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Formation and emission of linalool in tea (*Camellia sinensis*) leaves infested by tea green leafhopper (*Empoasca* (*Matsumurasca*) *onukii* Matsuda)

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ABSTRACT

Famous oolong tea (Oriental Beauty), which is manufactured by tea leaves (*Camellia sinensis*) infected with tea green leafhoppers, contains characteristic volatile monoterpenes derived from linalool. This study aimed to determine the formation mechanism of linalool in tea exposed to tea green leafhopper attack. The tea green leafhopper responsible for inducing the production of characteristic volatiles was identified as *Empoasca* (*Matsumurasca*) *onukii* Matsuda. *E.* (*M.*) *onukii* attack significantly induced the emission of linalool from tea leaves ($p < 0.05$) as a result of the up-regulation of the *linalool synthases* (*CsLIS1* and *CsLIS2*) ($p < 0.05$). Continuous mechanical damage significantly enhanced *CsLIS1* and *CsLIS2* expression levels and linalool emission ($p < 0.05$). Therefore, continuous wounding was a key factor causing the formation and emission of linalool from tea leaves exposed to *E.* (*M.*) *onukii* attack. This information should prove helpful for the future use of stress responses of plant secondary metabolism to improve quality components of agricultural products.

1. Introduction

In response to environmental stresses, plants, especially vegetative parts, synthesize and emit volatile compounds. These stress-induced volatiles function in multiple ways to reduce the negative effects of stresses on plants (Dong, Fu, Watanabe, Su, & Yang, 2016; Pichersky & Gershenzon, 2002). Additionally, these stress-induced volatiles can be used as quality components of agricultural plants. As an example, tea (*Camellia sinensis*) aroma (volatile compounds) is an essential component in the evaluation of sensory scores and commercial description of tea, and the formation of tea aroma can be induced by the various stresses from the preharvest or postharvest processes (Yang, Baldermann, & Watanabe, 2013). During the postharvest process (i.e. tea manufacturing process), mechanical damage and low-temperature stresses led to the accumulation of indole, jasmine lactone, *trans*-nerolidol, and volatiles derived from fatty acids (Gui et al., 2015; Katsuno et al., 2014; Zeng et al., 2016). During the preharvest process (i.e. tea plant growth process), shading treatment increased volatile phenylpropanoids and benzenoids (Yang et al., 2012). Furthermore, blue light and red light increased most volatiles including volatile fatty acid derivatives, phenylpropanoids, benzenoids, and terpenes (Fu et al., 2015). Besides abiotic stresses, biotic stresses such as insect attacks significantly affected volatiles emitted from tea plants (Dong et al., 2011). As a classical example of the utilization of insect attacks to induce the formation of tea aroma, a famous oolong tea (Oriental Beauty) is manufactured using the tea leaves infected by tea green leafhoppers with a piercing-sucking mouthpart. This special process endows the oolong tea with characteristic volatile monoterpenes (Cho et al., 2007), which are derived from linalool. However, the effect of tea green leafhopper attack on the formation and emission of linalool is unknown.

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The formation and emission of linalool in some plants, especially flowers and fruits, have been characterized (Lücker et al., 2001; Nagegowda, Gutensohn, Wilkerson, & Dudareva, 2008). Linalool is derived from geraniol diphosphate (GPP) via the action of terpene synthases (TPS) (Negre-Zakharov, Long, & Dudareva, 2009). Moreover, linalool can be converted to linalool glycosides by the action of glycosyltransferases (Nagegowda et al., 2008). Furthermore, environmental stresses such as insect attack can induce accumulation and emission of linalool from plants (Dong et al., 2011; Kessler & Baldwin, 2001; Martin, Gershenzon, & Bohlmann, 2003). As the genetic transformation system of tea is not successfully established yet, it is not easy to characterize the functions of the enzymes involved in the biosynthesis of metabolites in tea plants. Two aroma-related enzymes have been isolated, identified, and functionally characterized in tea leaves. One is β-primeverosidase (β-Pri), which hydrolyzes β-primeverosidically bound volatiles to free volatiles (Mizutani et al., 2002; Zhou et al., 2014) and the other is the glycosyl transferases that are involved in the transformation of free volatiles into glycosidically bound volatiles (Ohgami et al., 2015). Currently, our knowledge of the formation and emission of volatiles in tea, especially under stresses, is mainly derived from the findings reported for other plant species. Because of the complexity of the networks involved in plant volatile formation and emission, there may be variations among species, although some of the networks are shared. Therefore, direct investigation of volatile synthesis and emission in tea plants is required (Yang et al., 2013). In the present study, we characterized the functions of linalool synthases (CsLISs) in tea leaves, and elucidated the effect of tea green leafhopper (*Empoasca* (*Matsumurasca*) *onukii* Matsuda) attack on the formation and emission of linalool in tea leaves, including internal linalool, emitted linalool, their key precursors, GPP and linalool glycosides, and the genes involved such as *CsLISs*, *glycosyltransferases*, and *β-Pri*. Furthermore, an automatic machine was employed to simulate continuous mechanical damage from *E.* (*M.*) *onukii* attack on tea leaves to probe whether it is a key factor involved in the formation and emission of linalool in response to *E.* (*M.*) *onukii* attack. The aim of this study was to discover the formation and emission mechanism of linalool in tea leaves exposed to *E.* (*M.*) *onukii* attack.

2. Materials and methods

2.1. Chemical compounds

Ethyl *n*-decanoate was purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. (±)-Linalool, GPP ammonium salt and isopropyl β-D-1-thiogalactopyranoside were purchased from Sigma-Aldrich, St. Louis, MO, USA. Amberlite XAD-2 column and polyvinylpolypyrrolidone (PVPP) were purchased from Sigma-Aldrich Co. Ltd., USA. Quick RNA Isolation Kit was purchased from Huayueyang Biotechnology Co., Ltd., China. β-Pri was purchased from Amano Enzyme Inc., Japan. β-Glucosidase (Almond) was purchased from USBiological, MA, USA. Ni Sepharose 6 Fast Flow and PD-10 desalting column were purchased from GE Healthcare, Waukesha, WI, USA. 2× SYBR Green Universal PCR Mastermix, coomassie blue R250, 30% acrylamide/bis solution, and iTaqTM Universal SYBR® Green Supermix were purchased from Bio-Rad Laboratories, Hercules, CA, USA. Dichloromethane, methanol, pentane, and chemicals of extraction buffer Tris-HCl were purchased from Quanshuo Co., Ltd, Guangzhou, China.

2.2. Plant materials and treatments

Tea (*C. sinensis* cv. Jinxuan) leaves and tea green leafhoppers were obtained from Yingde Tea Experimental Station of Tea Research Institute, Guangdong Academy of Agricultural Sciences (Yingde city, China). The tea green leafhoppers were identified as *Empoasca* (*Matsumurasca*) *onukii* Matsuda. In field experiments, the tea leaves (one bud and three leaves, which was one bud with three leaves on the same branch and generally used for tea manufacturing as a whole) infested by *E.* (*M.*) *onukii*, which appearance showed internode shortening, yellow and curling leaves (Cho et al., 2007), were obtained. The control was the intact tea leaves (one bud and three leaves) without any insect attacks. In the laboratory control experiments, the following two treatments were used: (1) insect treatment: fifteen shoots of intact tea leaves (one bud and three leaves) were treated by 30 insects (*E.* (*M.*) *onukii*) for 3 days. The control was intact tea leaves without treatment. (2) Mechanical damage treatments: intact tea leaves were treated by continuous pricking damage (one pricking/6 s, 3.5 h) performed by an automatic machine, which was manufactured in our lab. Single damage on intact tea leaves was performed by pricking damage at the beginning and then stood for 3.5 h. The control was intact tea leaves without treatment. The laboratory control experiments were carried out under room conditions of 25 °C and 70% humidity.

2.3. Collection and analyses of emitted linalool from tea samples

After treatments, emitted linalool from tea samples was collected by solid-phase microextraction (SPME, 2 cm-50/30 μm DVB/CarboxenTM/PDMS Stable FlexTM) at 25 °C for 1 h. The SPME was then analyzed by gas chromatography-mass spectrometry (GC–MS) QP2010 SE (Shimadzu Corporation, Japan) equipped with a SUPELCOWAX™ 10 column (30 m × 0.25 mm × 0.25 μm, Supelco Inc., Bellefonte, PA, USA). The injector temperature was 230 °C, splitless mode was used with a splitless time of 1 min, and helium was the carrier gas with a velocity 1.0 mL/min. The GC temperature started at 60 °C for 3 min, ramp of 4 °C/min to 150 °C, followed by 30 °C/min to 240 °C, and then 240 °C for 15 min. MS was performed in full scan mode (mass range *m*/*z* 40–200) (Fu et al., 2015).

2.4. Extraction and analyses of internal linalool of tea samples

To extract internal volatiles that are stored in tea leaves, finely powdered tea sample (1 g) was extracted with 3 mL dichloromethane containing 5 nmol ethyl *n*-decanoate as an internal standard for 8 h. The extract was dried over anhydrous sodium sulfate. Then, 1 μL of the extract was subjected to a GC–MS analysis as described above (Fu et al., 2015).

2.5. Extraction and analyses of glycosidically conjugated linalool of tea samples

The methods were described by our previous studies (Dong et al., 2012; Gui et al., 2015; Zhou et al., 2014). Fresh tea leaves (500 mg) were finely powdered and extracted with 2 mL of cold methanol by vortexing for 2 min and then ultrasonic extraction for 10 min. For phase separation, the methanol extracts were mixed with 2 mL of cold chloroform and 0.8 mL of cold water. The resultant upper layer was dried and dissolved in 1 mL of water. The resulting solution was mixed with 30 mg of PVPP, left standing for 90 min, centrifuged (16400×*g*, 4 °C, 10 min), and then repeated with the supernatant. The final supernatant was loaded to an Amberlite XAD-2 column (1 mL) and eluted with 5 mL of water, 5 mL of pentane:dichloromethane (2:1), and 5 mL of methanol, successively. The obtained methanol eluent was dried under nitrogen gas, and redissolved in 400 μL of 50 mM citric acid buffer (pH 6.0) containing β-Pri (1.12 unit) and β-glucosidase (from almonds, 3.12 unit). The solution was reacted at 37 °C for 16 h. Afterward, 144 mg of sodium chloride was added to the reaction solution, and left for 15 min followed by addition of 5 nmol of ethyl decan-