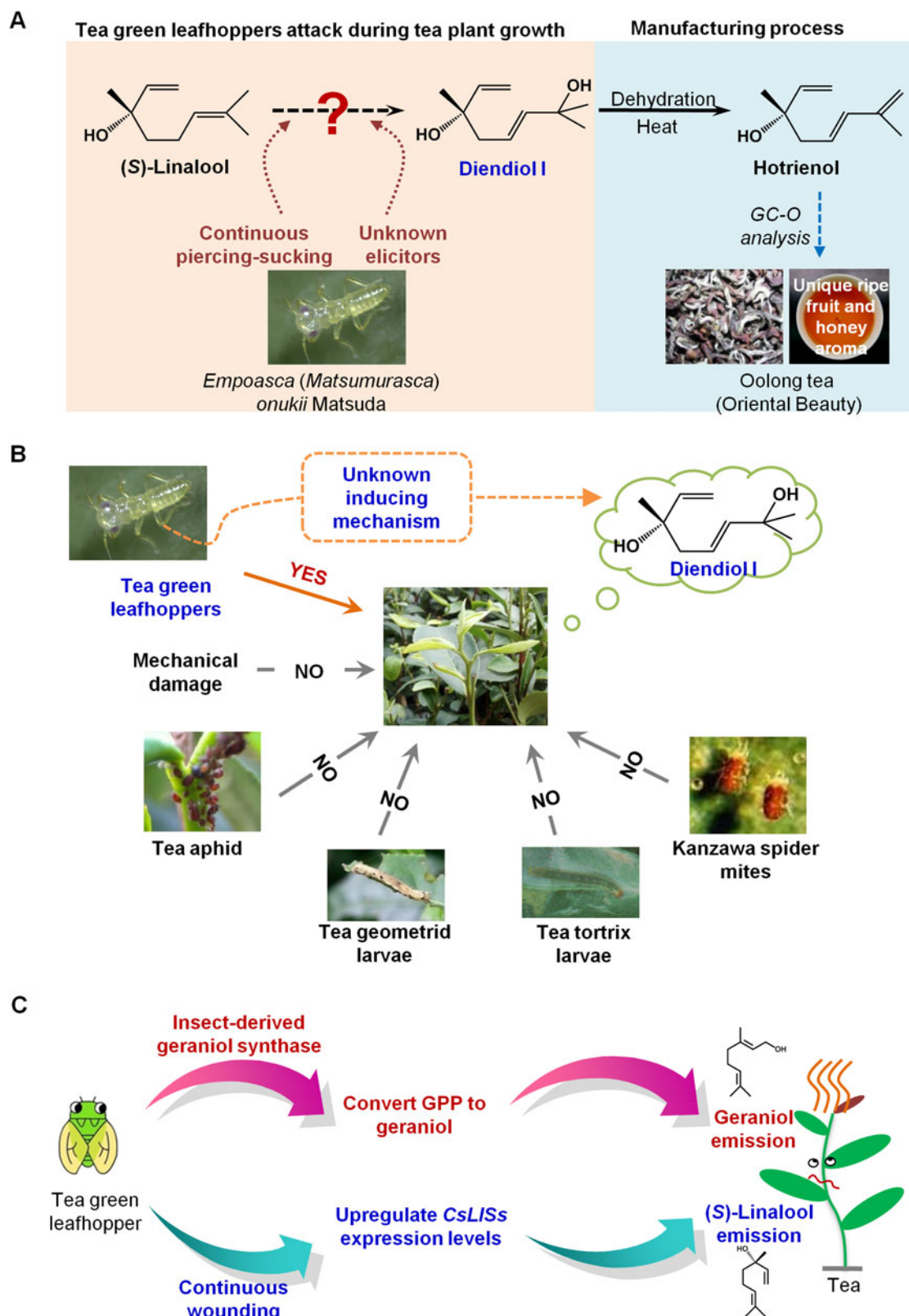
Figure 3. Classical example of oolong tea made using tea-green-leafhopper-infested tea leaves as raw materials. (A) Application of tea-green-leafhopper-infested tea leaves in the manufacturing process. (B) Unique formation of diendiol I induced by tea green leafhopper. (C) Mechanisms of formation and emission of (S)-linalool and geraniol in tea-green-leafhopper-infested tea leaves. GPP, geranyl diphosphate. LIS, (S)-linalool synthase.

Formation of tea aromas from enzymatic reactions where a live tea leaf is exposed to abiotic stress

Compared with biotic factors, more abiotic factors can potentially be used to positively modify aromas in tea leaves during preharvest and postharvest treatment. During plant growth (preharvest leaves), abiotic factors, such as soil conditions, temperature, and light, can result in multifarious plant responses (Mittler 2006). A crucial environmental

factor, light, not only provides plants with essential energy, but also acts as an external signal regulating plant growth and development (Smith 1982). Studies have shown that light intensity and wavelength are both likely to regulate the formation of most tea volatiles. In practical applications, dark treatment (almost no light) has been used to improve tea flavor quality. For example, Gyokuro and Tencha, known as the finest teas in Japan, are produced from tea leaves under shading treatment, because temporary cultivation without light (shading treatment for 3 weeks) could significantly enhance the contents of many aromas in tea leaves, especially VPBs (Yang et al. 2012). Furthermore, the transformation of related upstream metabolites, especially shikimic acid, prephenic acid, and phenylpyruvic acid (PPA), into VPBs is accelerated by shading treatment (Yang et al. 2012). Shade management is a common approach to regulating light conditions during tea plant growth (Beer 1987). However, in response to prolonged shading, tea plants may accelerate their reproductive development, potentially leading to decreased biomass (Franklin and Whitelam 2005). Therefore, more studies are needed to determine an optimal balance between flavor quality and biomass to acquire the best economic benefits in production.

To date, attempts have also been made to investigate changes in tea aroma profiles of tea leaves during treatment with different light wavelengths. In contrast to natural light or dark treatment, blue light (470 nm) and red light (660 nm) dramatically enhanced most volatiles, including VFADs, VPBs, and VTs, in preharvest tea leaves (Fu et al. 2015). Blue or red lights can activate many important genes, such as 9/13-LOX, phenylalanine ammonialyase (PAL), and terpene synthases (TPSs), involved in VFAD, VPB, and VT formation, respectively, which results in tea aroma enrichment (Fu et al. 2015). In contrast to shading treatment (dark), red and blue light treatment do not significantly affect the biomass of tea leaves and further improve tea aroma. These results suggest that blue and red light are promising technologies for remodeling the aroma of preharvest tea leaves. Furthermore, single-light wavelengths also enhanced the amount of tea volatiles in postharvest leaves, but had a less significant effect on volatile formation compared with preharvest leaves (Fu et al. 2015). In short, both shading and irradiation with blue or red light can increase the amounts of most volatiles in tea leaves. This information will enrich our knowledge of the effects of environmental factors, such as light, on metabolites in tea leaves, and presents an opportunity to manipulate the aroma in tea raw materials without adding transgenes, treating with hormones, or affecting plant nutrition.

Light has little effect during postharvest processing because the treatment time is too short for light to make a significant difference. However, other abiotic factors are involved in the various manufacturing processes (postharvest treatment). Under normal circumstances, fresh tea leaves are virtually odorless or have a slight green note, with most aroma compounds produced during the tea manufacturing process (Mizutani et al. 2002). In general, teas can be classified into six types according to the manufacturing process, including non-fermented green tea, slightly fermented white tea, semi-fermented oolong tea, fully fermented black tea, post-fermented yellow tea, and dark tea (Baldermann et al. 2014). Based on the comparison and analysis of the manufacturing processes of the six tea types, oolong tea manufacture involved the most stresses (Fig. 4A) (Zeng et al. 2017, 2018), namely, wounding (plucking and turn over), drought (solar and indoor withering), and ultra-violet (UV) irradiation (solar withering) (Cho et al. 2007; Gui et al. 2015; Zeng et al. 2016, 2017; Zhou et al. 2017c). Usually, oolong tea is considered to possess more pleasant or characteristic aroma qualities than other teas, such as black tea and green tea. (Yang, Baldermann, and Watanabe 2013). Furthermore, oolong tea manufacture keeps the tea leaves alive for the longest (Fig. 4A). Therefore, the characteristic aroma formed during the manufacturing process of oolong tea is proposed to be due to the stress-responsive biochemical reactions of tea plants.

Recently, the volatile constituents present in oolong tea and their odor attributes have been characterized (Gui et al. 2015). Many characteristic volatile compounds, such as indole, (E)-nerolidol, and some VFADs (including jasmine lactone), were found to have their contents significantly increased during the oolong tea manufacturing process, especially at the turnover stage involved in wounding (Gui et al. 2015; Yang et al. 2010). The study also showed that wounding stress could enhance the accumulation of these volatiles by activating the key genes involved in their syntheses during the turnover stage (Gui et al. 2015). Indeed, the CsTSB2 gene involved in indole formation and CsNES gene involved in (E)-nerolidol formation are activated during continuous mechanical damage (wounding stress), triggering the accumulation of these two aromas (Zeng et al. 2016; Zhou et al. 2017c). The results further confirmed that these two aromas were wounding stress-responsive. In addition to wounding stress, other stresses involved in the oolong tea manufacturing process have also been found to regulate the formation of many volatiles. For example, different degrees of drought stress can induce various types of aromatic constituents, with the number of aromatic constituents induced in fresh leaves increasing as drought stress increased (Cao, Liu, and Liu 2007). Furthermore, UV-B irradiation of postharvest tea leaves was found to stimulate the expression of β-primeverosidase and β-glucosidase genes, leading to the release of volatiles from the leaves (Jang et al. 2010). Ai et al. (2017) found that green light irradiation markedly damaged the aroma and taste of the tea, leading to a strong greenish flavor and astringent taste. Furthermore, monochromatic yellow, orange, and red lights were proposed for withering black tea to improve its overall quality. Although these studies suggest that UV irradiation, different light qualities, and drought stresses regulate the formation of many volatiles, wounding stress is the key factor controlling the formation of the characteristic aroma of oolong tea. This was demonstrated by the amounts of indole, jasmine lactone, and (E)-nerolidol, identified as relatively potent odorants in oolong tea, being nearly identical after solar and indoor withering, while a significant increase was observed at the turnover stage (that involves wounding stress) (Gui et al. 2015). These results help provide

direct evidence of volatile formation in postharvest leaves, especially in oolong tea production.

Multiple stresses act on tea leaves during the manufacturing process. Once picked, tea leaves suffer from wounding stress, with the occurrence of another stress, such as low temperature, water losing, resulting in multiple stresses during tea manufacture (Fig. 4B). In practical applications, the postharvest tea leaves are manufactured at relatively low temperatures. Therefore, low temperature stress is also considered a regulating factor involved in the tea manufacturing process. Indeed, a study has demonstrated that low temperature stress could induce the formation of various aromatic constituents in tea leaves (Cao et al. 2006). Furthermore, the combination of low temperature and mechanical damage

(wounding stress) has a synergistic effect on volatile formation in postharvest tea leaves. The indole level in tea leaves increases rapidly after plucking (Yang et al. 2010, Gui et al. 2015), but the degree of increase is lower than that of leaves stored at $15\,°C$ for $16\,h$ after plucking (Fig. 4C) (Katsuno et al. 2014), which involves multiple stresses, such as wounding, low temperature, and water losing. To exclude the effect of water losing stress on indole formation, preharvest tea leaves (i.e. tea leaves on the tea plants that had no water losing stress) were used for investigations. Wounding and $15\,°C$ treatment on the preharvest tea leaves resulted in more accumulation of indole, compared with the preharvest tea leaves exposed to $25\,°C$ with the same wounding or exposed to $15\,°C$ without wounding (Zeng
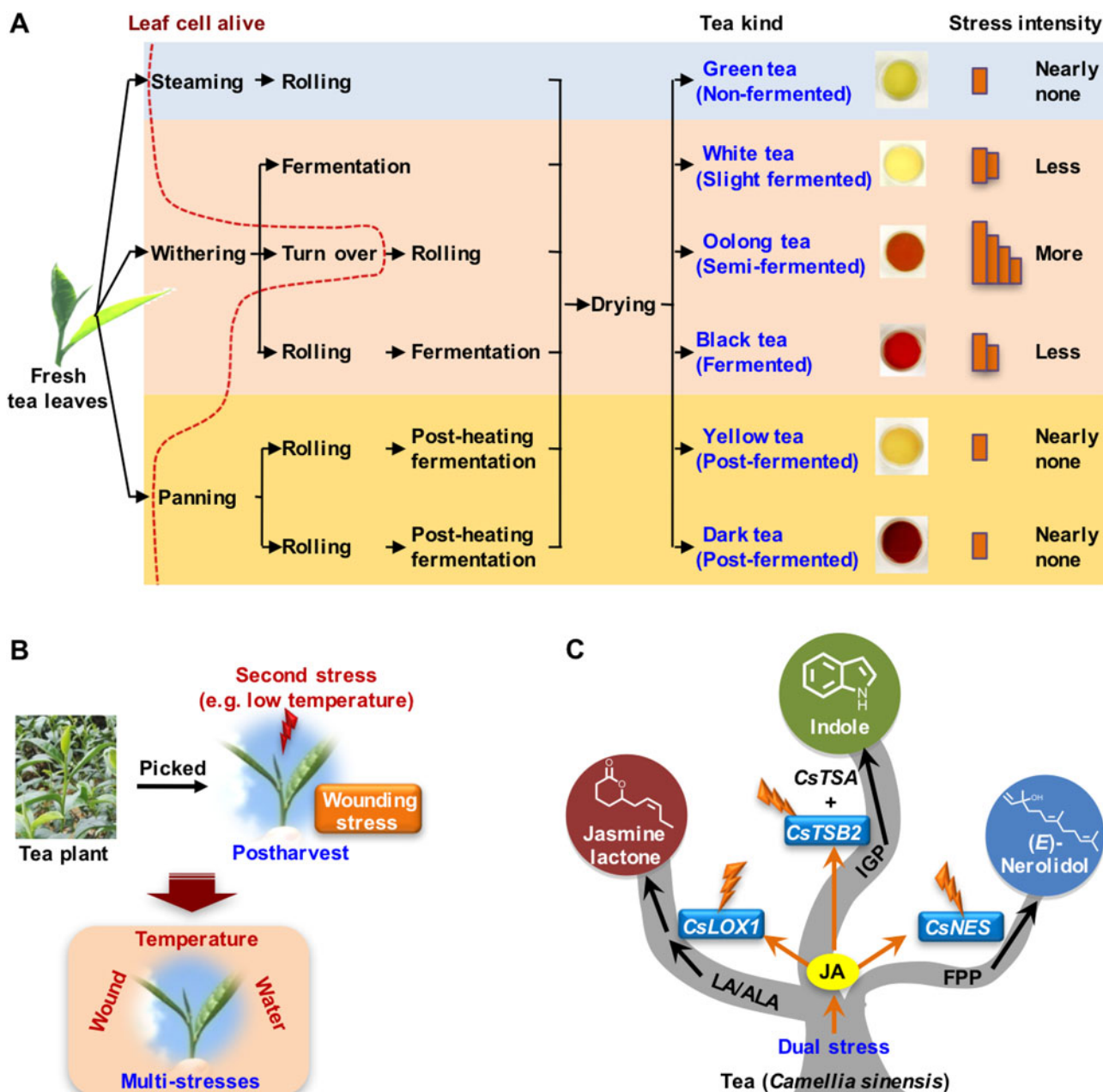

Figure 4. Stress involved in postharvest processing of six tea types. (A) Manufacturing process of six tea types and their related stress intensities. (B) Multi-stresses in postharvest processing. (C) Formation mechanism of characteristic floral aromas under dual stress of wounding and low temperature. CsTSA, tryptophan synthase α-subunit; CsTSB2, tryptophan synthase β-subunit 2. CsLOX1, lipoxygenase 1; CsNES, (E)-nerolidol synthase; CsMYC2, myelocytomatosis 2; LA, linoleic acid; ALA, α-linolenic acid; IGP, indole-3-glycerol phosphate; FPP, farnesyl pyrophosphate; JA, jasmonic acid.

2018). This interesting phenomenon indicated that dual stress (wounding and low temperature) not only regulated, but also had a synergistic effect on, volatile formation. Furthermore, these two stresses also had a synergistic effect on the formation of (E)-nerolidol and jasmine lactone, other odorant compounds (Fig. 4C) (Zeng et al. 2018; Zhou et al. 2017c). The underlying mechanism is the same as that induced by continuous mechanical damage, namely, triggering (E)-nerolidol and jasmine lactone formation by enhancing the expression level of CsNES and CsLOX1 (Zeng et al. 2018; Zhou et al. 2017c). Indole, (E)-nerolidol, and jasmine lactone are synthesized from three different metabolic pathways, namely, shikimate, terpenoid, and fatty acid deviated pathways, respectively. This suggests that tea leaves may have unknown transcription factors activated by dual stress that regulate genes involved in multiple biosynthetic pathways of tea aromas. Several studies have demonstrated that phytohormones regulate the biosynthesis of volatiles and act as important upstream signal chemicals (Dudareva et al. 2013; Kessler, Halitschke, and Poveda 2011; Underwood et al. 2005). According to our preliminary experiments, jasmonic acid might be a key upstream factor. These results contribute to identifying a unified mechanism of formation for characteristic aromas in response to multiple stresses in the tea manufacturing process. These studies indicate that low temperature should be introduced into the tea manufacturing process to improve the floral aroma quality of tea.

Formation of tea aromas from enzymatic reactions where the tea leaf cell is disrupted

In addition to enzymatic reactions when the leaf cell is alive, enzymatic reactions when the tea leaf cell is disrupted also contribute to tea aroma formation. These reactions mainly occur during the tea manufacturing process, especially the rolling process in black tea manufacture. When the tea leaf cell is disrupted, the concentration of RNA is reduced from several hundred to $20 \, ng/\mu L$ (Zeng et al. 2016), which might not lead to the activation of genes involved in tea aroma biosyntheses, but terminates tea aroma syntheses. For example, wounding stress was involved in the manufacturing processes of both oolong tea and black tea, but there was a marked difference in the indole amounts in these two kinds of tea. This was not due to tea leaf cell disruption in the rolling process (wounding stress) of black tea leading to indole conversion, but the termination of indole synthesis (Zeng et al. 2016). Although activation of tea aroma-related genes may not occur in disrupted tea leaf cells, the disruption of tea leaf cells increases the availability of interactions between substrates and enzymes, which are located in different subcellular organs. The most-studied example is the enzymatic hydrolysis of glycoside-bound aromas to free aroma compounds under the action of glycosidases.

As most glycoside-bound aromas in tea leaves are beta-type, the corresponding hydrolases are β-glycosidases (Yang, Baldermann, and Watanabe 2013). Furthermore, as β-primeveroside-bound aromas are major glycoside-bound aromas in tea leaves (Yang, Baldermann, and Watanabe 2013),

β-primeverosidase attracted initial research interest. Mizutani et al. (2002) first reported that β-primeverosidase was N-glycosylated and had an N-terminal signal sequence, and was, therefore, presumed to be located in the cell wall. Subsequently, Gui et al. provided direct evidence of the subcellular localization of β-primeverosidase, which was indeed located in the leaf cell wall (Gui et al. 2015). The glycoside-bound aromas are present within vacuoles. This compartmentation of glycoside-bound aromas (substrates) and β-primeverosidase (enzyme) in plant cells implies that interactions between the enzyme and substrate do not occur in intact tea leaves (Fig. 5A). In addition to β-primeverosidase, β-glucosidases are other hydrolyases in tea leaves involved in the transformation of β-glucoside-bound aromas into free aromas. Tea leaves only contain one β-primeverosidase gene, while many β-glucosidases are present. Recently, Zhou et al. (2017a) reported that the three β-glucosidases belonging to GH1, GH3, and GH5 families of plants, CsGH1BG1, CsGH3BG1, and CsGH5BG1, had functions in the hydrolysis of β-glucoside-bound aromas to free aromas via a yeast expression system, suggesting that there were no specific β-glucosidases for the hydrolysis of β-glucoside-bound aromas. Interestingly, the three β-glucosidases had different subcellular localizations in tea leaves. CsGH1BG1 and CsGH3BG1 were located in the cytosol and vacuole, respectively, while CsGH5BG1 was located in the cell wall (Fig. 5A) (Zhou et al. 2017a). This also suggested that CsGH3BG1 might be responsible for the hydrolysis of β-glucoside-bound aromas, even in intact tea leaves (Fig. 5A). As β-glucoside-bound aromas occur in tea leaves in relatively low amounts, the CsGH3BG1-catalyzed hydrolysis of β-glucoside-bound aromas might not contribute significantly to tea aroma formation.

No β-glycoside-bound aromas showed reduced contents during the enzyme-active process of oolong tea, namely from the plucking process to fixing process (enzymatic reaction termination), which has been validated in different tea cultivars (Cui et al. 2016; Gui et al. 2015; Wang et al. 2001a, 2001b). Gui et al. (2015) investigated the cell structures of tea leaves from the oolong tea manufacturing process. From plucking to the first turnover process, the leaf cells were complete and unwounded. The content was divided into several parts and showed a clear boundary. From the second turnover to the fifth turnover process, the cells had shrunk and distorted extensively, while the cell walls remained in good condition and unwounded (Gui et al. 2015). This suggested that only CsGH1BG1 located in the cytosol and CsGH3BG1 located in the vacuole might interact with β-glucoside-bound aromas to release free aromas, while β-primeverosidase and CsGH5BG1 located in the cell wall had no interaction with the β-glycoside-bound aromas (Fig. 5B). As β-primeveroside-bound aromas are major glycoside-bound aromas in tea leaves (Yang, Baldermann, and Watanabe 2013), the hydrolysis of β-glycoside-bound aromas did not contribute significantly to tea aroma formation during the enzyme-active process of oolong tea.

During the manufacturing process, when tea leaf tissues and cells are disrupted, interactions occur between
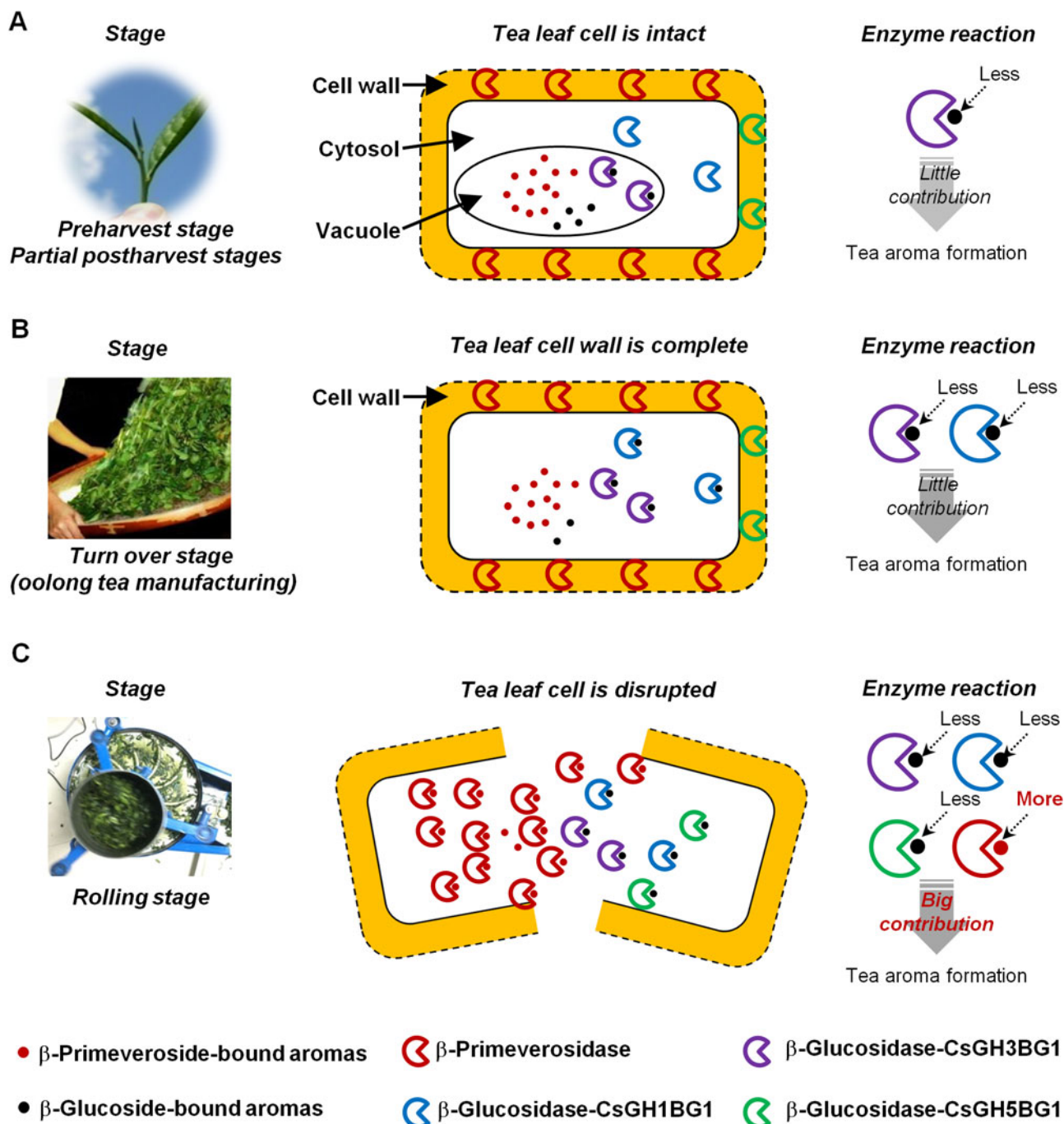
Figure 5. Effects of tea leaf cell conditions on enzymatic hydrolysis of glycoside-bound aromas under the action of β-glycosidases, including β-primeverosidase and β-glucosidases. (A) Enzyme reaction of β-glycosidases when tea leaf cell is intact. (B) Enzyme reaction of β-glycosidases when tea leaf cell wall is complete. (C) Enzyme reaction of β-glycosidases when tea leaf cell is disrupted.

β-glycosidase and the β-glycoside-bound aromas, and the free aromas are liberated. For example, during the black tea manufacturing process, levels of β-primeveroside-bound aromas decreased significantly after rolling, suggesting that enzymatic hydrolysis of β-glycoside-bound aromas mainly occurred during the rolling stage (Gui et al. 2015; Wang et al. 2001b). In this situation, all β-glycosidases, including β-primeverosidase and β-glucosidases, were able to interact with β-glycoside-bound aromas and release many free aromas (Fig. 5C). Among these, β-primeveroside-bound aromas as major glycoside-bound aromas in tea leaves were also hydrolyzed by β-primeverosidase. Therefore, the hydrolysis

of β-glycoside-bound aromas contributed to tea aroma formation during the enzyme-active process of black tea.

Concluding remarks and perspectives

In this review, we have summarized current knowledge of the biosynthesis of tea aromas and their stress-response mechanisms. The enzymes/genes involved in the final steps of formations of some characteristic tea aroma compounds have been functionally characterized using *E. coli* and yeast expression systems, and a model plant transgenic system. In preharvest

leaves, attack by a characteristic insect and light wavelength are major factors affecting tea aromas. In postharvest leaves, wounding and temperature stresses can effectively affect tea aromas. Therefore, utilizing stress-response mechanisms is a safe and effective approach to improving aroma compounds in tea leaves. However, the large variety of aroma compounds contributing to tea aroma, and their complex and diverse flavor properties, suggest that more remains to be learned. The simultaneous regulation of multiple aroma compounds is a significant challenge. Furthermore, high tea aroma quality is not solely determined by the high concentration of aroma compounds, but also by the reasonable design of compositions of different aroma compounds and their concentrations.

Several important questions should be addressed in future studies. Firstly, the basic understanding of tea aroma biosyntheses needs to be further improved. At the gene level, *in vivo* evidence of tea aroma biosyntheses is required and a tea genetic transformation system, even for transient systems, is urgently needed to allow characterization of functions of tea aroma synthetic genes. The recent successful isolation of protoplasts from tea leaves (Peng et al. 2018) will promote the establishment of transient overexpression or RNAi systems in tea protoplasts. Furthermore, the *Agrobacterium*-mediated transgenic hairy root induction of tea may be an alternative approach (Alagarsamy, Shamala, and Wei 2018). The third tentative approach is pollen magnetofection for genetic modification with magnetic nanoparticles as gene carriers (Zhao et al. 2017). This method can transform many crop species, such as cotton, that are difficult to regenerate, which is similar situation to tea plants. At the metabolite level, current studies are mostly focused on tissue levels. Understanding of metabolites at the cellular and subcellular levels will provide more precise regulation targets for tea aromas. Recently, matrix-assisted laser desorption/ionization-imaging mass spectrometry and desorption electrospray ionization-imaging mass spectrometry have been successfully applied to elucidate the cellular spatial distribution of metabolites in some medicinal plants (Heskes et al. 2018; Tocci et al. 2018). This technique may be suitable for elucidating cellular levels of metabolites involved in tea aroma formation. Furthermore, a nonaqueous fractionation method (Arrivault et al. 2014; Farré et al. 2001; Tohge et al. 2011) can be used to determine subcellular metabolic changes in non-model plant teas in response to biotic and abiotic stresses. At the upstream signal level, as volatile metabolites are highly sensitive to environmental stresses, epigenetic regulation might possibly be responsible for tea aroma formation. However, the related information is unknown.

Secondly, applications of stress-response based techniques to improve tea aroma quality need further reasonable design. The balance between tea aroma quality and yield or appearance needs be considered. For example, tea green leafhopper can enhance the tea aroma quality, but negatively affects the tea yield and appearance. Furthermore, due to large variations in the damage intensity of each insect, tea aroma quality is difficult to keep stable to obtain uniform quality. Therefore, studies on the underlying mechanism of tea green leafhopper-induced high tea aroma quality will provide essential information for the future manual simulation of insect stress to produce tea products with stable and uniform aroma quality, without affecting the yield and appearance. Studies on multiple stress effects and stress intensity control will contribute to future orientation and precise improvements in tea aroma composition, ratio, and concentrations.

Acknowledgements

Abbreviations

AAATs	aromatic amino acid aminotransferases
AP	acetophenone
CsNES	(*E*)-nerolidol synthase
CoA	coenzyme A
CsLIS	linalool synthase
CsHPL	hydroperoxide lyase
CsOS2	*β*-ocimene synthase 2
CsPESs	1-phenylethanol synthases
CsSAMT	salicylic acid carboxyl methyltransferase
CsTSA	tryptophan synthase *α*-subunit
CsTSB2	tryptophan synthase *β*-subunit 2
E. coli	*Escherichia coli*
E. (M.) onukii	*Empoasca (Matsumurasca) onukii* Matsuda
FPP	farnesyl diphosphate
GC	gas chromatography
GC–MS	gas chromatography–mass spectrometry
GPP	geranyl diphosphate
GBVs	glycosidically bound volatile compounds
GT	glycosyltransferase
HPL	hydroperoxide lyase
13-HPOT	13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)octadecatrienoic acid
IGP	indole-3-glycerol phosphate
LOX	lipoxygenases
MEP	methylerythritol phosphate
MVA	mevalonic acid
NMR	nuclear magnetic resonance
PAL	phenylalanine ammonialyase
PD	*β*-primeverosidase
1PE	1-phenylethanol
Phe	phenylalanine
PPA	phenylpyruvic acid
TPSs	terpene synthases
UV	ultra-violet
VFADs	volatile fatty acid derivatives
VPBs	volatile phenylpropanoids/benzenoids
VTs	volatile terpenoids

Author contributions

Yang Z. constructed the manuscript outline. Zeng L. and Yang Z. wrote the manuscript. Yang Z., Zeng L., and Watanabe, N. contributed to the tea aroma-related research aspects done by the authors. All authors reviewed the manuscript.

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