RESPONSE MECHANISM OF TEA LEAVES TO BLISTER BLIGHT AND PROTECTIVE EFFECT OF EXOGENOUS SALICYLIC ACID AND SAPONIN AGAINST *Exobasidium vexans*

XIAOLU ZHOU

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กลไกการตอบสนองของใบชาต่อโรคใบพุพองและประสิทธิภาพของกรดซาลิไซลิกและซาโปนินต่อการยับยั้งเชื้อรา *Exobasidium vexans*

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปรัชญาดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee

(Asst. Prof. Dr. Tiyakhon Chatnaparat)
Chairperson

(Asst. Prof. Dr. Kumrai Buensanteai)
Member (Thesis Advisor)

(Prof. Dr. Xin Mei)
Member (Thesis Co-advisor)

(Dr. Toan Le Thanh)
Member (Thesis Co-advisor)

(Dr. Wanploy Jinagool)
Member

(Assoc. Prof. Dr. Yupaporn Ruksakulpiwat)
Vice Rector for Academic Affaire and Quality Assurance

(Prof. Dr. Neung Teaumroong)
Dean of Institute of Agricultural Technology

เสี่ยวลู่ โจว : กลไกการตอบสนองของใบชาต่อโรคใบพุพองและประสิทธิภาพของกรดซาลิไซลิกและซาโปนินต่อการยับยั้งเชื้อรา *Exobasidium vexans* (RESPONSE MECHANISM OF TEA LEAVES TO BLISTER BLIGHT AND PROTECTIVE EFFECT OF EXOGENOUS SALICYLIC ACID AND SAPONIN AGAINST *Exobasidium vexans*) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.กำไร เบือนสันเทียะ, 217 หน้า

โรคใบพุพอง/*Camellia sinensis*/*Exobasidium vexans*/เอนไซม์ป้องกัน/ปฏิกิริยาระหว่างพืชกับเชื้อโรค/ซาโปนิน

โรคใบพุพองเกิดจากเชื้อรา *Exobasidium vexans* สาเหตุของโรคทางใบที่เข้าทำลายซึ่งส่งผลเสียต่อผลผลิตและคุณภาพของชาอย่างมาก การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจสอบกลไกการตอบสนองของชาต่อการติดเชื้อ *E. vexans* และควบคุมโรคนี้เพิ่มเติมโดยใช้กรดซาลิไซลิกและสารซาโปนินจากชา การใช้โครมาโทกราฟีของเหลวสมรรถนะสูงแบบผสมผสาน (UPLC-MS/MS) และการวิเคราะห์ทางชีวเคมีเพื่อตรวจสอบการเปลี่ยนแปลงของสารเมตาบอไลต์ องค์ประกอบทางเคมี และฤทธิ์ของสารต้านอนุมูลอิสระในใบชาที่มีสุขภาพดี และใบชาที่ติดเชื้อรา *E. vexans* ที่มีลักษณะอาการของโรคใบพุพอง ผลการวิจัยพบว่าพบมีสารประกอบทั้งหมด 1,166 ชนิด โดยถูกตรวจพบภายหลังการติดเชื้อสาเหตุโรค โดยมีสารประกอบทั่วไปที่แตกต่างอย่างมีนัยสำคัญสะสมอยู่จำนวน 73 ชนิด ได้แก่ ฟลาโวนอยด์, กรดฟีนอลิก รวมถึง kaempferol (3,5,7,4' - tetrahydroxyflavone), kaempferol-3-*O*-sophoroside-7-*O*-glucoside, โฟลเรติน, กรด 2,4,6-trihydroxybenzoic, galloylprocyanidin B4 และ procyanidin C1 3'-*O*-gallate ซึ่งบ่งชี้ว่าสารเมตาบอไลต์เหล่านี้อาจมีอิทธิพลในทางบวกที่สามารถต้านทานต่อเชื้อรา *E. vexans* ยิ่งไปกว่านั้นพบว่าการสังเคราะห์ ฟลาโวนและฟลาโวนอลมีความสัมพันธ์กับการต้านทานต่อเชื้อรา *E. vexans* อีกด้วย และยังพบว่าปริมาณฟลาโวนอยด์รวม, ฟีนอลิก, อัลคาลอยด์ และเทอร์พีนอยด์ มีการสะสมอย่างมีนัยสำคัญในระยะที่ 2 ของการติดเชื้อ และปริมาณของ DPPH อาจสัมพันธ์กับฟลาโวนอยด์มากที่สุด ในขณะที่ FRAP และ ABTS อาจมีความสัมพันธ์เชิงบวกกับเทอร์พีนอยด์, อัลคาลอยด์ และฟีนอลิก จากนั้นทำการตรวจสอบทรานสคริปโตมิกส์และเมแทบอลิซึมของฮอร์โมนที่มีเป้าหมายอย่างกว้างขวาง เพื่อตรวจสอบความแตกต่างในการแสดงออกของยีนและเครือข่ายการส่งสัญญาณฮอร์โมนพืชของใบชาที่มีสุขภาพดี และใบชาที่ติดเชื้อรา ผลการวิจัยพบว่ายีนต้านทานโรคส่วนใหญ่ถูกกระตุ้นเพื่อตอบสนองต่อการติดเชื้อของเชื้อสาเหตุโรค โดยเฉพาะอย่างยิ่งยีนที่เกี่ยวข้องกับการส่งสัญญาณฮอร์โมนพืชและปฏิสัมพันธ์ระหว่างเชื้อโรคกับพืช ส่วนใหญ่ของยีนที่เกี่ยวข้องกับกลไกการป้องกันตัวเองของพืช (เช่น CDPK, RBOH, CaM/CMLs, FRK1 และ PR1), ทรานสคริปชันแฟคเตอร์ (WRKY, MYB, bHLH และ AP2/ERF) และโมเลกุลส่งสัญญาณของพืชที่เกี่ยวข้องกับ SA, กรดแจสโม

นิก (JA), เอทิลีน (ETH) และบราสเซียนสเตียรอยด์ (BR) ที่แสดงออกในใบชาหลังการติดเชื้อรา *E. vexans* นอกจากนี้ฮอร์โมนพืช โดยเฉพาะ SA, กรดแจสโมนิก และสตริโกแลกโทน มีการสะสมอยู่ในใบชาที่ติดเชื้อรา *E. vexans* โดยเส้นทางการส่งสัญญาณของ JA อาจทำงานร่วมกันกับเส้นทางการส่งสัญญาณของ BR, ETH, auxin (AUX) และจิบเบอเรลลิน (GA) เพื่อปรับปรุงการแสดงออกของยีนที่เกี่ยวข้องกับการป้องกันตัวเองในต้นชาหลังจากการติดเชื้อ *E. vexans* ที่ทำงานเป็นปฏิปักษ์กับวิถีการส่งสัญญาณของไซโตไคนิน (CK) การฉีดพ่นทางใบด้วย SA, ซาโปนิน (TS) และการผสมร่วมกันของสารทั้งสองที่ความเข้มข้นที่เหมาะสม เพื่อควบคุมโรคใบพุพอง การใช้ร่วมกันของ SA และ TS ได้เพิ่มประสิทธิภาพในการควบคุมโรคใบพุพองได้ถึง 49.89% ภายใต้การทดลองภาคสนาม ซึ่งสูงกว่าการใช้ SA และ TS เพียงอย่างเดียวที่ 19.06% และ 30.25% ตามลำดับ การเพิ่มขึ้นอย่างมีนัยสำคัญในการทำงานของเอนไซม์ป้องกันตัวเอง เช่น โพลีฟีนอลออกซิเดส, คาตาเลส, ฟีนิลอะลานีนอะมิโนเลส, เปอร์ออกซิเดส, ซูเปอร์ออกไซด์ดิสมิวเตส, β-1,3-กลูคาเนส และไคติเนส ถูกพบในต้นชาที่ได้รับการรักษาด้วยการใช้ร่วมกันของ SA และ TS เมื่อเทียบกับพืชที่ไม่ได้รับการรักษา ดังนั้น การศึกษานี้จึงให้ข้อมูลเชิงลึกที่ครอบคลุมเกี่ยวกับกลไกการตอบสนองของชาต่อโรคใบพุพองในระดับกระบวนการลอกรหัสและเมแทบอลิซึมซึ่งอาจส่งเสริมการเพาะพันธุ์ชาพันธุ์ต้านทาน นอกจากนี้ การใช้ SA และ TS ภายนอกยังเป็นกลยุทธ์สำคัญในการควบคุมโรคใบพุพอง

สาขาวิชาเทคโนโลยีการผลิตพืช ลายมือชื่อนักศึกษา Xiaohe Zhou

ปี การศึกษา 2566 ลายมือชื่ออาจารย์ที่ปรึกษา .....

XIAOLU ZHOU : RESPONSE MECHANISM OF TEA LEAVES TO BLISTER BLIGHT AND PROTECTIVE EFFECT OF EXOGENOUS SALICYLIC ACID AND SAPONIN AGAINST *Exobasidium vexans*. THESIS ADVISOR : ASST. PROF. DR. KUMRAI BUENSANTEAI, 217 PP.

BLISTER BLIGHT DISEASE/*Camellia sinensis*/*Exobasidium vexans*/DEFENSE ENZYME/ PLANT-PATHOGEN INTERACTION/TEA SAPONIN

Blister blight caused by obligate biotrophic pathogen *Exobasidium vexans* is a destructive foliar disease affecting quality and yield of tea. The aim of this study was to investigate the response mechanism of tea plants against *E. vexans* infection and further control this disease using exogenous salicylic acid (SA) and tea saponin (TS). Firstly, ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)-based metabolomics and biochemical analysis were employed to investigate the changes in metabolites, chemical components, and antioxidant activities in healthy and *E. vexans*-infected leaves as blister symptoms developed. Results showed that 1,166 compounds were identified following *E. vexans* infection, among which 73 common compounds were significantly accumulated including flavonoids and phenolic acids, e.g., kaempferol (3,5,7,4'-tetrahydroxyflavone), kaempferol-3-*O*-sophoroside-7-*O*-glucoside, phloretin, 2,4,6-trihydroxybenzoic acid, galloylprocyanidin B4, and procyanidin C1 3'-*O*-gallate, indicating that these metabolites might positively dominate resistance to *E. vexans*. Furthermore, flavone and flavonol biosynthesis was found to be closely related to resistance to *E. vexans*. Additionally, total flavonoids, phenolics, alkaloids, and terpenoids were significantly accumulated in the second stage of infection. The value of DPPH might be most correlated with flavonoids; whereas FRAP and ABTS might correlate positively with terpenoids, alkaloids, and phenolics. Subsequently, transcriptomics and widely-targeted hormone metabolomics were performed to investigate the differences in gene expression and phytohormone signaling networks of healthy and infected leaves. The results showed that most disease-resistant genes were activated in response to pathogen infection, particularly those involved in plant hormone signal transduction and plant-pathogen

interaction. The majority of defense-related genes (e.g., CDPK, RBOH, CaM/CMLs, FRK1, and PR1), transcription factors (WRKY, MYB, bHLH, and AP2/ERF), and phytohormone signaling molecules involved in SA, jasmonic acid (JA), ethylene (ETH), and brassionsteroid (BR) were highly expressed in tea leaves following *E. vexans* infection. Additionally, phytohormones, especially SAs, jasmonates (JAs), and strigolactones (SLs), were accumulated in *E. vexans*-infected leaves. Furthermore, the JA signaling pathway might work synergistically with BR, ETH, auxin (AUX), and gibberellin (GA) signaling pathways to enhance the expression of defense-related genes in tea plants following *E. vexans* infection, whereas it functions antagonistically with the cytokinin (CK)-mediated pathway. Finally, foliar spraying of SA, TS, and their combination were employed to control blister blight. The combined use of SA and TS improved the control effect by 49.89% under field trials, which were 19.06% and 30.25%, respectively, higher than using SA and TS alone. A significant increase in defense enzymes activity like polyphenol oxidase, catalase, phenylalanine aminolase, peroxidase, superoxide dismutase, β-1,3-glucanase, and chitinase was observed in tea plants treated with the combination of SA and TS compared to those untreated. Accordingly, this study provides comprehensive insights into the response mechanisms of tea against blister blight at the transcriptional and metabolic levels, which may advance the breeding of resistant tea cultivars. In addition, application of exogenous SA and TS provides an important strategy for controlling blister blight.

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Student's Signature Xiaolu Zhou
Advisor's Signature

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LIST OF ABBREVIATIONS

ABA	=	Abscisic acid
ANOVA	=	Analysis of variance
BAK1	=	BRI1-associated receptor kinase 1
BR	=	brassinosteroid
BKI1	=	BRI1 kinase inhibitor 1
BRI1	=	Brassinosteroid insensitive 1
BSK	=	Brassinosteroid-signaling kinase
BZR1/2	=	Brassinozole-resistant l/2
$CaCl_2$	=	Calcium chloride
CaM	=	Crassulacean acid metabolism
CAT	=	Catalase
CMLs	=	Calmodulin-like proteins
DR	=	Disease reduction
DS	=	Disease severity
ERF1/2	=	Ethylene response factor 1/2
ETH	=	Ethylene
ETI	=	Effective-triggered immunity
ETR	=	Ethylene receptor
GA	=	Gibberellin
GI	=	Geographical indication
GID1	=	Gibberellin insensitive dwarf 1
H2JA	=	Dihydrojasmonic acid
H_2O_2	=	Hydrogen peroxide
HR	=	Hypersensitive response
ISR	=	Induced systemic resistance
JA	=	Jasmonic acid
JA-ILE	=	Jasmonoyl-L-isoleucine

LIST OF ABBREVIATIONS (Continued)

JA-Phe	=	5N-[(-)-jasmonoyl]-(l)-phenalanine
JA-Val	=	N-[(-)-jasmonoyl]-(L)-valine
JAZ	=	Jasmonate ZIM-domain
LIT	=	Linear ion trap
MEJA	=	Methyl jasmonate
NOS	=	Nitric oxide synthase
OPDA	=	Cis (+)-12-oxophytodienoic acid
PAL	=	Phenylalanine ammonia-lyase
PAMP	=	Pathogen-associated molecular patterns
PCA	=	Principal component analysis
PCD	=	Programmed cell death
PDI	=	Percentage disease index
PGPR	=	Plant growth-promoting rhizobacteria
POD	=	Peroxidase
PPO	=	Polyphenol oxidase
PR	=	Pathogenesis-related
PTI	=	Pattern-triggered immunity
RCBD	=	Randomized complete block design
ROS	=	Reactive oxygen species
SA	=	Salicylic acid
SAR	=	Systemic acquired resistance
SOD	=	Superoxide dismutase
SL	=	Strigolactone
TF	=	Transcription factor
TS	=	Tea saponin
UPLC-MS	=	Ultra-performance liquid chromatography tandem mass spectrometry

CHAPTER I
INTRODUCTION

1.1 Background

Tea [*Camellia sinensis* (L.) O. Kuntze] is a perennial dicotyledonous plant classified within the family Theaceae, which is widely cultivated in plantations across Asia, Africa, and South America. The fresh shoots and tender leaves, an economically essential component of tea, are processed to produce one of the most popular non-alcoholic beverages worldwide with various beneficial values (Liu et al., 2019; Fatemeh et al., 2021). However, tea plants are commonly challenged by various biotic and abiotic factors that significantly impact their growth and cultivation. Among the numerous diseases that afflict tea plants, fungal diseases account for a significant portion (Pandey et al., 2021). Tea plants thrive in warm and moist conditions, providing an optimal environment for the growth and reproduction of fungi. Given that the economic value of tea plants lies primarily in the leaves, fungal diseases in the foliage directly lead to a decrease in the quantity and quality of tea leaves. Among these, blister blight, which is caused by an obligate parasitic fungus *Exobasidium vexans* Massee, is one of the most destructive foliar fungal diseases in tea plants (Sen et al., 2020; Liu et al., 2021). This disease typically affects tea buds and young leaves, posing threats to both the yield and quality of tea (Baby et al., 2004). Chemical management strategies are commonly employed for controlling blister blight, yet these chemical fungicides frequently pose adverse issues concerning food safety and environmental pollution (Karunarathna et al., 2020). Recently, several strategies such as adoption of proper cultivation techniques, the introduction of resistant cultivars (Ran et al., 2021), application of bio-agents (Saravanakumar et al., 2006), and employment of plant-derived defense enhancers (Yang et al., 2023), have alleviated the damage caused by blister blight on tea plant.

However, there is still a lack of comprehensive scientific understanding regarding the immune mechanisms of tea plants in response to blister blight, which poses certain challenges to its prevention and control efforts. Therefore, this study focused on the molecular mechanisms and regulatory networks of tea leaves defense against blister blight at the transcriptional and metabolic levels, exploring the interaction between pathogens and host tea plants. Additionally, tea saponin (TS), a terpenoid compound derived from tea plants, was employed as a synergist in combination with salicylic acid (SA) to enhance the control effect of blister blight. This research will have significant scientific and practical implications for addressing the actual challenges faced by tea production.

1.2 Research objectives

1. To investigate the changes in metabolites and chemical components in tea leaves as blister symptoms develop.

2. To identify the response mechanism of tea leaves against blister blight infection at the transcriptional and phytohormone levels.

3. To evaluate the resistance of tea leaves induced by combination of SA and TS against blister blight.

1.3 Research significance

Blister blight, caused by an obligate parasitic fungus *E. vexans*, is a serious foliar disease. It is widely prevalent in tea cultivation of India (Singh et al., 2020), China (Zhang et al., 2022), Japan, Malaysia (Mohktar and Nagao, 2019), and Sri Lanka (Punyasiri et al., 2005; Sinniah et al., 2016). This disease mainly damages the new shoots and tender leaves of tea plants, resulting in 20%-50% yield loss. Additionally, blister blight invasion can disrupt the metabolic balance within the tea plants, which significantly impacts the tea quality. Fuding Dabaicha is a designated standard control variety for regional tea experiments in China, also being one of the earliest approved asexual tea varieties at the national level (Wang et al., 2019). Around 80%

of tea varieties in the tea plantations of Guizhou is Fuding Dabaicha. Whereas, blister blight frequently infects this variety, significantly affecting the utilization rate of fresh tea leaves and the comprehensive value. Therefore, this study explores the mechanisms by which tea leaves respond to blister blight infection at the transcriptional and metabolic levels. This could be exploited in breeding programs of screening for tea germplasm. Meanwhile, exogenous SA in combination with TS could serve as a potential enhancer in integrated management strategies in tea cultivation.

1.4 Research scopes and limitation

This study focuses primarily on the immune mechanism of tea leaves in response to blister blight and control effects of enhancers on pathogens. Firstly, the samples of Fuding Dabaicha were collected from the tea plantations of Maojian town (Duyun, China) in August 2021. Then, the healthy and infected leaves were used to perform the metabolites, chemical components, and antioxidant activity using UPLC-MS/MS based widely targeted metabolome and biochemical analysis. Secondly, the same samples as the metabolome analysis were used to perform transcriptomics and targeted hormone metabonomics. Finally, foliar spraying of SA in combination with TS was used to evaluate the control effects of tea leaves against blister blight.

However, the limitation of this study is that there is currently no suitable method for natural isolation and identification of *E. vexans* pathogen for inoculation.

1.5 References

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CHAPTER II

LITERATURE REVIEW

2.1 Cultivation and germplasm resources of tea plants

Tea [*Camellia sinensis* (L.) O. Kuntze] is a perennial crop cultivated in Asia, Africa, and South America (**Figure 2.1**), with an economic lifespan of 50 to 60 years, the leaves and buds are typically processed into a beverage consumed by people worldwide. Tea plants belonging to the genus *Camellia* in the family *Theaceae* are divided into 12 morphological species: *C. sinensis*, *C. taliensis*, *C. costata*, *C. sealyama*, *C. ptilophylla*, *C. kwangsiensis*, *C. leptophylla*, *C. grandibracteata*, *C. crassicolumna*, *C. gymnogyna*, *C. fengchengensis*, and *C. tachangensis*. Tea plants have been described by taxonomists as three distinct taxa based on their heterogeneous origins: *C. sinensis* var. *sinensis* (L.) O. Kuntze (China-type small-leaf variety), *C. sinensis* var. *assamica* (Masters) Wight (Assam-type large-leaf variety), and *C. sinensis* var. *lasiocalyx* (Planex ex Watt) Wight (Cambod-type variety) (Pandey et al., 2021). The economies of numerous nations within the latitudinal range of 41°N to 16°S are significantly reliant on tea production.

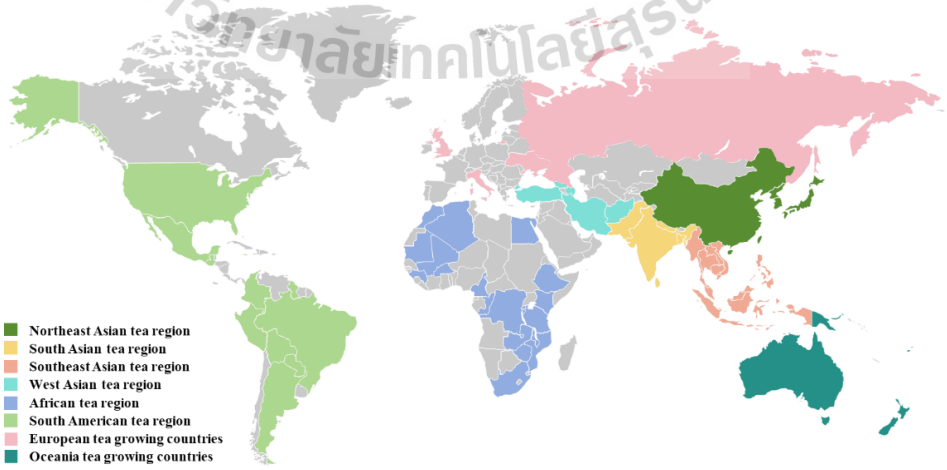

Figure 2.1 Distribution of tea regions in the world.

The quality of an excellent tea cultivar is characterized by its suitability for manufacture, including the physical and chemical properties of leaves. Several excellent *Camellia* germplasm resources, such as 'Fuding Dabaicha' (*C. sinensis* var. *sinensis*), 'Zijuan' (*C. sinensis* var. *assamica*), 'Anji Baicha', 'Tie Guanyin', and 'Wu Niuzao', have been identified from most of tea plantations with the unique geographical and ecological environments in China (Xu et al., 2017; Huang et al., 2022; Li et al., 2023). Fuding Dabaicha, originated from Fuding City, Fujian Province, was introduced to Zhejiang, Anhui, and Guizhou provinces in 1965. Fuding Dabaicha was approved as a geographical indication (GI) product by General Administration of Quality Supervision, Inspection and Quarantine of China in June 2004. 'Fuding Dabaicha' variety contains the rich concentration of amino acids (4.3%), polyphenols (16.2%), catechins (11.4%), and caffeine (4.4%), tea shoots have been generally used to manufacture white tea including 'Baihao Yinzhen', 'White Peony', 'Gong Mei', and 'Shou Mei'. Besides that, 'Fuding Dabaicha' tea leaves are suitable for processing into green, oolong, and black teas, which is primarily attributed to a great deal of good quality characteristics (Fang et al., 2019; Li et al., 2021; Hao et al., 2023). Additionally, this variety serves as a positive role in physiological activities such as antioxidants, anti-inflammatory, anti-obesity, and disease resistance owe to the abundant beneficial bioactive ingredients including flavonoids, alkaloids, terpenoids, and phenolic acids (Yi et al., 2020; Zhou et al., 2021).

2.2 Major fungal diseases of tea plants

Biotic factors involved in diseases and pests seriously damage the growth and performance of tea plants, with fungal diseases being the most prominent. Fungal pathogens probably present in the foliage, stems, and roots of tea plants. In general, foliar diseases directly affect the harvestable leaves and shoots, while stem and root diseases pose a threat to the survival of tea plants.

2.2.1 Foliar diseases

Tea plant, a perennial species, is susceptible to a variety of foliar diseases that impose significant adverse effects on both the yield and quality of tea. Foliar diseases occur during different periods of the tea growing season because of variations in the biological characteristics of the pathogens. The most impactful fungal diseases that damage tender leaves and new shoots of tea plants include blister blight (*Exobasidium vexans* Massee) (Chaliha et al., 2020), white scab (*Phyllosticta theaefolia* Hara) (Zhou et al., 2020), bud blight (*Phyllosticta gemmiphliae* Chen et Hu) (Dong, 2018), and gray blight (*Pestalotiopsis theae* (Sawada) Steyaert) (Maharachchikumbura et al., 2013) (**Figure 2.2A-D**). Among these, blister blight and white scab pose significant threats to tea production and quality, particularly in southern China and high mountain tea areas. Predominant diseases affecting mature and older leaves include brown blight [*Guignardia camelliae* (Cooke) Butler] and anthracnose (*Colletotrichums gloeosporioidesis*) (**Figure 2.2E-F**). Most of these diseases are caused by fungi, which overwinter in diseased leaves on trees or fallen leaves on the soil surface through mycelium, conidial discs, or ascomycete shells. After winter, conidia or basidiospores develop in the following spring under moist conditions and disperse through rain and dew. The incidence of blister blight, white scab, and bud blight is promoted by moderate temperature, high humidity, and prolonged leaf wetness. Additionally, the prevalence of blister blight is closely associated with sunshine exposure, which can occur for less than 4 h over 5 consecutive days. Generally, the peak period of tea white scab occurs during spring and autumn (May, June, and September), while bud blight usually occurs in spring (April and May). Nevertheless, brown blight and anthracnose exhibit a preference for high temperature and high humidity (Liu and Tang, 2015). The incidence of brown blight is observed throughout the year except for the winter, whereas anthracnose is significantly prevalent during the rainy season (May and June) and the autumn rainfall period (September and October).

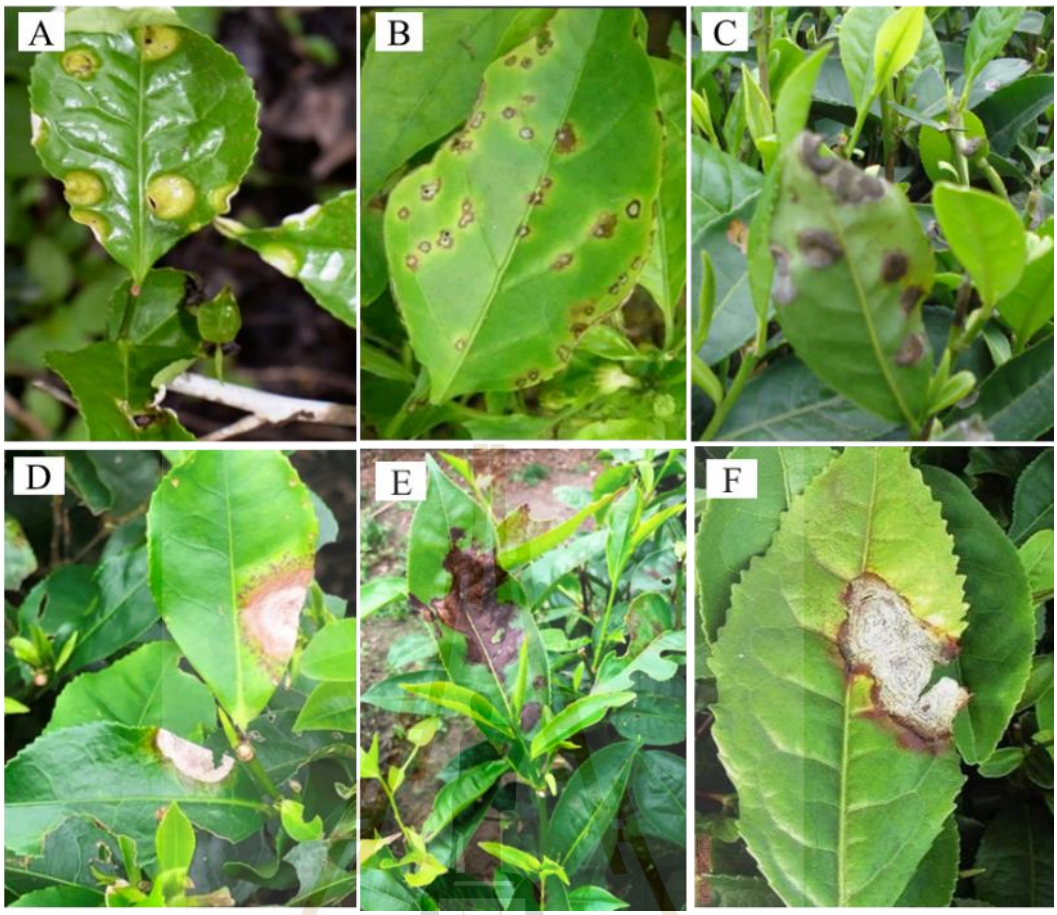

Figure 2.2 Symptoms of foliar diseases on tea leaves. (**A**) blister blight, (**B**) white scab, (**C**) bud blight, (**D**) gray blight, (**E**) brown blight, and (**F**) anthracnose (Pandey et al., 2021).

2.2.2 Stem diseases

The various stem diseases, including collar canker, branch canker, wood rot, and thorny stem blight, are responsible for approximately 40% of mortality among tea plants. The most prevalent and serious types involved in collar canker, also namely *Phomopsis* canker, and branch canker, also known as *Macrophoma* canker, are infected with the parasitic fungus *Phomopsis theae* and *Macrophoma theicola*, respectively (Ponmurugan and Baby, 2007). Previous studies from China, India, Sri Lanka, and Kenya have reported that *Phomopsis* canker attacked stem and twigs, and killed branches, resulting in the death of around 2- to 8-year-old shrubs and a

significant reduction in the tea plantations (Ahmad et al., 2016). Whereas several studies from India, Sri Lanka, Taiwan, and Southeast Asia have shown that branch cankers caused by *Macrophoma theicola* and twig dpieback caused by *Fusarium solani* (Mart.) Sacc resulted in a yield loss of more than 40% to 50% (**Figure 2.3A and B**) (Barthakur 2011; Ahmad et al., 2016; Sinniah et al., 2017).

It is well known that the prevalent types of tea stem diseases in China include tea red rust (*Cephaleuros parasiticus Karst*), sclerotium black rot (*Corticium invisum Petch*), and branch black spot (*Cenangium sp.*). Tea red rust and sclerotium black rot damage the branches and leaves and secrete toxins, leading to significant leaf loss in severe cases. In general, the occurrence of tea stem diseases is closely associated with the vitality of tea plants. In addition to the prevalence of branch black spot disease in rejuvenated and mature tea gardens, other stem diseases are more common in tea gardens with poor management and weak tree vigor.

Figure 2.3 Symptoms of tea stem diseases (**A**) *Macrophoma* canker and (**B**) twig dieback (Pandey et al., 2021).

2.2.3 Root diseases

The most prevalent root rot pathogens involve *Poria hypolateritia*, *Ustulina zonata*, *Armillaria mellea*, *ordariomycete Rosellinia arcuata*, *R. bunoides*, and *Phellinus noxius*, of which *Armillaria* root rot caused by *Armillaria mellea* and red root rot caused by *Poria hypolateritia* (**Figure 2.4**) in tea plants are considered as the primary root diseases in Kenya, India, Sri Lanka, and African tea plantations (Onsando et al., 1997; Ponmurugan et al., 2016). These pathogens harm the entire root system of tea plant, ultimately resulting in the death of the shrubs.

In China, the major tea root diseases recorded are *Meloidogyne* spp., *Pellicularia rolfsii* (Sacc.) West, *Agrobacterium tumefaciens* (E. F. Smith Tounsend), *Poria hyperpolarita* Berk, and *Helicobasidium mompa* Tanaka. Among these, *Meloidogyne* spp. is caused by nematodes, while *Agrobacterium tumefaciens* is caused by bacteria, and other root diseases are caused by fungi. When the tea plantations are established on mature land, the pathogens and nematodes present in the soil can infect tea seedlings with diseases. In the tea plantations cultivated within the virgin forest, the stumps and roots remaining in the soil can serve as parasitic sites for root rot pathogens, leading to subsequent infections of the root system and causing fungal diseases in tea plants.

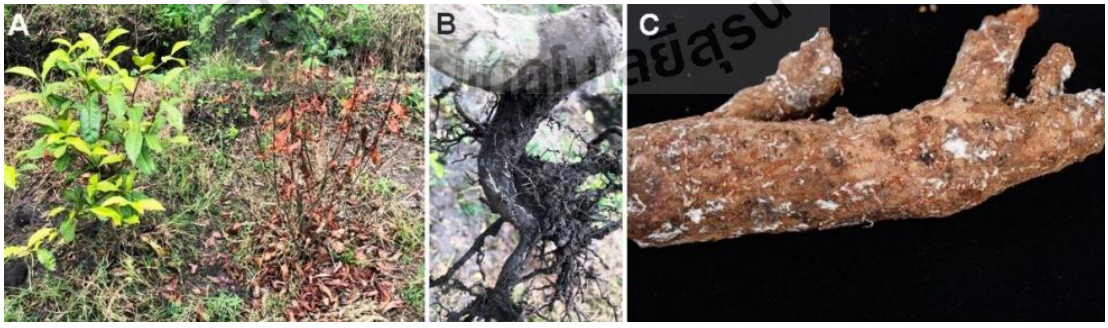

Figure 2.4 Symptoms of red root rot (**A**- Aboveground symptoms of a tea bush, **B**- root showing whitish rhizomorphs at the early stage, and **C**- reddish rhizomorphs at the later stages) (Pandey et al., 2021).

2.3 The epidemiology of blister blight disease

Blister blight widely occurs in most tea-growing regions, causing a yield loss of 20-50% in China, India, Japan, Sri Lanka, and Indonesia (Barthakur, 2011). The occurrence of blister blight was initially documented in Assam region of India in 1855 (Watt and Mann, 1903), and subsequently caused destructive damage in 1906. Prior to the year 1908, this disease was believed to be confined only to the Assam region; while it unexpectedly emerged in the Darjeeling tea region in 1908 (Montemartini, 1910), after which it became a significant issue for tea plantations across Asian countries. Afterwards, the disease spread successively to China in 1903 (Xiao et al., 2020), Japan in 1912, Vietnam in 1930, Sri Lanka in 1947 (Tubb, 1947), Indonesia in 1949 (de Weille, 1960), and Sumatra in 1959 (**Figure 2.5**). As for the tea-producing regions in China, blister blight occurs in all areas except for the north of the Yangtze River, with particularly severe occurrences in the high mountain tea plantations of the southwest and central-southern regions (Yang et al., 2019).

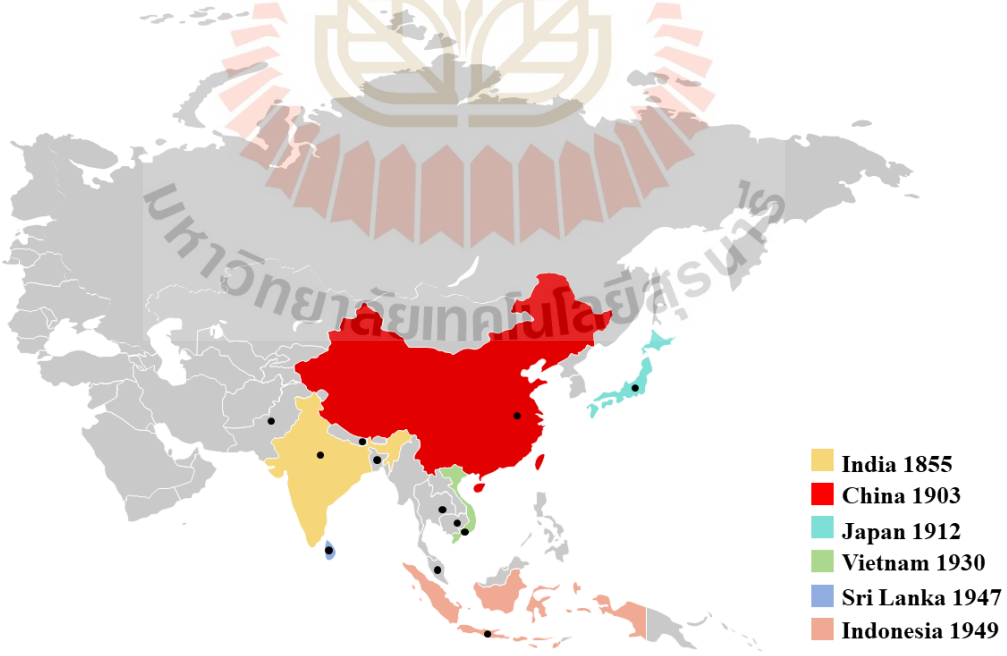

Figure 2.5 The geographical distribution of tea blister blight worldwide. Black dots represent the locations where blisters occur (Sen et al., 2020).

The survival of blister pathogen depends on favorable weather conditions characterized by low temperature and high humidity, with humidity exerting the most significant influence on the development of this disease (Jiang et al., 1985). Wind-borne spores settle on the surface of susceptible host tissues and germinate in humid atmospheres with a minimum relative humidity of 80%. After invasion, the hymenium forms during an incubation period of 3-18 days, followed by the formation of the lesions (Tan et al., 2015). Additionally, sunlight is another major factor affecting the prevalence of blister blight. Exposure to sunlight for less than four hours can promote the occurrence of blister blight during the onset period of the disease; conversely, it can inhibit the spread of the disease. The moderate temperature for the occurrence of blister blight is between 15°C and 25°C (Tan et al., 2015), while high temperatures above 31°C can suppress the disease (Gunasekera and Paul, 2007). Therefore, the presence of abundant tender shoots and leaves on the tea tree, along with the mild and humid climatic conditions, are two important factors required for the prevalence of this disease.

2.4 The pathogen, life cycle, and symptoms of blister blight disease

E. vexans Massee is an obligate parasitic fungus classified within the taxonomic categories of Exobasidiaceae, Exobasidiales, Exobasidiomycetes, and Basidiomycota. The cross section of blister on the diseased leaf reveals that mycelium grows between plant cells and forms finger-like haustoria structure that invades the parenchymatous cells of the leaf. The mycelium grows for a period of times and develops a hymenial layer on the lower epidermal cells of the diseased leaf, where numerous clavate, single-celled, colorless basidia are densely arranged, forming a palisade tissue structure. The size of basidia ranges from 30 μm × 5 μm to 35 μm × 6 μm, with 3-4 small stalks growing at the apex, each of which bears solitary basidiospore. The basidiospores are reniform or elliptical in shape, with a slightly rounded tip and pointed base, measuring from 9 μm × 3.5 μm to 16 μm × 6 μm in size. Basidiospores are frequently present on the surfaces of diseased areas, attached

to small structures, and easily detachable. It typically consists of a single chamber, while it develops into three chambers and emerges 2-4 germ tubes during the germination process (Mohktar and NagaoGadd, 2019). The tip of germ tube expands to create appressoria, through which the newly generated protoplasm is transported to plant cells (**Figure 2.6**).

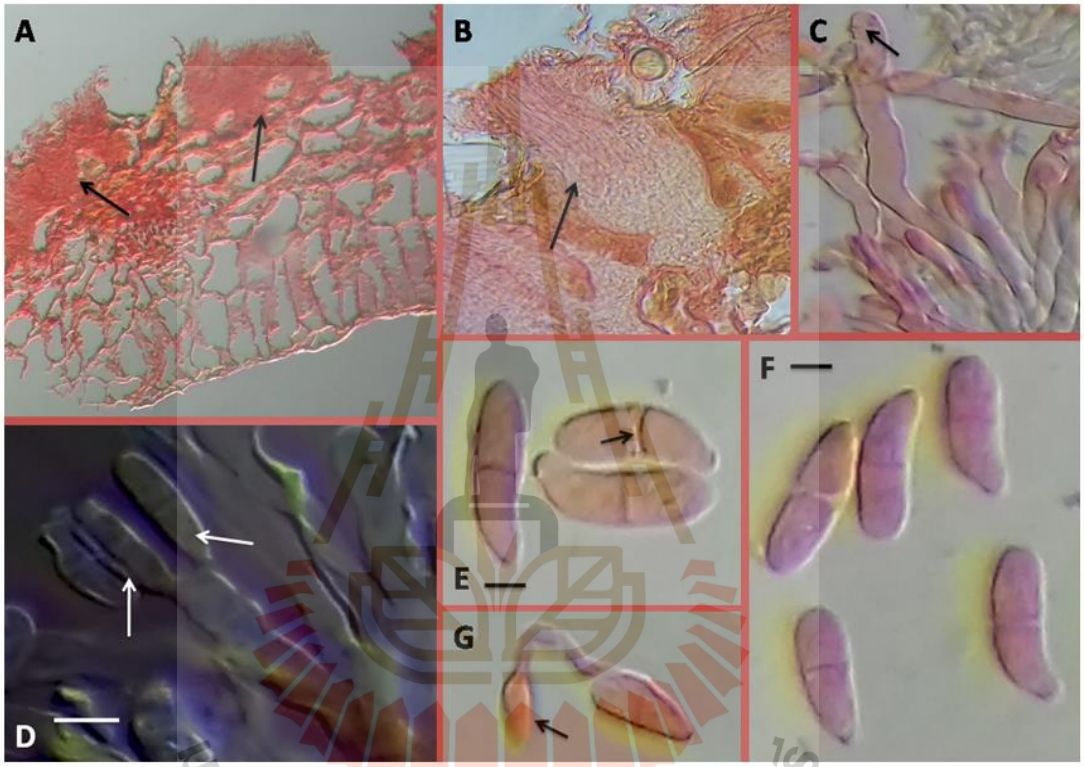

Figure 2.6 Morphological characteristics of blister blight caused by *E. vexans*. (**A-B**) Cross section of infected leaves. The hymenium contains numerous paraphyses and basidia which exert pressure on the epidermis (black arrows). (**C**) Finger-like haustoria structures in the hymenial layer (black arrows). (**D**) Structures of basidium and basidiophores. The white arrows indicate basidiospores attached to basidium; scale bar = 10 µm. (**E-F**) The mature basidiospores exhibiting clear septation; scale bar = 5 µm. (**G**) Spore structure during germination. Spore contains germ tubes equipped with appressoria at the tip (Sen et al., 2020).

The habitat of *E. vexans* in a tea plant is predominantly within the leaf spongy tissues. Blister blight is characterized by a complex disease cycle with a relatively short fungal life span of 11-28 days, divided into four phases. During the initial 0-24 hours of spore invasion, the basidiospores penetrate the epidermal cells of the lower leaf surface through the cracks in the cuticle layer and proliferate between the cells of the mesophyll tissue via the stomata; meantime, the diameter of pale yellowish-green or pink translucent spots appearing on the tea leaf is approximately 3-6 mm (**Figure 2.7A**). During the 1-7 days of spore infection, tea leaf tissue maintains a relatively intact cellular structure at the spore germination stage; from the phenotype of the diseased leaf, the lesion begins to expand, with a smooth and bright front part forming a depression, and a dark or gray part with thickened protrusions resembling a powdery appearance (**Figure 2.7B**). Within 7-14 days of spore infection, a large number of basidiospores are produced along with the formation of appressoria, resulting in the formation of typical blister lesion on the back of the leaves with abundant white fuzz (**Figure 2.7C**). During the 14 to 28 days of spore invasion, the cells of the infected tissue deplete the nutrients from the leaf, simultaneously inducing host resistance reactions; then blisters begin to become necrotic, browning, drying, and canker, with limited expansion of the lesion (**Figure 2.7D**). As the nutrients of the sponge tissue cells are gradually depleted, the vitality of the basidiospores and mycelium declines, and the leaf cells tend towards death (Zhao et al., 2018; Barman et al., 2020). Blister blight also affects the tender and young stems. Infection of young stems leads to an increase in the severity of damage, as the infected stems are prone to breaking off and eventual death, thereby hampering growth and reducing crop yields.

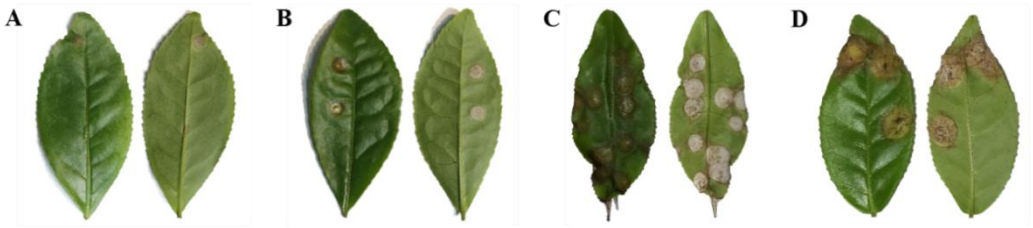

Figure 2.7 Symptoms of blister blight on upper and lower leaf at different phases of *E. vexans* infection. (**A**) Transparent spots on the tea leaf from the first phase (the initial 0-24 hours of spore invasion). (**B**) Enlarged lesion on the tea leaf from the second phase (1-7 days of spore infection). (**C**) Typical blister lesion on the tea leaf from the third phase (7-14 days of spore infection). (**D**) Necrotic lesion on the tea leaf from the fourth phase (14-28 days of spore invasion).

2.5 Biochemical changes during the process of tea infection with blister blight

The response of tea plants to *E. vexans* infection is a complex process. Many antimicrobial metabolites and disease-resistant genes are either activated or suppressed. Several metabolic pathways involved in disease resistance such as monoterpene biosynthesis, plant hormone signal transduction, flavonoid biosynthesis, phenylpropanoid metabolism, diterpene biosynthesis, and plant-pathogen interaction were significantly expressed following *E. vexans* infection (Sun et al., 2020). At different stages of *E. vexans* infection, it has various effects on pathogenesis-related (PR) proteins [chitinase (CsCHIT), glucanase (CsGLUC), and phenylalanine ammonia-lyase (CsPAL)], genes in flavonoid metabolism, and antioxidant enzymes such as peroxidase (POD), ascorbate peroxidase (APX), and superoxide dismutase (SOD), suggesting that flavonoids, lignin, anthocyanins, and reactive oxygen species (ROS) could potentially serve antifungal roles in the defense or resistance response of tea (Nisha et al., 2018).

2.5.1 Changes in enzyme activity

Different levels of enzyme activity were observed between resistant and susceptible varieties of tea plants following blister blight infection. Nisha et al. (2018) have demonstrated that the levels of the anti-oxidative enzymes POD and APX significantly accumulated in both resistant and susceptible varieties during blister blight infection, with a more significant increase observed in the susceptible varieties compared to the resistant one; whereas the activity of SOD was observed to be notably lower in the diseased leaves of both susceptible and resistant varieties than in their healthy leaves. A similar observation was discovered by Ran et al. (2021) who showed that SOD activity was significantly decreased in the tolerant cultivars following blister blight infection compared to the susceptible ones, whereas PAL activity showed the opposite phenomenon. Substantial changes in enzyme activity have been observed following the implementation of management practices aimed at controlling blister blight disease. The foliar application of biotic enhancer calcium chloride ($CaCl_2$) at 1%, chemical enhancer acibenzolar-S-methyl (ASM) at 0.14%, and botanic enhancer isobavachalcone (IBC) at 0.2% significantly induced the activity of defense enzymes including POD, PAL, polyphenol oxidase (PPO), catalase (CAT), SOD, β-1,3 glucanase, and chitinase against tea blister blight (Ajay and Baby, 2010; Chandra et al., 2014; Yang et al., 2023). Additionally, the levels of defense enzymes such as POD, PPO, PAL, chitinase, and β-1,3-glucanase were significantly higher in plants treated with *Pseudomonas fluorescens* Pf1 than in untreated ones during blister blight infection (Saravanakumar et al., 2006). The constitutive expression of chitinase genes (Singh et al., 2015) and endo-1,3-β-*D*-glucanase genes formed a hypersensitivity reaction zone (Singh et al., 2018), which was involved in signal transduction and pathogen recognition along with activated the activity of defense enzymes such as PAL against blister blight infection (Singh et al., 2019).

2.5.2 Changes in polyphenols and alkaloids

Tea contains many significant compounds involved in polyphenols and alkaloids, which contribute to taste and flavor. In addition, these substances also

process several bioactivity properties, such as antioxidant, antibacterial, and anti-inflammatory (Jiang et al., 2019; Li et al., 2023). However, polyphenols, alkaloids, and their associated metabolites in tea leaves exhibit significant changes during the process of blister blight infection. Several studies were performed by Punyasiri et al., (2001, 2005) who revealed that the higher levels of (-)- epicatechin (EC) were observed in resistant cultivars as compared to susceptible ones, conversely, the opposite was observed for (-)- epigallocatechin gallate (EGCG); while, no significant differences were found in the levels of (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), catechin, and caffeine between resistant and susceptible varieties. Punyasiri et al., (2001) also reported that the levels of two important catechins, ECG and EGCG, increased during the translucent stage of the infection, followed by a decrease as the infection progressed to the mature blister stage. In addition, the reduction in EC, EGC, and their esters upon infection, along with the production of cyanidin and delphinidin during the oxidative depolymerization of the blisters, indicated a potential involvement of proanthocyanidins in the defense mechanisms (Punyasiri et al., 2001). Simultaneously, tea leaves infected with blister blight caused a shift in the proanthocyanidin stereochemistry from 2,3-*trans* such as catechin and gallocatechin (GC) to 2,3-*cis* including EC and EGC (Punyasiri et al., 2004). Mur et al., (2015) have demonstrated that the levels of quercetin and kaempferol glucosides, along with kaempferol triglycosides and certain catechin-class antioxidants were observed to increase, whereas apigenin, myricetin glycoside, especially caffeine, decreased to significant level as the aggravation of blister infection. It has been observed that the susceptible cultivars contained higher contents of total phenol, flavonoids, and EGCG compared to tolerant genotypes (Anjan et al., 2022). Foliar application of *Ochrobactrum anthropi* BMO-111 improved the levels of catechins such as EGC, EC, EGCG, ECG, gallic acid, and catechin, along with polyphenol and caffeine compared to plants treated with chemical fungicides and those infected with blister (Sowndhararajan et al., 2013a).

2.5.3 Changes in amino acid and volatile compounds

It has been observed that tea plants with different resistant varieties showed various changes in both concentrations and types of free amino acids during blister blight infection, among which theanine had the highest concentration, followed by theanine γ-aminobutyric acid, methionine, and cysteine (Shan et al., 2017). Lysine, γ-aminobutyric acid, and arginine are physiologically active compounds in plants, contributing to metabolic regulation, growth promotion, and resistance against pathogen infections (Gogoi et al., 2016; Wang et al., 2019). Hence, the concentrations of these three amino acids in high resistant varieties were significantly higher than in high susceptible varieties, which might be due to the formation of γ-aminobutyric acid by glutamate and transformation into putrescine and spermidine; lysine and arginine are decarboxylated to form cadaverine and putrescine, respectively. However, spermidine, cadaverine, and putrescine can inhibit the growth of pathogens and improving resistance. Amino acids were reported to decrease initially and then increase during the blister blight infection (Zhang, 2022). Plant volatile compounds have been reported to be associated with plant disease resistance (Gogoi et al., 2016). It is considered that pathogen infection can induce the release of volatile compounds in plants that differ from those in healthy plants (Frost et al., 2007), which may serve as a direct chemical defense factor to prevent pathogen spread and act as a warning signal or deterrent involved in the dissemination of pathogens by plants (Farmer, 2001). *C. sinensis* var. *assamica* of tea plant infection with *E. vexans*, where more types of volatile compounds were identified in susceptible leaves than in healthy leaves (Zhang et al., 2012). Furthermore, the types of azotic, sulfur, terpenes, aromatic, and green leaf volatiles were dominant in volatile compounds of infected tea plants.

2.6 Plant defense mechanism against pathogen infection

2.6.1 Plant hormones

Plant hormones, also known as phytohormones or endogenous plant hormones, are involved in various processes of normal growth, development, differentiation, and reproduction in the plant life cycle (Castroverde and Dina, 2021). Additionally, they serve as disease-resistant signal molecules in triggering responses to pathogens, heterotrophic microorganisms, and herbivorous insects (Syrova et al., 2022). Plant hormones constitute a complex signaling regulatory network, which enable plants to survive and grow in a limited resource space by quickly adapting to the complicated biotic and abiotic environment. Plant hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) act as signaling molecules to mediate plant defense responses upon infection by pathogens, with SA and JA particularly considered as the primary defense response enhancers (Koo, 2018; mam et al., 2021). Hormones associated with growth regulation, including auxins, cytokinins (CK), gibberellins (GA), abscisic acid (ABA), brassinosteroids (BR), are not only fundamental to plant physiology and development but also play a positive or negative regulatory role in plant immune response to varying degrees, enabling plants to grow normally and respond rapidly to external stimuli (De Vleesschauwer et al., 2013).

SA is a potent signaling molecule naturally involved in plant systemic acquired resistance (SAR) concerning stress conditions. It inhibits the activity of plant cell wall degradation enzymes that are secreted by pathogens to reduce their pathogenicity, and it stimulates the expression of disease-related genes (Kaur et al., 2022). In *Arabidopsis*, SA signal can be recognized by the NPR1 receptor protein, which triggers plant immune response. Tea plants treated with 250 ppm of SA resulted in a lesser disease severity of *E. vexans* infection and enhanced the activities of β-1,3-glucanase, PAL, and POD compared to those untreated (Ajay and Baby, 2010). JA is a type of lipid-derived phytohormone, in which JA and methyl jasmonate (MeJA) play significant regulatory roles in plant growth and development, as well as in response to biotic and abiotic stress, especially in defense against

necrotrophic pathogens (Poonam et al., 2021). Recent findings have suggested that JA signaling pathway was able to positively regulate the resistance of tea plants to geometrid caterpillars mediated by PPO and synergistically interact with GA, thereby prioritizing rice (Li et al., 2015). Importantly, the levels of SA and JA were observed to be significantly decreased in the second stage of *E. vexans* infection, undoubtedly impairing the resistance to pathogen (Mur et al., 2015). Whereas the incidence and disease severity of blister blight treated with exogenous SA and JA were significantly lower than those in untreated plants, suggesting that SA and JA could induce resistance of tea plants against blister blight (Ran et al., 2022). Therefore, SA-JA defense network might be targeted by the utilization of exogenous chemicals that trigger SAR, which could be an efficient mechanism for the reduction of production and economic losses caused by blister blight.

2.6.2 Phytoalexins

Phytoalexins, a type of small molecule compound, can be biosynthesized *de novo* and accumulated rapidly by plant's metabolic pathways in response to pathogens and various abiotic stresses (Ahuja et al., 2012). Phytoalexins contribute to plant defense response by delaying and inhibiting the reproduction of pathogens or directly disrupting the normal metabolic activity of pathogens with their toxicity. The intention of phytoalexins was proposed more than 70 years ago regarding potato (*Solanum tuberosum*) tuber tissues infected with incompatible races of *Phytophthora infestans* induced resistance to compatible races of *P. infestans*. To date, over 200 phytoalexins identified from the diverse plant species have been evidenced to regulate agriculture and human health, among which phenolics, flavonoids, terpenes, alkaloids, and lignin were phytoalexins that have been extensively investigated (Ahmed and Kovinich, 2020).

Phenolic compounds, as the inducible antimicrobial metabolites in plants, have been suggested to be an important component of the plant immune system, acting as a crucial role in the responses of diverse plants to pathogen infections. Phenolic compounds were reported to inhibit the incidence of anthracnose caused

by *Colletotrichum gloeosporioides* in avocado fruit (Osondu et al., 2022), anthracnose caused by *C. gloeosporioides* in poplar (Zhang et al., 2022), and gray mold infected with *Botrytis cinerea* in table grapes via activating the phenylpropanoid pathway (Xu et al., 2019). Phenylpropanoids are precursors for phenolic compounds involved in flavonoids, isoflavones, and cumin apigenin, and the significant accumulation of their content following treatment with exogenous compounds could reduce plants' susceptibility to pathogens (Martínez et al., 2017; Maria et al., 2019). Flavonoids, a vital part of phenolic metabolites, have been indicated to possess antimicrobial activities that inactivate cell membrane transport proteins, stimulate hormone signal transduction, enhance *Arabidopsis* resistance to *Botrytis cinerea*, and disrupt microbial membranes and respiratory chains (Hong et al., 2015; Lu et al., 2017). In addition, previous reports have provided some evidence demonstrating the accumulation of various secondary metabolites including kaempferol, apigenin, quercetin triglycosides, proanthocyanidins, and myricetin-glycosides following blister blight infected with *E. vexans* (Punyasiri et al., 2004; Mur et al., 2015), which suggested that these phytoalexins could serve as antimicrobial agents in tea cultivars against blister disease. Furthermore, exogenous application of antimicrobial phytoalexins has been reported to contribute to tea plants defense against *E. vexans* infection (Chaliha et al., 2022).

2.6.3 Plant pathogenesis-related genes

Many genes are induced to express in plant-pathogen interaction, and their coding products directly or indirectly participate in plant defense responses. These genes include those related to pathogenesis mechanisms, cell wall modification, and secondary metabolites (Anisimova et al., 2021). Following the invasion signals of pathogens, PR proteins transcribed and translated from PR genes belong to a category of acidic and secreted small molecular proteins with antimicrobial functions, typically serving as defense signaling pathways and SAR markers. It was first identified in the susceptible tobacco plants infected with *Tobacco mosaic virus* (TMV) (Van Loon and Van Strien, 1999), since then, PR proteins were defined as intracellular and

extracellular proteins induced by pathogen infection or related conditions in plants. Nowadays, PR proteins are classified into 17 families with multiple functions, including PR1, β-1,3-glucanase (PR2), chitinases (PR3, PR4, PR8, PR11), protease inhibitor (PR6), peroxidase (PR9), lipid transfer proteins (PR14), and oxalate oxidase proteins (PR16) (Van Loon et al., 2006).

Historically, PR1 was first known in 1970 as the most important PR protein induced by pathogen or SA and SAR marker genes (Van Loon and Van Strien, 1999). Some findings provided evidence that the constitutive expression of PR1a in transgenic tobacco plants enhanced the resistance to *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae* (Alexander et al., 1993), and the crucial role of PR1 protein defense against *Phytophthora capsica* in black pepper (*Piper nigrum* L.) (Divya et al., 2021). Recently, PR1 genes have been found to positively participate in the response to *E. vexans* infection, which offered new opinions in response to blister blight and established a fundamental basis for following research on the function of PR1 in tea plants (Zhang et al., 2022). In addition, β-1,3-glucanase and chitinases might contribute to the defense against malformation in mango (*Mangifera indica* L.) (Ebrahim et al., 2011) and *Macrophomina phaseolina* in *Arachis hypogaea* (Iwuala et al., 2020). Similarly, numerous findings have revealed that the significant accumulation of enzymes involved in chitinase and β-1,3-glucanase in tea plants induced by plant growth-promoting rhizobacteria (PGPR) bioformulation *P. fluorescens* Pf1 (Saravanakumar et al., 2006), the higher increase in GH18 and GH19 families of chitinases challenged by *E. vexans* (Bordoloi et al., 2021), and class I chitinase gene (AF153195) from potato (*Solanum tuberosum*) inoculated by *E. vexans* could enhance the disease resistance against blister blight (Singh et al., 2015). Because of the potential antimicrobial function of PR proteins, they may elicit defense responses and mediate plant immunity against abiotic and biotic stresses in tea plants and other crops.

2.6.4 Plant disease resistance-related transcription factors

The activation of signal transduction process and the variation of considerable gene expression in plants induced by pathogen infection are largely modulated by specific transcription factors (TFs) (Dortje et al., 2011). Generally, TFs act as multifunctional regulatory proteins in the process of signal interaction in plants. They regulate gene expression by binding to the specific DNA sequence of target genes, thereby modulating the activity of transcription initiation ribonuclease (Amorim et al., 2017). Recently, TFs (e.g., WRKY, MYB, and bHLH) associated with plant disease have been reported to be involved in almost all biological processes of plants and play a crucial role in the transcription regulation of stress response genes related to biotic and abiotic stresses (Eulgem and Somssich, 2007; Meng et al., 2020).

WRKY proteins, a specific superfamily, are one of the largest TF families in plants, which play a vital role in regulating diverse processes from plant growth and development to multiple stress responses and hormone signal transduction (Ramamoorthy et al., 2008). Particularly, WRKY factors are DNA-binding proteins that specifically recognize the W-box (TTGACC/T) element in the promoter region of various plant disease-resistant genes. This suggests that WRKY proteins regulate the expression of disease-resistant genes and also interact with each other among different WRKY TFs to mediate plant defense responses (Cheng et al., 2019). After first being identified in the sweet potato, WRKY proteins have since been reported in diverse plant crops including *Arabidopsis thaliana* (Imran et al., 2018), rice (*Oryza sativa*) (Lilly and Subramanian, 2018), cotton (*Gossypium hirsutum*) (Lilly and Subramanian, 2018), and tobacco plants (*Nicotiana tabacum*) (Yao et al., 2020). Most WRKY genes act as positive or negative regulatory factors involved in plant immune responses to biotic and abiotic stresses and play significant roles in leaf senescence, seed formation, dormancy, and germination (Yao et al., 2020). Recently, CsWRKY14, a disease resistance gene, isolated from tea leaves was found to contribute to the activation of SA-induced pathogen-related gene expression and the regulation of

defense enzymes (POD, SOD, and CAT) against *E. vexans* infection. In addition, 56 CsWRKY regulatory factors (e.g., CsWRKY31, CsWRKY31, and CsWRKY48) identified from tea plants might provide potential information for breeding high-quality tea varieties in response to various stresses (Luo et al., 2018; Chen et al., 2019).

2.7 Tea defense mechanism against *E. vexans* pathogen infection

2.7.1 Leaf morphological characteristics of tea plants in resistance to pathogens

Tea plants have traditionally been classified as small-, medium-, and large-leaf varieties based on morphological features. These different leaf types are associated with varying levels of disease resistance (Zhang et al., 2018). It has been reported that the resistance of medium-leaf tea varieties to brown blight was relatively stronger than that of large-leaf ones (Hu, 2005). The surface of mature tea leaves is covered with a thick waxy layer and cuticle, as well as a complex cell wall and mesophyll tissue structure, contributing to the morphological resistance of tea leaves. The epidermal cells of tea leaves are coated with an uneven cuticle layer predominantly made of unsaturated fatty acids, which plays a positive role in anti-microbial pathogens. In addition, the palisade tissue cell density in tea plants differs among various varieties, and the growth and spread of pathogen mycelium within plant tissues are constrained by the dense cell arrangement (Yang, 2009). Moreover, the leaf cell wall is predominantly composed of cellulose, hemicellulose, and pectin. These substances play an important role in enhancing cell wall strength, hardness, and flexibility to prevent pathogens from invading and toxic substances from diffusing through the cell wall.

2.7.2 Physiological and biochemical components of tea plants disease resistance

Following infection by various pathogens, tea plants will undergo a series of physiological changes with common characteristics. Alterations in cell membrane

permeability and electrolyte leakage are significant physiological lesions in the early stage of infection, followed by the increase of protein content and total nitrogen content, enhancement of respiration, and weakness of photosynthesis. Yang (2009) demonstrated that the significant accumulation of free amino acid was observed in many susceptible tea varieties, while the protein synthesis capability rapidly declined. In addition, the high resistance of tea variety to pathogens such as fungi, bacteria, and viruses depends on the presence of inhibitory compounds in tea cells, including polyphenols, alkaloids, tea saponins, and various defense enzymes. Polyphenols such as flavonoids (catechins and epicatechins), flavonols (kaempferol and quercetin), and phenolic acids are the essential secondary metabolites in tea leaves, accounting for about 22% of dry matters, which are mainly stored in the vacuole of mesophyll cells (Neveu et al., 2010). It has been reported that catechin was able to inhibit diverse hydrolase secreted by pathogens, including β-1, 3 glucanase, pectinase, cutinase, and cellulose degrading enzymes, thereby preventing the invasion of pathogens (Deng and Tan, 2005). Caffeine is the predominant alkaloid in tea leaves, primarily synthesized in within chloroplasts. Caffeine is considered to be a broad-spectrum compound with disease-resistant properties that can inhibit the growth of various pathogens at low concentrations (Waldhauser and Baumann, 1996; Kato et al., 1998). Methyl salicylate and green leaf volatiles in tea exhibited inhibitory effects on the normal growth of *C. theae sinensis* mycelium. Besides that, pathogen inhibitors secreted on the surfaces of tea leaf can inhibit the germination of fungal spores and the bursting of conidia, especially in high-resistant varieties.

2.7.3 Molecular mechanism of tea plants resistance to pathogen invasion

Biotrophic pathogens propagate within the intercellular spaces upon entering through stomata and derive nutrition from specialized haustoria (Jayaswall, et al., 2016). These pathogens can lower plant immunity by conveying effectors into plant cells. Plants indirectly activate multiple resistance (R) genes by relying on innate or acquired immunity of individual cell and systemic signal transduction from the

infected sites (Jayaswall et al., 2016). In the plasma membrane, plants can recognize pathogen/microbial-associated molecular patterns (PAMP/MAMP) via pattern recognition receptors (PRR) to trigger pattern-triggered immunity (PTI) (Guillaume, 2021). The occurrence of plant disease resistance is regulated through the eruption of ROS, activation of mitogen-activated protein kinase (MAPK), and induction of several resistance proteins and defensive enzymes as main immune modulators to control various physiological and biochemical reactions in response to plant pathogen (Jones and Dangl, 2006; Mendes et al., 2021). In addition, WRKY and NAM transcription factors, molecular chaperone proteins, and cysteine proteases are primarily involved in the process of plant disease resistance, of which the regulation of gene expression associated with disease resistance is mediated by the binding of WRKY and NAM transcription factors to the promoter elements of disease-resistant genes in plant immunoreaction (Pandey and Somssich, 2009). Currently, several R genes related to resistance against blister blight have been identified in tea plants to reveal their response mechanisms to the disease.

Along with the establishment of tea genome database and the rapid development of sequencing technology, several scientific strategies have been widely used to reveal some R genes related to blister blight defense and their response mechanisms to pathogens. A study has suggested that the number of differentially expressed genes (DEGs) and GO terms in susceptible varieties infected with tea blister pathogen were higher than those in resistant varieties; besides that, some genes such as R protein, hydrolase, cell wall thickening, transcription factors, plant hormones, and antioxidant enzymes were observed to be up-regulated in these two varieties following *E. vexans* infection (Ran et al., 2022). Another transcriptome analysis has revealed that nucleotide binding leucine rich repeat (NB-LRR) mediated effective-triggered immunity (ETI) was activated to trigger the R gene-mediated SA signaling pathway to induce the production of secondary metabolites and programmed cell death (PCD) for defending against the invasion of blister blight pathogen; meantime, 149 disease resistance-related genes, such as defense enzymes

(e.g., SOD and MPKK6), transcription factors (e.g., WRKY and NPR), transporters (e.g., MDR), R genes (e.g., RIN4 and RPM1), and retrotransposons (e.g., LTR), were identified from the protein-protein interaction network of *Arabidopsis thaliana* as candidates (**Figure 2.8**), which provided potential regulatory mechanisms for tea or other crops to resist various biotic stresses (Siemens et al., 2006). *CsWRKY14* was reported to participate in the SA signaling pathway, with its expression level being higher in resistant cultivar 'Zhongcha 108' (ZC) compared to susceptible cultivar 'Longjing changye' (LJ); simultaneously, the content of endogenous hormone SA and the expression of SA signaling pathway-associated genes (NPR1, PR1, PR2, and PR4) were observed to accumulate significantly in ZC than in LJ, suggesting that *CsWRKY14* could serve as a dependable genetic resource for resistance to blister blight in tea plants (Liu et al., 2021). Additionally, cDNA-AFLP and SSH methods have been used to identify new genes associated with disease resistance between resistant and susceptible cultivars during blister blight infection; at the same time, some genes involved in energy metabolism, oxidative stress response, protein modification, cell wall fortification, and signal transduction were closely related to plant defense or resistance response (Bhorali et al., 2012).

Figure 2.8 Hypothetical defense mechanisms of tea against blister blight (Jayaswall et al., 2016).

The host plants can recognize certain conserved substances, such as chitin and β-glucan, in the fungal cell wall during the pathogen invasion to trigger the plant's immune mechanism and induce the expression of PR genes in response to the invasion process (Atkinson and Urwin, 2012; Rejeb et al., 2014). Chitinase and β-1,3-glucanase are typical PR proteins with hydrolytic capability. Specifically, chitinase can catalyze the hydrolysis of β-1,4 linkages between chitin *N*-acetylglucosamine oligomers in the fungal cell wall, while β-1,3-glucanase can hydrolyze polysaccharides on the cell wall to form oligosaccharides, thereby destroying the fungal cell wall and preventing invade (Jalil et al., 2015; Iwuala et al., 2020). A previous report has demonstrated that *Agrobacterium*-mediated *Solanum tuberosum* class I chitinase gene and endo-1,3-β-*D*-glucanase gene could enhance the resistance of tea plants to *E. vexans* infection (Singh et al., 2015, 2018, 2020). Additionally, *CsPR-1* genes identified via transcriptome analysis and tea genome database were found to be closely associated with signal transduction pathways, such as TCA, NPR1, EDS16, BGL2, PR4, and HCHIB, suggesting their significant role in defense mechanism against blister blight (Zhang et al., 2022).

The invasion of pathogens into plants induces the occurrence of the hypersensitive response (HR), which activates SAR to prevent the spread of pathogens (Ajay and Baby, 2010). In addition to pathogens, some biological or chemical enhancers applied to plants can also induce SAR during host-pathogen interaction. For instance, certain enhancers involved in SA, $CaCl_2$, S-methyl ester (SAM), chitosan, IBC, and biocontrol bacteria/fungus (*O. anthropi* BMO-111 and *Trichoderma*) can promote the activity of defense enzymes, increase the content of lignin to strengthen cell wall function, promote the biosynthesis of some antibacterial/fungal substances (e.g., flavonoids, anthocyanins, and polyphenols), and regulate downstream functional gene expression through the nitric-oxide (NO) signaling pathway (Saravanakumar et al., 2006; Ajay and Baby 2010; Sowndhararajan et al., 2013b; Chandra et al., 2014; Adhikari and Rai 2022; Yang et al., 2023). This provides a selection of bioagents for controlling blister blight and establishes a

foundation for revealing the molecular mechanism by which tea defense against *E. vexans* infection.

2.8 Integrated control managements for blister blight disease

The occurrence and severity of blister blight are primarily determined by environmental conditions, tea varieties, and cultivation management practices. More importantly, the selection of disease-resistant tea cultivars is an essential strategy to control *E. vexans* infection. In addition, chemical practices to manage blister blight should be performed during the initial stages of the outbreak; however, the excessive application of chemical agents may cause phytotoxicity and residual effects. Collectively, the intervention approaches to tea plantation management such as cultural practices, good cultivation or agronomic measures, the utilization of fungicides and microbial agents, deployment of disease-resistant varieties, and defense enhancers can effectively reduce the damage of fungal diseases to tea plants (Pandey et al., 2021).

2.8.1 Cultural practices

The primary goal for controlling blister blight is to prevent *E. vexans* pathogen from spreading to disease-free areas and to reduce the severity of the disease. Many cultural strategies have been attempted to control this disease, such as proper pruning and picking, weed removal, fertilizer management, shade thinning, and intercropping in tea plantations. Prompt pruning of tea branches with uneven growth or infection points and removal of diseased leaves have been beneficial for maintaining ventilation and light penetration between plantations, thereby reducing the incidence of pathogen (Ahmad et al., 2016). The shade trees are essential parts of tea plantations as they protect against the adverse effects of high temperatures on the yield and quality of tea plants (Barthakur, 2011; Li et al., 2020). On the contrary, higher humidity under the shade trees tends to promote the occurrence and development of foliar diseases. Shade thinning during the initial period of

disease occurrence and infected shoots plucking have been implemented as strategies to reduce the severity of blister blight (Barthakur, 2011). In addition, proper application of phosphorus, potassium, and organic fertilizers has been practiced for enhancing the growth vitality of tea bushes, improving plant disease resistance, and maintaining the yield and quality of tea. Furthermore, previous investigations have revealed that intercropping of tea plants with camphor trees, light pruning of tea bushes after spring harvesting, and closure of the tea garden in winter could effectively reduce the incidence of blister blight (Ran et al., 2021).

2.8.2 Host resistance cultivars

The breeding and cultivation of disease-resistant cultivars contribute to the avoidance of pesticide residues caused by improper application of chemical fungicides and reduction of labor costs, which provides essential measures for preventing the occurrence and development of blister blight.

Recently, molecular marker based on PCR has been confirmed to be an effective technique for intervening resistance genes into breeding plants. EST-SSR 073 sequence can serve as a molecular marker for tea resistant varieties (tri2043), the protein of which was characterized by CT repeat extension and C-terminal truncation, resulting in the formation of hydrogen bonds that interact with other subunits of PSAD I and photosystem I within a simulated three-dimensional protein structure (Karunarathna et al., 2020). Meanwhile, the association between the marker traits obtained from the F1 population of blister blight and the diagnostic marker EST-SSR 073 gained by detecting alleles through fragment analysis matched with the subunit II of photosystem I reaction center, which could be effectively used in tea breeding to enhance resistance to blister disease (Karunarathna et al., 2020).

Numerous disease-resistant genes in tea plants are effective in controlling blister blight. Transcriptome analysis revealed that R genes (RIN4, RPM1, and RPS2), defense-related enzymes (SOD and chitinase), transcription factors (WRKY and NAM), retrotransposons (LTR/Copia), and resistance transporters (MDR) were considered as

candidates against blister blight in tea plants (Jayaswall et al., 2016). Additionally, *CsWRKY14* has been reported to contribute to an increase in SA concentration and activate the expression level of pathogen-related genes induced by SA, which could serve as an effective genetic resource for breeding tea resistant to blister blight (Liu et al., 2021).

In recent years, resistant cultivars involved in Zhongcha 108, TRI 2043, and Ambei-Valai-2 (AV-2) were identified as resistance against blister blight from China, Sri Lanka, and India. Briefly, the activity variations of PAL and SOD were closely correlated with disease resistance. The PAL activity in disease-resistant varieties was higher than that in susceptible varieties; in contrast, the tolerant varieties exhibited lower SOD activity compared to susceptible varieties (Ran et al., 2021). In addition, TRA, Jorhat, and Assam-viz showed that the contents of acid phosphatase, catechol oxidase, PO, and SOD in resistant variety AV-2 were higher than those in susceptible variety B-157, and the accumulation of secondary metabolites such as total phenols, proanthocyanidins, and total soluble proteins served as antimicrobial compounds defense against plant diseases (Hazra et al., 2018). Besides that, the accumulation of EC levels was significantly higher in resistant varieties compared to susceptible ones, while the opposite was true for EGCG, and high resistance of purple green-leaf tea varieties against disease was also due to extra catechins derived from high level of anthocyanins (Punyasiri et al., 2005).

2.8.3 Chemical fungicides

It has been found that the incidence of blister blight exceeding 35% can lead to significant yield losses, which is considered as a threshold level of disease incidence (Shanmuganathan and Arulpragasam, 1966). The appropriate application of fungicides has been proven to be effective in preventing and controlling prevalent tea diseases. However, certain potent fungicides cannot be frequently used in tea cultivation due to concerns about their potential adverse effects on the environment, and the possibility of chemical residues in tea leaves affecting human health (Dixon, 2014). Foliar application of fungicide Nativo 75 WG (25% trifloxystrobin

+ 50% tebuconazole) at 125 g/ha showed 60% reduction in the incidence of *E. vexans* pathogen (Thakur and Masand, 2011), and even the symptoms of chlorosis, necrosis, and epinasty were not observed on the young and tender shoots treated with high-concentration fungicide (Thakur and Masand, 2015). Pyracarbolid formulations containing 50% moisture agent (Hoe 2989 and Hoe 6052) at 140 g/ha or 15% dispersion (Hoe 13764) at 75 g/ha were recommended as effective foliage sprays at conventional 7-day intervals, which has achieved excellent disease control under various agricultural climatic conditions (Venkata, 1975). Similarly, pyracarbolid completely inhibited spore germination after inoculation and provided better protection against tea blister blight at 0.28 kg/ha compared to use of 50% cuprous oxide fungicide (Shanmuganathan and Saravanapavan, 1978). Ergosterol biosynthesis inhibiting (EBI) fungicides were applied to tea plants infected with blister blight to restrain spore germination, reduce spore size and vigor, and inhibit disease incidence (Baby et al., 2004). Wettable powder formulation mixed with 1.5% plant fungicide of *Cynanchum auriculatum* have been found to be effective in controlling *E. vexans* infection, with an average efficacy of 31.29% (Rayati, 2015). Therefore, strategic application of fungicides with ecological safety and effective active ingredients help manage fungicide resistance to pathogens of tea plants.

2.8.4 Biocontrol practices

Although fungicides with various modes of action have shown some promising effects in controlling multiple foliar diseases of tea plants, phytotoxicity and chemical residues of fungicides pose a threat to environmental pollution and human health (Ajay and Baby, 2010). Recently, more attention has been paid to biocontrol methods for managing fungal diseases in tea (Sanjay et al., 2007). Several potential antagonists like *Bacillus subtilis, P. fluorescens,* and *Trichoderma harzianum* have been identified as microbial agents to control tea blister blight.

In the case of severe disease (index > 50%), the application of *Monascus* or pile-fermentation was effective in reducing the occurrence of *E. vexans* pathogen by 22% (Rayati 2011). Fauziah et al. (2016) performed nursery experiments with local-

code microbes, Azoto II-1, Endo-5, and Endo-76, which indicated that the combination of 75% Azoto II-1 with 25% Endo-5 was able to inhibit the disease incidence of *E. vexans* (1.27%). In addition, *O. anthropi* BMO-111 and its liquid culture filtrate exhibited superior efficiency in suppressing the mycelial growth of fungal pathogen and reducing the incidence of blister blight compared to chemical fungicides (copper oxychloride and hexazole) (Sowndhararajan et al., 2013b). Additionally, they have enhanced the resistance of tea to *E. vexans* infection by increasing biochemical indicators like chlorophyll, polyphenols, and catechins (Sowndhararajan et al., 2013a). PGPR of *P. fluorescens* Pf1 spraying on the leaves at intervals of 7 days reduced the incidence of blister blight under field conditions (Saravanakumar et al., 2006). Two biofungicides derived from the *Trichoderma viride* and *Trichoderma harzianum* have recently been reported to exhibit significant inhibitory effects on mycelial growth of *E. vexans*, with inhibition rates of 70.87% and 66.98%, respectively (Adhikari and Rai, 2022).

2.8.5 Defense enhancers

Enhancer-mediated enhancement of plant innate immune system defense against pathogen infection has been believed to be a substitutable and eco-friendly strategy for crop plant protection (Chakraborty and Acharya, 2016). Application of chemical enhancers or microbial inoculants, including chitinase genes (Singh et al., 2015, 2020), 1,3-*β*-*D*-glucanase genes (Singh et al., 2018), and *P. fluorescens* Pf1 (Saravanakumar et al., 2006), has been confirmed to activate the activity of defense enzymes (PAL) and induced systemic resistance (ISR) or SAR in tea plants, effectively reducing the disease incidence of *E. vexans* pathogen and significantly improving tea production.

Foliar spraying of biotic enhancers 0.01% chitosan (Chandra et al., 2017), 1% $CaCl_2$ (Chandra et al., 2014) and chemical enhancer 0.18% ASM applied alternately with standard fungicides (hexaconazole + copper oxychloride) (Ajay and Baby, 2010) at 15-day intervals under field experiments provided approximately 68 %, 71%, and 47% protection against blister blight. Meanwhile, the application of these enhancers

also showed a significant accumulation of phenolic substances and defense enzymes such as PO, PPO, PAL, β-1, 3-glucanase, and NO (Ajay and Baby, 2010; Chandra et al., 2014, 2017). Recently, Anjan et al. (2022) have demonstrated that ROS mediated response of tea plants against blister blight, and the higher levels of ROS, lipid peroxidation, and total protein were observed in resistant genotypes compared to susceptible ones. Treatment of botanic fungicide isobavachalcone (IBC) combined with biotic enhancer chitosan oligosaccharides (COSs) exhibited superior efficiency in controlling blister blight than individual application of IBC or COSs and could enhance the disease resistance of tea plants by improving the activity of defense-related enzymes (Yang et al., 2023).

2.9 Omics overview and application

Omics is a formidable approach to analyze large sets of data at a type, individual, organ, cellular or molecular levels, commonly including genomics, transcriptomics, metabolomics, and proteomics (Campos et al., 2020). Omics has been widely applied in multitudinous subjects of life sciences such as medicine, human health, animal sciences, and environmental sciences (Paredi et al., 2013). Recently, omics has been extensively used in agricultural sciences involved in plant-pathogen interactions as well as plant growth and development following with the rapid development of biotechnology, for instance, mechanism of mycorrhizal interaction (Larsen et al., 2016), senescence process of wheat leaf, and heat stress tolerance of tomato (Joon-Yung et al., 2022).

2.9.1 Application of transcriptomics analysis in the research of plant-pathogen interactions

Transcriptomics is the study of transcriptome, that is, a set of RNA transcripts produced by genome in specific environments or cells using DNA direct sequencing technologies such as microarray analysis, serial analysis of gene expression (SAGE), massively parallel signature sequencing (MPSS), and RNA-sequencing (RNA-Seq)

(VanGilder et al., 2012). The identification of DEGs is allowable by the analysis and comparison of transcriptome in diverse cell populations, or in response to different treatments. Generally, RNA-Seq is an increasingly common strategy for deep sequencing of transcriptome based on the second-generation sequencing platform, which can identify transcriptional interferences possibly caused by gene variations using functional genomic information (Byron et al., 2016). This approach reveals the patterns and mechanisms of life activities at the transcriptional level.

Plant diseases have always been the principal consideration reducing the crop yield and affecting the quality of agricultural products (Song et al., 2016). For a long time, researchers have devoted a considerable amount of effort to investigate the mechanisms of plant disease resistance to reduce agricultural production losses caused by plant diseases. In the long-term interactions with pathogens, plants have gradually developed a series of complex defense response mechanisms, whereas transcriptomics can provide a large amount of gene expression information at the whole genome level, stands as an ideal approach for studying the mechanisms of plant disease resistance (Ge et al., 2022). For instance, RNA-seq technology was used to reveal the early defense response mechanism of button mushrooms (*Agaricus bisporus*) to pathogens (Yang et al., 2022), resistance mechanism of soybean to anthracnose (*Colletotrichum truncatum*) (Zhu et al., 2022), GbABR1 gene of cotton against *Verticillium wilt* (Liu et al., 2018). Additionally, the application of transcriptomics has been conventionally reported to reveal resistance mechanisms of tea plants against *E. vexans* (Jayaswall et al., 2016; Liu et al., 2021), *C. truncatum* (Xiong et al., 2020), *Didymella segeticola* (Huang et al., 2022), and *Pestalotiopsis trachicarpicola* (Xia et al., 2022).

2.9.2 Application of metabolomics analysis in the research of plant-pathogen interactions

Metabolomics is derived from the metabolic profiling, which focuses on the quantitative, qualitative, and fluctuations of small molecular compounds in organisms, organs, tissues, and cells at a specific time or under specific

environmental conditions overall (De Souza Leonardo et al., 2020). At present, several techniques in metabolomics research mainly include liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), capillary electrophoresis-mass spectrometry (CE-MS), nuclear magnetic resonance (NMR), flourier transform infrared spectroscopy (FTIR), high performance liquid chromatography (HPLC), and ultra-high performance liquid chromatograph (UHPLC). Among these, LC-MS, GC-MS, NMR, and FTIR have become the four main platforms for metabolomics analysis that was attributed to the good comprehensive performance involved in applicability, sensitivity, accuracy, and completeness of relevant databases (Chen et al., 2019). MS analysis is to determine the content of specific ions by ionizing the isolated compounds to obtain the qualitative and quantitative results of the samples. LC-MS analysis possesses the absolute superiority in the qualitative analysis of metabolites and the access of structural information (Kohler et al., 2020), which can detect non-volatile, thermally unstable, polar, and macromolecular metabolites such as inorganic ions, carbohydrates, vitamins, organic acids, amino acids, nucleotides, and nucleosides. Whereas, GC-MS analysis can be used for the detection of volatile and intermediate compounds, including organic acids, amino acids, aromatic amines, sugars, and fatty acids owing to its advantages of high separation efficiency and retention time with good reproducibility (Roessner et al., 2000).

Metabolomics is currently widely utilized in research of plant-pathogen interaction, with a specific focus on investigating the functions and pathways of metabolites in the pathogenesis mechanism and the developmental processes of interactions with plants. Metabolomics can be employed to determine the genetic developmental patterns and the variations in metabolic features induced by host stimulation, comprehensively reflecting the phenotypic changes of phytopathogens (Nakabayashi and Saito, 2015). In addition, metabolomics can also be used to acquire small-molecule metabolites produced by plants following pathogen infection, contributing to the screening and breeding of resistant varieties (Chen et al., 2017).

^{1}H NMR and GC-MS analysis were performed to investigate the differences of metabolites between four different types of *Fusarium* strains, indicating that the influence of nutrient conditions on fungal metabolism was relatively greater than that of genotypes (Lowe et al., 2010). Sade et al. (2015) performed a combine analysis of GC-MS/LC-MS and target gene qRT-PCR to study the resistance mechanism of tomato to *Tomato yellow leaf curl virus* (TYLCV), which revealed that metabolic pathways of phenylpropanoid, tryptophan/nicotinate, urea, polyamine, and SA played important roles in disease resistance. Li et al. (2023) used a widely targeted metabonomic analysis based on UHPLC-MS/MS to study the mechanism of the active compounds of kiwifruit in exogenous IAA treatment and *B. cinerea* infection, which found that IAA could induce the resistance of kiwifruit against *B. cinerea* and reduced the occurrence of disease. Mur et al. (2015) used metabolomic approach to reveal that the occurrence and development of *E.vexans* infection in tea plants were associated with the reduction of several antimicrobial metabolites and some phytohormones (SA and JA).

2.10 References

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CHAPTER III

ANALYSIS OF WIDELY TARGETED METABOLOME AND BIOCHEMICALS IN TEA INFECTED WITH BLISTER BLIGHT DISEASE

ABSTRACT

Blister blight infection with *E. vexans* is one of the most destructive foliar diseases that seriously affect the quality and yield of tea. This research investigated the changes in metabolites of healthy and infected leaves on tea cultivar 'Fuding Dabaicha' and further explored the important chemical substances against *E. vexans* infection. In total, 1,166 compounds were identified during the entire course of infection, among which 73 common compounds were significantly accumulated involved in the important antimicrobial substances of flavonoids and phenolic acids, including kaempferol (3,5,7,4'-tetrahydroxyflavone), kaempferol-3-*O*-sophoroside-7-*O*-glucoside, phloretin, 2,4,6-trihydroxybenzoic acid, galloylprocyanidin B4, and procyanidin C1 3'-*O*-gallate, which indicated that these metabolites might positively dominate resistance to *E. vexans*. Furthermore, flavone and flavonol biosynthesis was found to be closely related to resistance to *E. vexans*. Additionally, total flavonoids, phenolics, alkaloids, and terpenoids contributing to antimicrobial and antioxidant capacity were significantly altered in four different infection periods, especially LeafS2 (the second stage of infection), in which the most concentration accumulated. The leaves affected by *E. vexans* infection at LeafS2 had the relatively highest antioxidant activity. Accordingly, this study provided a theoretical support for and comprehensive insights into the effects on the metabolite changes, chemical components, and antioxidant activities of blister blight caused by *E. vexans*.

Keywords: antioxidant activity; blister blight; *Camellia sinensis*; *Exobasidium vexans*; metabolite; plant-pathogen interaction; tea

3.1 Introduction

Tea [*Camellia sinensis* (L.) O. Kuntze] is an important economic crop cultivated in tropical and subtropical regions (Wangnow et al., 2018). The popular nonalcoholic beverage processed from the fresh and tender leaves of tea plants is consumed by a vast number of people globally. Tea contains active components, including polyphenols, caffeine, flavanones, catechins, terpenes, theanine, and carbohydrates (Wei et al., 2021; Zhou et al., 2021). *C. Sinensis* 'Fuding Dabaicha' is the standard comparison species in the regional test of Chinese tea and one of the earliest clonal tea cultivars approved at the national level (Huacha No. 1, GSCT1) (Wang et al., 2019). Fuding Dabaicha is a variety of small arbor tea characterized by bulging leaf surfaces, numerous hairs, green leaves, and yellow-green buds with the high concentrations of amino acids (4.3%), tea polyphenols (16.2%), catechins (11.4%), and caffeine (4.4%); its freshly harvested tea shoots have generally been processed into white, green, oolong, and black teas (Fang et al., 2019). This tea variety, as one of the most commonly used parents in tea breeding, has been widely promoted nationwide due to its early germination, heavy buds, high yield of more than 200 kg/667 m^3, as well as strong stress resistance and adaptability. Guizhou Province has the largest tea plantation area in China, with more than 7 million acres of tea gardens, with approximately 80% of tea varieties in these gardens being Fuding Dabaicha.

However, Fuding Dabaicha variety frequently suffers from blister blight disease from May to August, significantly affecting the utilization rate of fresh tea leaves and the comprehensive value (Liu et al., 2021). Blight blister disease caused by *E. vexans*, an obligate pathogen, is one of the severe foliar diseases of tea plants, mainly occurring in major tea-planting countries, such as China, India, Japan, South Korea, Thailand, Indonesia, and Sri Lanka (Nagao, 2012; Mur et al., 2015; Sinniah et al., 2016). Currently, the application of chemical reagents and fungicides is the main strategies to prevent and control *E. vexans* infection in tea (Sowndhararajan et al., 2013). However, the phytotoxicity and fungicide residues caused by the application

of these control methods have created a major problem and great challenge to food safety and environmental pollution (Karunarathna et al., 2020). Although several kinds of plant endophytic fungi, including endophytic *Trichoderma* spp. (Amperes et al., 2020), *Fusarium* spp. (Sayed and El-Sayed, 2020), and *Colletotrichum* spp. (Shi et al., 2021), have become important available biocontrol resources for fungal diseases, their efficacy is not particularly significant (Jacques et al., 2018). Thus, several effective strategies, such as breeding and cultivation of resistant varieties, may fundamentally solve the problems of plant disease control and increase tea production in terms of quantity as well as quality.

Approaches to disease control and prevention based upon host-resistance mechanism, however, have not been systematically investigated. Plant responses to pathogen infection may be related to massive changes in metabolite biosynthesis and gene expression. It was reported that the infection of tea plants caused by *E. vexans* resulted in the accumulation of polyphenol oxidase and peroxidase activities, as well as the reduction of catechin, chlorophyll, and carotenoids (Punyasiri et al., 2005). Flavonoids, the most abundant chemical class of plant and fungal secondary metabolites, including flavone and flavonol, flavanone and flavanonol, flavane and flavanol, are the main contributors of tea's refreshing taste and bright yellow color in soup, as well as its antioxidant, anti-inflammatory, and other functions (Huang et al., 2022). In addition, gallocatechin, kaempferol, flavan-3-ols, and quercetin have been shown to be involved in a certain antimicrobial activity against diseases (Da Silva et al., 2014; Aboody and Mickymaray, 2020) and may act as a passive or induced barrier to resist pathogens (Manoela et al., 2007), whose main targets include protein kinase, p-diphenyl ethylene oxidase, fatty acid synthase, and lipoxygenase of pathogens. With the exception of these compounds, proanthocyanidins, plant defense compounds, also have been reported to inhibit macerating fungal enzymes of *Botrytis cinerea* in grapes (Pezet et al., 2003) and to be used as potent antimicrobial agents (Anastasia et al., 2011; Zang et al., 2013). Proanthocyanidins in tea plants are synthesized through epicatechin pathway, the levels of which were observed to

increase approximately 10-fold during infection, whereas epigallocatechin and free epicatechin showed a decreasing trend after infection. Moreover, the contents of epigallocatechin gallate, gallate esters, and epicatechin gallate were relatively high at the early stage of infection but decreased with the maturation of disease spots (Nimal Punyasiri et al., 2004). In addition to the inhibitory effects of proanthocyanidins on *E. vexans* infection, a series of other internal changes occurred in the leaves, including a decrease of several antimicrobial metabolites, such as polysaccharides, quercetin, apigenin, and myricetin, while the synthesis of signal transduction substances, including SA and JA, was interfered to facilitate the colonization and development of *E. vexans* in tea plants. Thus, pathogen resistance to blister blight probably depends on antimicrobial metabolites because of the rich chemical composition of tea, which can be identified if applied to breeding programs.

Metabolomics provides a comprehensive viewpoint on the dynamic changes in chemical components, and the mechanism of plant defense against pathogen infection in terms of small molecular substances and cellular levels (Feussner and Polle, 2015). For instance, the alteration of several nutritional and functional ingredients or potential antimicrobial substances (e.g., caffeine and flavonoids) and important plant defense hormones (e.g., SA and JA) were associated with tea blister disease infection. In current study, ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)-based metabolomics and biochemical analysis were employed to investigate the metabolic changes, chemical components, and antioxidant activities in healthy and *E. vexans*-infected leaves of Fuding Dabaicha as blight blister symptoms develop. At the same time, metabolomics revealed differential metabolic profiles with potential antimicrobial efficacy.

3.2 Materials and methods

3.2.1 Tea samples collection

Fuding Dabaicha was planted in the tea plantations of Maojian town in Duyun city, China (107°36′E, 26°18′N; altitude 1738m) under natural conditions. The samples of this variety were collected from the same plot with identical field management conditions in August 2021. Plant samples (the third leaf) of uniform size were randomly harvested based on the natural susceptibility status of tea leaves, which were divided into non-infected leaves (Healthy leaves; LeafCK) and infected leaves showing early symptoms with translucent spot formation (Stage 1; LeafS1), disease spots with typical blisters (Stage 2; LeafS2), blister spots depressions (Stage 3; LeafS3), and blisters causing necrotic spots on leaves (Stage 4; LeafS4) (**Figure 3.1**). To avoid any deviation occurring within a stage of blister blight conversion process, five subsamples were collected for each individual sample. The samples were then immediately frozen in liquid nitrogen and stored at -80°C for refrigeration until further metabolomic analysis and the determination of biochemical components and antioxidant activities. Three biological replicates in each treatment were performed for the detections.

Figure 3.1 Various stages of blister blight disease on tea (*Camellia sinensis*) leaves. Healthy leaves (CK) and tea leaves infected with tea blister blight (*E. vexans*) at designated infection stages 1 (S1), 2 (S2), 3 (S3), and 4 (S4).

3.2.2 Sample preparation and extraction

The samples from either healthy or *E. vexans*-infected tea leaves were freeze-dried using a vacuum freeze-dryer (Scientz-100F) and ground to a fine powder at 30 Hz for 1.5 min using a mixer mill (MM 400, Retsch, Shanghai, China) with a zirconia bead. Then, 100 mg of lyophilized powder was dissolved in 1.2 ml of 70% methanol solution and vortexed six times (every 30 min for 30 s) overnight at 4°C. Following centrifugation at 12,000 × *g* for 10 min, the extracts were filtrated (SCAA-104, 0.22 μm pore size; ANPEL, Shanghai, China) before UPLC-MS/MS analysis.

3.2.3 UPLC-MS/MS conditions

The extracts were determined using an UPLC-ESI-MS/MS system (UPLC, SHIMADZU Nexera X2, https://www.shimadzu.com.cn/, accessed 22 February 2022; MS, Applied Biosystems 4500 QTRAP, https://www.thermofisher.cn, accessed 22 February 2022) equipped with Agilent SB-C18 column (1.8 μm, 2.1 mm × 100 mm). The analytical conditions were as follows: the mobile phase consisted of solvent A (pure water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). Metabolites detections were performed with a gradient program that employed the starting conditions of 95% A and 5% B; a linear gradient to 5% A and 95% B was programmed within 9 min, and a composition of 5% A and 95% B was held for 1 min; subsequently, a composition of 95% A and 5.0% B was adjusted within 1.1 min and held for 2.9 min. The flow velocity was set as 0.35 ml/min; the column oven temperature was maintained at 40°C; the injection volume was set as 4 μl. Later, linear ion trap (LIT) and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (Q TRAP) and AB4500 Q TRAP UPLC/MS/MS system equipped with an ESI Turbo Ion-Spray interface, which was operated with Analyst 1.6.3 software (AB Sciex, Framingham, Massachusetts, USA) in positive and negative ion modes using the ESI source operation parameters based on our previous report (Zhou et al., 2022).

3.2.4 Quality control samples

Quality control (QC) samples were prepared by mixing healthy and *E. vexans*-infected tea leaves to analyze the repeatability of samples under the same treatment method. In the process of instrument detection, one QC sample was inserted into every 10 detection samples to examine the repeatability of the entire analytic process.

3.2.5 Determination of chemical components

3.2.5.1 Total flavonoids content

The total flavonoids content was determined according to the method of $NaNO_2$-$AlCl_3$-NaOH with slight modifications (Sahar et al., 2016). Around 0.1 g of tea powders was extracted with 1.5 ml of 60% ethanol, and then vibrated in the warm water at 60℃ for 2 h. The solution was centrifuged at 12,000 × *g* for 10 min at 25℃, and the supernatant was subsequently collected in a 10-ml volumetric flask. The supernatant was diluted with 60% ethanol to a final volume of 10 ml. After that, 0.5 ml of extracted solution was mixed with 2 ml of distilled water and 0.15 ml of 5% $NaNO_2$ solution. Then, 0.15 ml of 10% $AlCl_3$ solution and 2 ml of 4% NaOH solution were added to the mixture at an interval of 6 min. Finally, 60% ethanol solution was added to the mixture to produce to a total volume of 5 ml and allowed to stand for 30 min. The enzyme-labeled instrument was preheated for more than 30 min and the wavelength was adjusted at 510 nm. Total flavonoids content was calculated based on the standard curve, which was obtained as $y = 2.2152x - 0.0298$ ($R^2 = 0.9932$), where x is the concentration of standard, and y is the difference value between the absorbance of the samples and the blank.

3.2.5.2 Total phenolic content

The total phenolics content was determined using the method of Folin-Ciocalteu with some modifications (Pinelo et al., 2003). About 2 g of ground tea sample was extracted with 20 ml of distilled water and then centrifuged at 3000 × *g* for 10 min to collect the supernatant. Subsequently, 1 ml of standard solution or

sample extract solution (primary extraction was diluted with 50-fold distilled water), 1 ml of Folin-Ciocalteu reagent, and 3 ml of 20% Na_2NO_3 solution were mixed in a 15-ml tube. After boiling in a water bath at 50°C for 30 min, the absorbance was adjusted to the wavelength of 760 nm with the distilled water as the blank control. Total phenolics content was calculated based on the standard curve of gallic acid, which was obtained as $y = 0.2563x + 0.0186$ ($R^2 = 0.9974$).

3.2.5.3 Total free amino acids content

The amino acid content was determined using the ninhydrin chromogenic method. Three grams of tea powder was added to 350 ml of boiling water and then soaked in the boiling water bath at 100°C for 45 min with a shake every 15 min. The solution was immediately centrifuged at 12,000 × *g* for 10 min at 4°C, and the residue was extracted two or three times with a small amount of hot distilled water. Subsequently, the filtrate was poured into a 500-ml volumetric flask and allowed to stand at room temperature. The extraction was standardized to a final volume of 500 ml with distilled water and shaken well for standby. The mixture with 4 ml of distilled water, 0.5 ml of ninhydrin solution, and 2 ml of phosphate buffer (pH 8.0) was sequentially added into 0.0, 1.0, 1.5, 2.0, 2.5, and 3.0 ml of 100 mg/liter of amino acid standard solution. After heating in the boiling water for 15 min, the mixtures were added to a final volume of 25 ml with the distilled water. The enzyme-labeled instrument was preheated for 30 min, and the absorbance was adjusted to the wavelength of 510 nm with the blank reagent as reference solution.

3.2.5.4 Total alkaloids content

The tea samples were dried at 60°C and then crushed and sieved. About 0.05 g of tea powder was weighed accurately and extracted with 1 ml of the mixture of dichloromethane/methanol/extract (40:10:1, *v*/*v*/*v*). The solution was extracted by shaking at room temperature for 30 min, and then by ultrasound for 30 min. After that, the solution was supplemented with the mixture to a final volume of 1 ml and subsequently centrifuged for 10 min at 4,000 × *g* at room temperature to collect the

supernatant. The procedure to determine alkaloids content was following the manufacturer's instructions (Norminkoda Biotechnology Co., Ltd. Wuhan, China). The absorbance was measured at the wavelength of 415 nm, and the standard curve was obtained as $y = 0.0388x + 0.0044$ ($R^2 = 0.9995$).

3.2.5.5 Total terpenoids content

First, 0.25 g of the ground tea powder was weighed accurately and soaked in 4.5 ml of saturated NaCl solution for 6 h. The mixture of tea powder/NaCl solution at a ratio of 1:18 was distilled for 8 h to collect the layer of volatile oil. Then, a moderate amount of Na_2SO_4 solution was added to the extraction to dry overnight, and the volatile oil was obtained after the removal of Na_2SO_4 solution. About 1 ml of volatile oil was mixed with absolute alcohol to a final volume of 10 ml and shaken well. Subsequently, the final extraction was operated in the same method with the above mixture. After that, 1 ml of the sample extraction was put in a water bath to evaporate all solvent, and then 3 ml of 5% vanillin-glacial acetic acid solution and 3.5 ml of perchloric acid were added to the mixture. Then, it was kept in a water bath at 71℃ for 28 min, and quickly cooled to room temperature. Finally, the mixture was supplemented with 1.5 ml of glacial acetic acid to a total volume of 8 ml. The absorbance was determined at a wavelength of 600 nm, and the standard curve of terpenoid content was obtained as $y = 31.614x + 0.0054$ ($R^2 = 0.9996$).

3.2.6 Determination of antioxidant activities

3.2.6.1 DPPH method

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity was determined using the method described by Sílvia et al. (2020). More specifically, the extraction and methanol DPPH solution were mixed in a 96-well microplate at a ratio of 1:19 (*v/v*) to a total volume of 200 µl. The microplates were covered with foil paper and allowed to stand for 30 min at room temperature. The absorbance was determined at a wavelength of 517 nm, and the standard curve was obtained as $y = 3.4642x + 28.443$ ($R^2 = 0.9909$).

3.2.6.2 FRAP method

The ferric reducing activity power (FRAP) was determined using the enzyme-labeled instrument following the method described by Sílvia et al. (2020) with some modifications. About 0.05 g of dry powder sample was mixed with 1 ml of 80% ethanol for homogenization. The mixture was ultrasonically extracted at 200 to 300 W for 30 min at 60℃ (shaken and mixed once at an interval of 5 min), and then centrifuged at 12,000 × *g* for 10 min to collect the supernatant for detection. Afterwards, 10 mM of 2,4,6-Tri(2-pyridyl)-S-triazine (TPTZ) was added to 40 mM of HCl solution and mixed with 20 mM of $FeCl_3 \cdot 6H_2O$ and 10-fold volume of buffer (pH 3.6). Subsequently, 275 µl of $Fe3^+$-TPTZ was mixed with 25 µl of the extraction. After incubation for 5 min, the absorbance was adjusted to a wavelength of 590 nm, and the standard curve was obtained as $y = 0.0179x + 0.0034$ ($R^2 = 0.9992$).

3.2.6.3 ABTS method

The 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) antioxidant activity was performed by enzyme-labeled instrument. ABTS was prepared by mixing 5 mM of NaH_2PO_4, 5 mM of Na_2HPO_4, and 154 mM of NaCl, and then the pH was adjusted to 7.4. Afterwards, 7 mM of ABTS was mixed with 2.5 mM of potassium persulfate to prepare ABTS radical solution, which was subsequently stored at room temperature for 16 h under dark conditions. The mixture was mixed with ethanol to adjust absorbance at a wavelength of 734 nm. Sample solution was detected using the previously described DPPH method. The absorbance was determined at a wavelength of 734 nm, and standard curve was obtained as $y = 0.4834x + 0.0091$ ($R^2 = 0.9868$).

3.2.7 Statistical analysis

Mass spectrometry data processing and quantitative analysis were performed using Analyst 1.6.3 software (AB Sciex) and triple quadrupole mass spectrometry multiple reaction monitoring (MRM), and all metabolites were subjected to peak extraction and peak correction. All data sets were processed and analyzed with

orthogonal partial least squares-discriminant analysis (OPLS-DA) model to evaluate the effectiveness of the model by predicting the values of the parameters R^2X, R^2Y, and Q^2. Differentially accumulated metabolites (DAMs) between groups were determined by variable importance in projection (VIP) ≥ 1 and absolute $\log_2$ fold change (FC) ≥ 1. VIP values were extracted from OPLS-DA results, which contained score plots and permutation plots generated using R package MetaboAnalystR. The data were log transform ($\log_2$) and mean centering before OPLS-DA. The identified metabolites were then annotated using Kyoto Encyclopedia of Genes and Genomes (KEGG) compound database (http://www.kegg.jp/kegg/compound/), and annotated metabolites were subsequently mapped to KEGG pathway database (http://www.kegg.jp/kegg/pathway.html). Pathways with significantly regulated metabolites were then fed into metabolite sets enrichment analysis (MSEA), with significance determined by *p*-values of the hypergeometric test.

3.3 Results

3.3.1 Full mass spectrometric analysis and data results evaluation

UPLC-MS/MS-based widely targeted metabolome analysis was conducted on samples from healthy leaves (LeafCK) and *E. vexans*-infected leaves (LeafS1, LeafS2, LeafS3, LeafS4). The overlay spectra of total ion current (TIC) and MRM diagram with different QC samples showed that the signal stabilization of the same sample was better when compounds were determined at different times (**Appendix Figure 1**). The principal component analysis (PCA) scores plot of all tea samples (including QC samples) revealed a definite trend of intergroup separation and intragroup aggregation, with 27.6% and 19.1% variation explained by the first two principal components (PC1 and PC2), respectively, indicating that QC samples exhibited similar metabolic profiles and were significantly distinguished from the other five groups and that the data processing analysis in this study was reliable and repeatable (**Figure 3.2**). OPLS-DA models were performed to further investigate and distinguish the intrinsic similarity of metabolic profiles in different comparison groups. The results

showed that R^2X, R^2Y, and Q^2 of OPLS-DA scores were greater than 0.717, 1, and 0.978, respectively, in all pairwise comparisons (**Figure 3.3 and 3.4**), which suggested that the model exhibited superior predictability and reliability, accurately reflecting the trend of the metabolites among the comparisons. In addition, the mutual verification of this model confirmed that it was feasible to compare the metabolites of different groups, and the obtained DAMs were statistically significant.

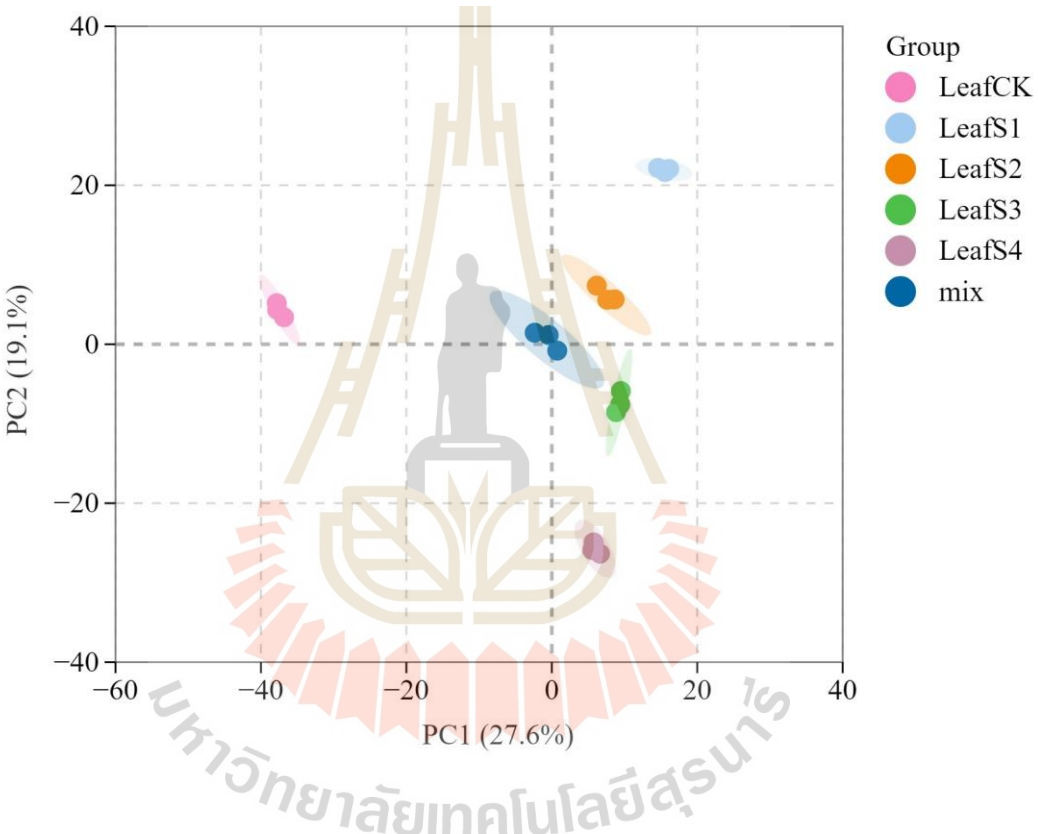

Figure 3.2 Principal component analysis (PCA) score scatter plot of total tea (*Camellia sinensis*) samples. CK, healthy leaves; designated *E. vexans* infection stages: 1 (S1), 2 (S2), 3 (S3), and 4 (S4).

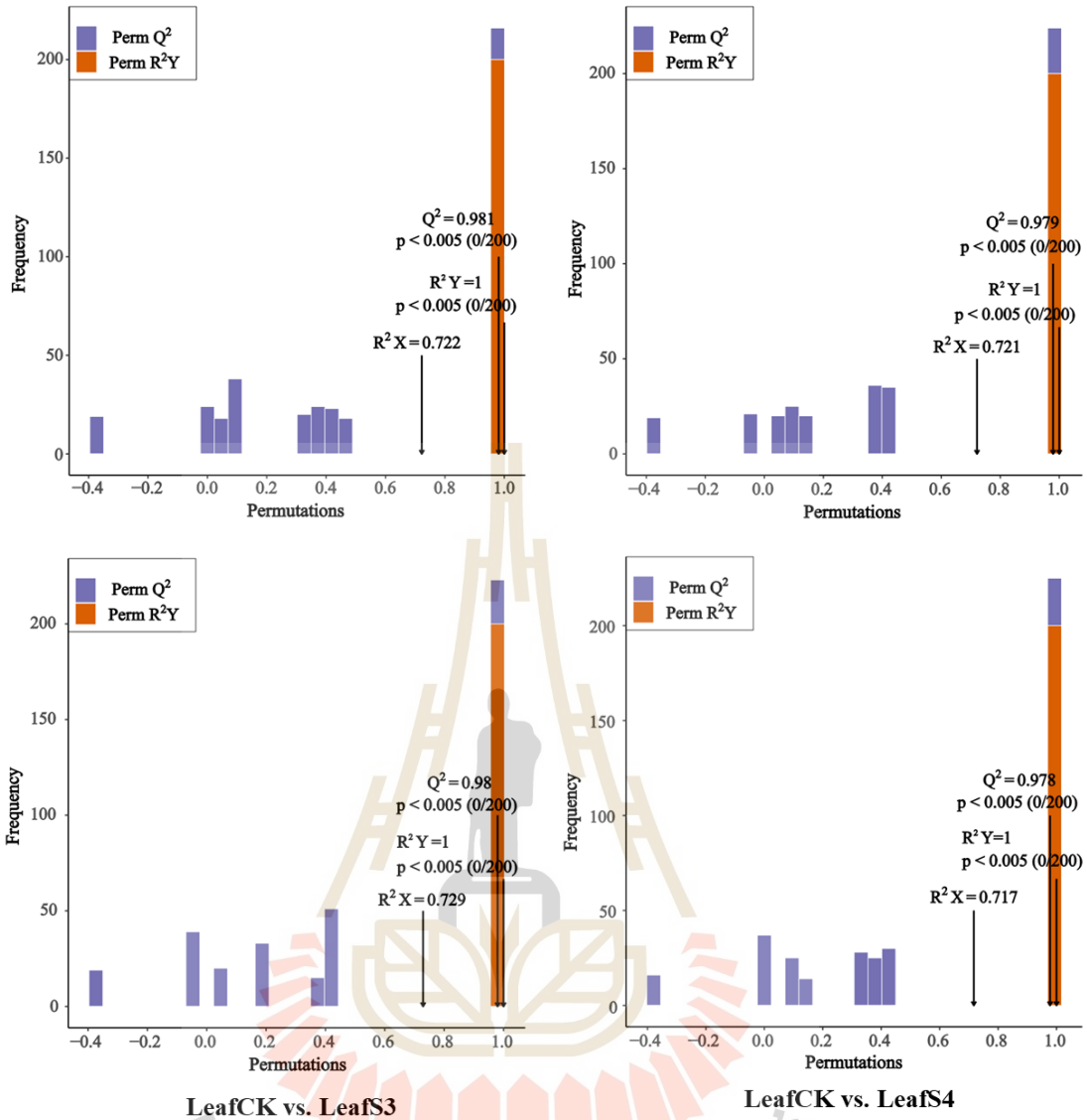

Figure 3.3 OPLS-DA permutation plots of DAMs in tea responsive to *E. vexans* infection among all pairwise comparisons. R^2Y scores and Q^2 values represent the interpretation rate of the model to the Y matrix and the prediction ability of the model, respectively. When $Q^2 > 0.5$, the model can be considered an effective model, and $Q^2 > 0.9$ is an excellent model.

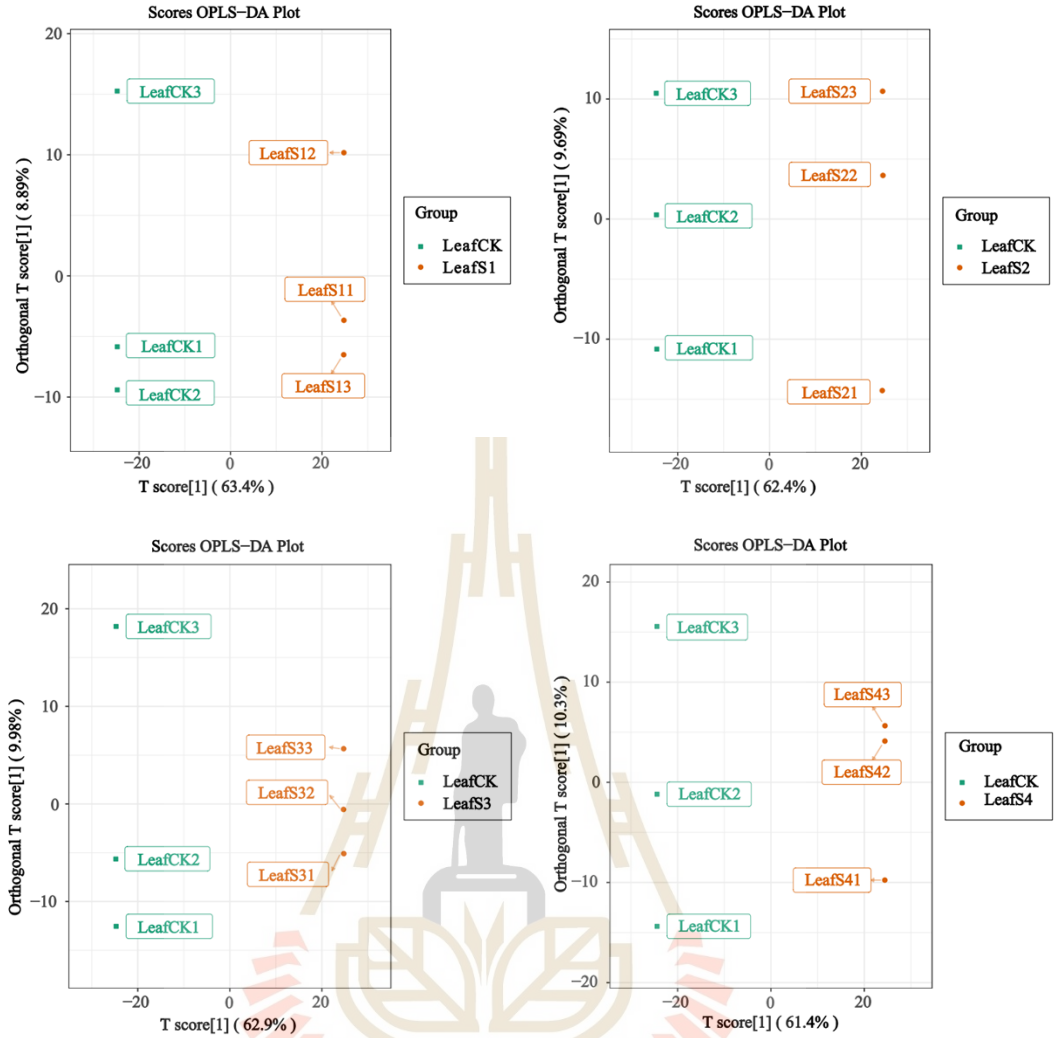

Figure 3.4 OPLS-DA Score plots of DAMs in tea responsive to *E. vexans* infection among all pairwise comparisons.

3.3.2 Metabolite analysis of tea in response to *E. vexans* infection

Healthy and susceptible tea leaves were determined and analyzed by UPLC-MS/MS-based on retention time, mass-to-charge ratio, and peak intensity matrix. A total of 1,166 metabolites were identified, as expressed in clustering heatmap, demonstrating that the relative changes in metabolite content obtained during the infection existed significant differences. Obviously, these DAMs identified were divided into four categories: LeafS2 and LeafS3 were clustered together into one category, whereas separated from LeafS1, LeafS4, and LeafCK (**Figure 3.5A**). In

addition, the different metabolites of tea in responsive to *E. vexans* infection could be mainly classified into 11 categories according to their chemical properties, including flavonoids (347), phenolic acids (182), lipids (111), amino acids and derivatives (97), organic acids (83), alkaloids (79), nucleotides and derivatives (62), lignans and coumarins (51), terpenoids (28), tannins (27), and others (99) (**Figure 3.5B**). Among these, flavonoids were predominant compounds, accounting for 29.8% of the total metabolites, followed by phenolic acids at 15.6%.

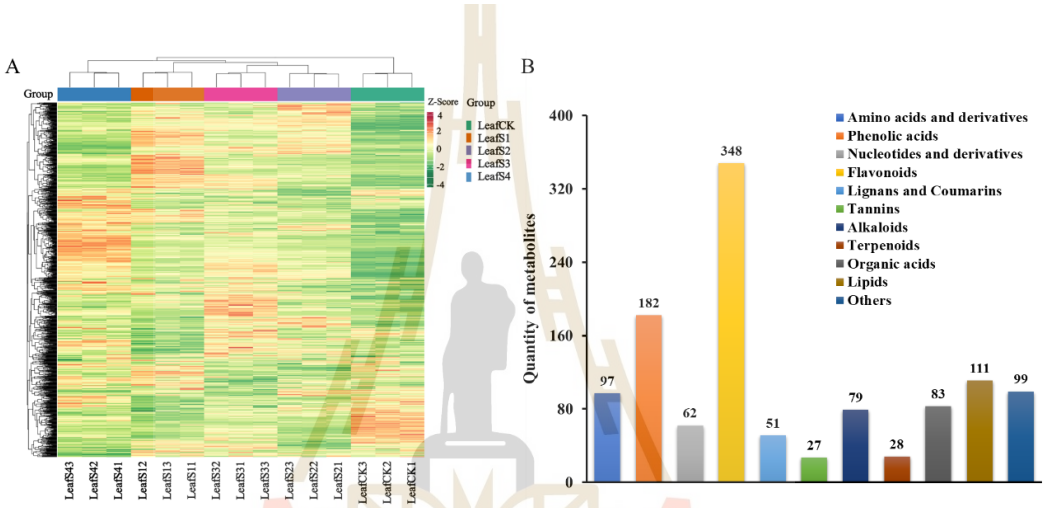

Figure 3.5 Overview of metabolome analysis of tea (*Camellia sinensis*) responsive to *E. vexans* infection. (**A**) Heat map of all metabolites. (**B**) Category and number of DAMs. "Others" includes vitamins, saccharides, and alcohols.

3.3.3 DAMs screening of tea leaves in response to *E. vexans* infection

To further clarify DAMs between healthy and infected leaves, combined univariate and multivariate statistical analysis was performed. Particularly, VIP ≥ 1 and FC ≥ 2 or FC ≤ 0.5 were used to screen DAMs in different comparisons according to the results of multivariate analysis OPLS-DA model. In detail, the most DAMs (284) were observed in LeafCK vs. LeafS1 (**Figure 3.6A**, 225 up-regulated and 59 down-regulated substances); the expression of 252 DAMs significantly altered was found in LeafCK vs. LeafS2 (**Figure 3.6B**, 171 up-regulated and 81 down-regulated substances); 259 DAMs were found in LeafCK vs. LeafS3 (**Figure 3.6C**, 223 up-regulated and 36

down-regulated substances) and 240 DAMs were found in LeafCK vs. LeafS4 (**Figure 3.6D**, 203 up-regulated and 37 down-regulated substances).

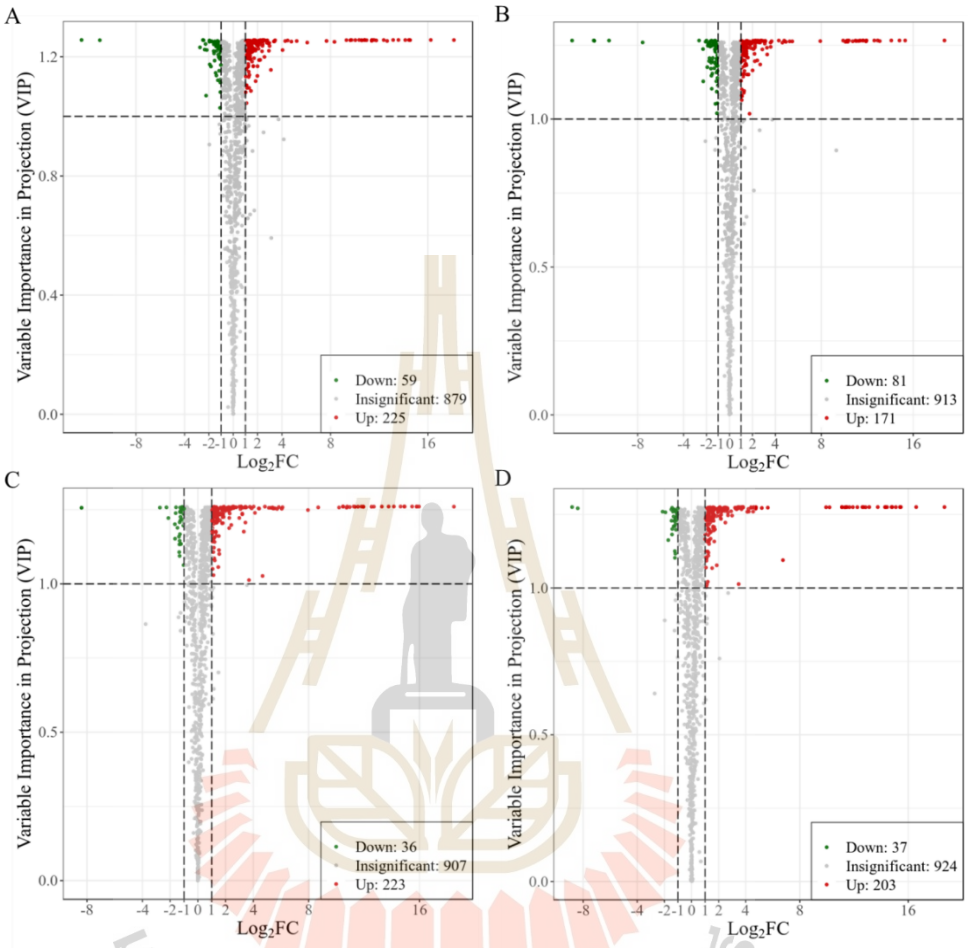

Figure 3.6 Volcano plot of DAMs in four pairwise comparisons. (**A)** LeafCK vs. LeafS1, (**B)** LeafCK vs. LeafS2, (**C)** LeafCK vs. LeafS3, and (**D)** LeafCK vs. LeafS4.

The metabolites with the highest values for fold change in the top 20 (up-regulation and down-regulation) showed that the fold changes of DAMs were related to infection severity of leaves (**Figure 3.7**). The largest up-regulation in fold change of DAMs was observed in LeafCK vs. LeafS4, including alkaloids (1), flavonoids (1), terpenoids (4), organic acids (2), lipids (1), and phenolic acids (1). Among them, the relative abundance of *O*-acetyl *L*-carnitine was up-regulated most ($log_2FC > 18.67$),

followed by kaempferol-3-*O*-rhamnoside (afzelin)(kaempferin) (log_2FC > 16.58) and isothankunic acid (log_2FC > 15.45) (**Figure 3.7D**), and fold changes of top 10 up-regulated DAMs were all above 14. However, the largest down-regulation in fold change of DAMs was found in LeafCK vs. LeafS2, including flavonoids (6), nucleotides and derivatives (1), lipids (1), lignans and coumarins (1), and others (1). Among them, 1-hydroxypinoresinol-4'-*O*-glucoside has a large fold change (log_2FC < -13.71), followed by 4-pyridoxic acid-*O*-glucoside (log_2FC < -11.82), and chrysoeriol-8-*C*-arabinoside-7-*O*-rutinoside (log_2FC < -11.77) (**Figure 3.7B**).

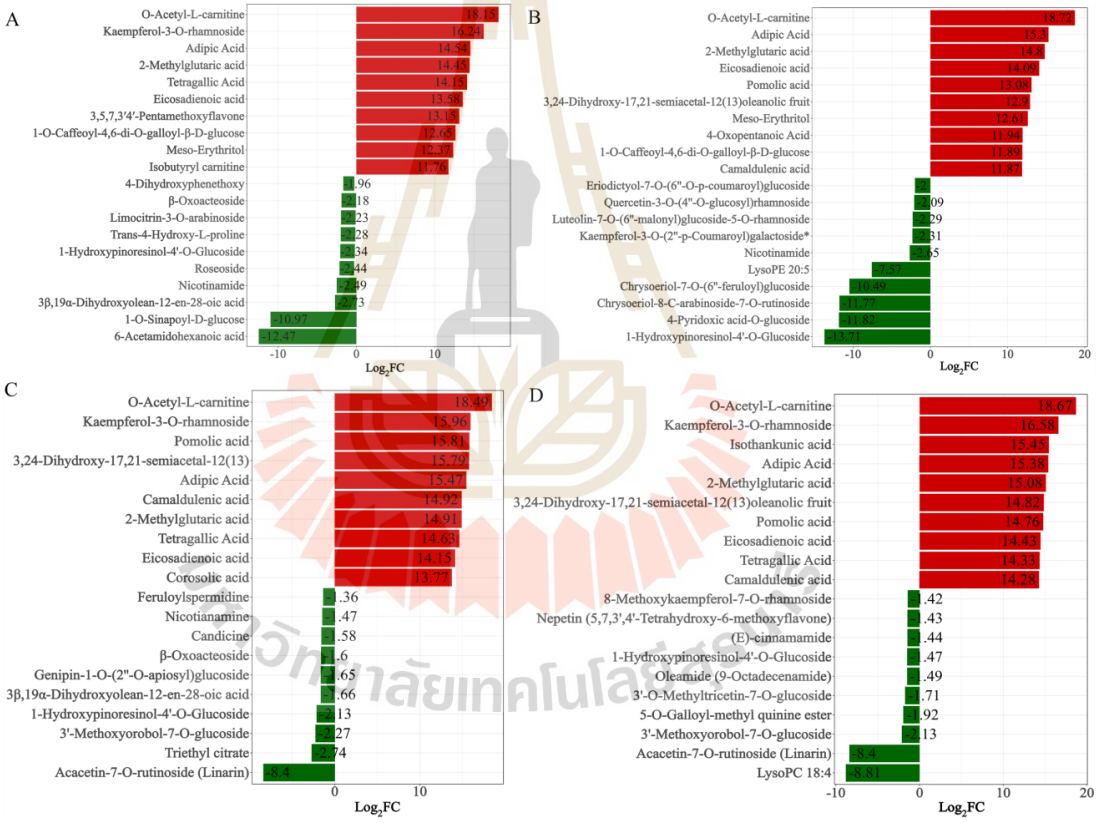

Figure 3.7 Difference multiple charts in four comparisons. (**A**) LeafCK vs. LeafS1, (**B**) LeafCK vs. LeafS2, (**C**) LeafCK vs. LeafS3, and (**D**) LeafCK vs. LeafS4. Red and green bars indicate up- and down-regulated DAMs, respectively.

Both common and unique DAMs exhibited in the comparison of different groups are shown in Venn diagram (**Figure 3.8**). A total of 493 substances were identified in LeafCK vs. LeafS1, LeafCK vs. LeafS2, LeafCK vs. LeafS3, and LeafCK vs. LeafS4, of which 77 common metabolites were found among these four comparison groups, including lipids (31), flavonoids (9), organic acids (9), phenolic acids (5), amino acids and derivatives (4), alkaloids (3), terpenoids (3), nucleotides and derivatives (3), lignans and coumarins (2), tannins (2), and others (6). In detail, the expression of DAMs in the susceptible leaves was significantly up-regulated, except for roseoside, trans-4-hydroxy-*L*-proline, nicotianamine, and 1-hydroxypinoresinol-4'-*O*-glucoside, which indicated that massive amounts of primary metabolites were required to be transformed into physiological active substances for the survival of pathogens. Thus, these primary metabolites, which included organic acids, lipids, amino acids, and their derivatives, were found to be significantly up-regulated. Besides that, *E. vexans* infection resulted in a significantly increased secretion of secondary metabolites that included alkaloids, phenolic acids, lignans and coumarins, flavonoids, terpenoids, and tannins (**Appendix Table 1**). In addition, a total of 68 DAMs specific to LeafCK vs. LeafS1 were only impacted by the incidence of *E. vexans* infection (**Figure 3.8**). Among the 47 substances that were significantly up-regulated, trimethyllysine, cinnamoylglycine, and lysoPC 16:1* showed relatively higher content, with $\log_2$FC > 2.04. Conversely, 21 differential compounds were observed to be significantly down-regulated, with example metabolites including 6-acetamidohexanoic acid ($\log_2$FC < -12.47), 1-*O*-sinapoyl-*D*-glucose ($\log_2$FC < -10.97), and secoisolariciresinol 4-*O*-glucoside ($\log_2$FC < -1.60). As the appearance of disease spots with typical blisters, a total of 48 metabolites were found in the comparison of LeafCK vs. LeafS2, of which tellimagrandin II and all four lignans and coumarins of 5'-methoxyisolariciresinol-9'-*O*-glucoside, eucommin A, isolariciresinol-9-*O*-glucoside, and 5'-methoxymatairesinoside decreased significantly. Also, 60% of flavonoids, such as chrysoeriol-7-*O*-(6''-feruloyl)glucoside, kaempferol-3-*O*-glucoside-7-*O*-rhamnoside*, and quercetin-3-*O*-robinoside*, were specifically down-regulated. Whereas the quantities of lipids (e.g.,

13-methylmyristic acid and palmitoleic acid) and phenolic acids (sinapinaldehyde, 1-*O*-salicyl-*D*-glucose, and salidroside) were significantly up-regulated. Furthermore, 31 DAMs were unique to LeafCK vs. LeafS3, among which seven phenolic acids and two lipids (myristic acid and palmitic acid) showed an upward trend; in contrast, *N*-methyl-trans-4-hydroxy-*L*-proline related to amino acids and derivatives significantly decreased; while the unique metabolites, which included flavonoids (10), alkaloids (8), and organic acids (2), were altered markedly. Except for benzyl salicylate, nepetin(5,7,3',4'-tetrahydroxy-6-methoxyflavone), 3'-*O*-methyltricetin-7-*O*-glucoside, hesperetin-7-*O*-neohesperidoside(neohesperidin)*, 10-dehydrogeniposide, quercetin-3-*O*-(6''-*O*-p-coumaroyl)galactoside, and quercetagetin, and the most DAMs obtained were significantly accumulated in LeafCK vs. LeafS4.

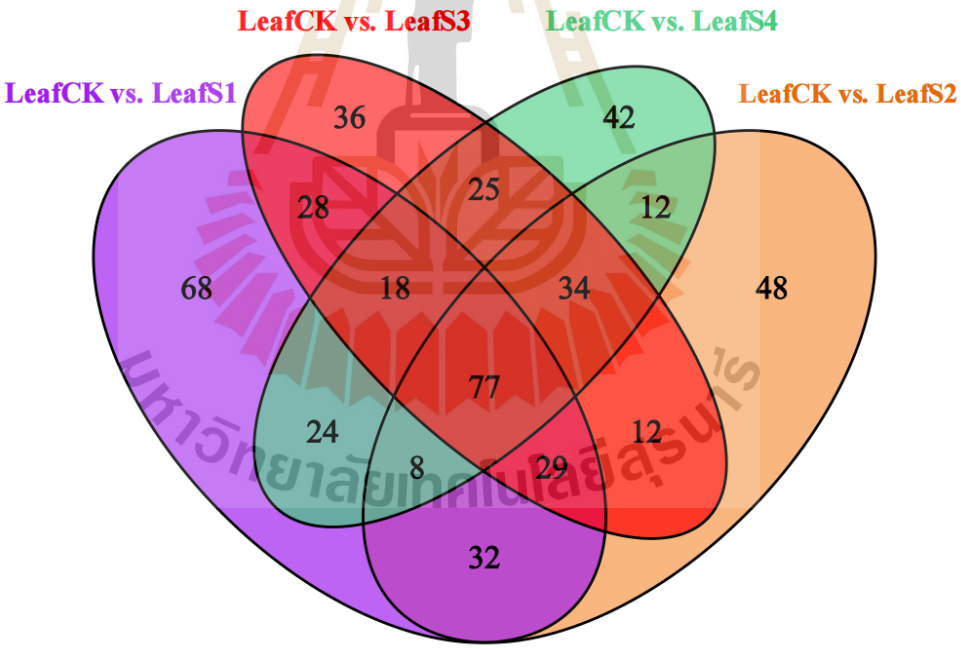

Figure 3.8 Venn diagram of DAMs in LeafCK vs. LeafS1, LeafCK vs. LeafS2, LeafCK vs. LeafS3, and LeafCK vs. LeafS4.

3.3.4 KEGG functional annotation and enrichment analysis of DAMs

Utilizing bioinformation databases such as KEGG enables understanding the mechanism involved in metabolic pathways, allowing the most correlative biological pathways and DAMs to be obtained. KEGG pathway enrichment analysis revealed that the identified metabolites were enriched in 85 metabolic pathways across four comparisons. Notably, these pathways included those crucial to disease resistance such as phenylalanine metabolism, arginine and proline metabolism, flavone and flavonol biosynthesis, flavonoid biosynthesis, isoflavonoid biosynthesis, plant hormone signal transduction, and caffeine metabolism (**Appendix Table 2**). In addition, a maximum of 78 substances identified in the comparison between LeafCK and LeafS1 were annotated into 80 KEGG pathways. This was followed by 73 and 71 metabolites observed in LeafCK vs. LeafS3 and LeafCK vs. LeafS4, which were respectively annotated to 70 and 56 KEGG pathways. Furthermore, 58 minimal metabolites obtained from the LeafCK vs. LeafS2 were annotated for 48 KEGG pathways (**Appendix Figure 2**). Thus, KEGG pathway analysis indicated that several metabolites were involved in multiple metabolic pathways, and these multiple pathways were consistent across the comparison groups.

KEGG pathway enrichment for analysis of the differential compounds revealed that a total of seven significantly enriched pathways involved in linoleic acid metabolism, fructose and mannose metabolism, 2-oxocarboxylic acid metabolism, caffeine metabolism, biosynthesis of unsaturated fatty acids, flavone and flavonol biosynthesis, and alpha-linolenic acid metabolism were screened with $p \leq 0.05$ as threshold in the pairwise comparison groups (**Table 3.1**). Among them, one common pathway of linoleic acid metabolism mentioned above was highly enriched in four pairwise comparisons, with the highest percentage of metabolites in LeafCK vs. LeafS2 being 18.97% (**Figure 3.9**). One common enriched pathway involved in biosynthesis of unsaturated fatty acids was obtained among three different comparisons of LeafCK vs. LeafS2, LeafCK vs. LeafS3, and LeafCK vs. LeafS4, with the percentage range from 5.63% to 6.9% of annotated metabolites (**Figure**

3.9B-D). Additionally, two common enriched pathways were found in the two comparisons of LeafCK vs. LeafS1 and LeafCK vs. LeafS3 (**Figure 3.9A and 3.9C**), of which fructose and mannose metabolism was highly enriched in LeafCK vs. LeafS3 with 5.48% of annotated metabolites (**Figure 3.9C**), and 2-oxocarboxylic acid metabolism was higher obviously enriched in LeafCK vs. LeafS1 with 13.7% of annotated metabolites (**Figure 3.9A**). Notably, flavone and flavonol biosynthesis was significantly assigned to LeafCK vs. LeafS2, with a proportion of 15.52% of annotated metabolites (**Figure 3.9B**), indicating that flavone and flavonol biosynthesis, an important downstream pathway of flavonoid biosynthesis, might be the most highly relevant pathway for tea leaves to respond to *E. vexans* infection, especially in the mid-stage. Besides that, caffeine metabolism was markedly obtained from LeafCK vs. LeafS4 with 5.63% annotated metabolites (**Figure 3.9D**).

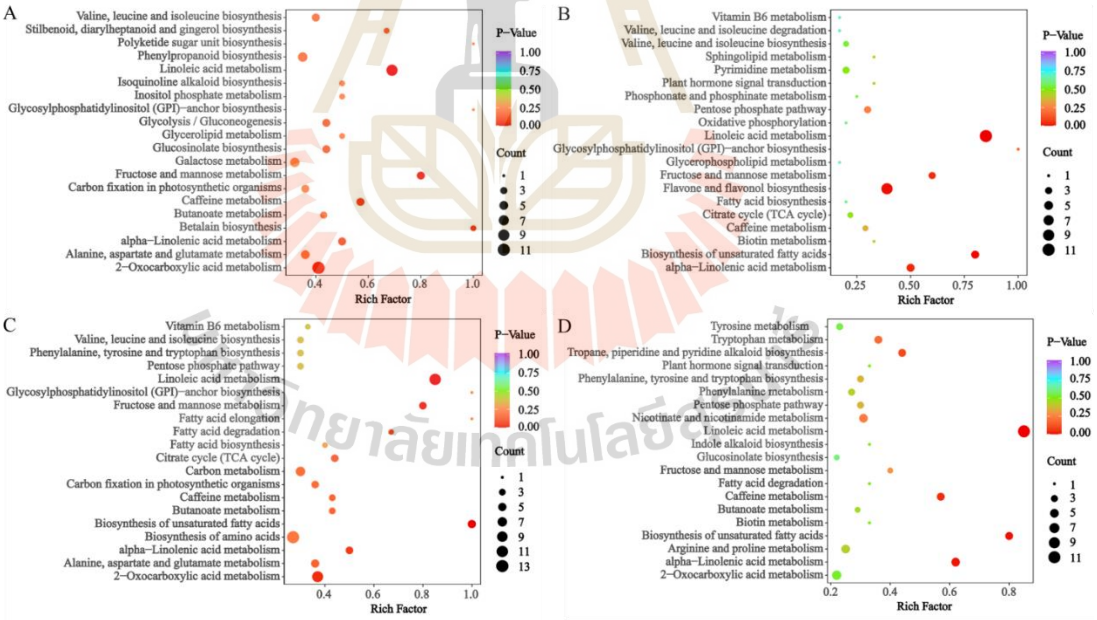

Figure 3.9 Top 20 KEGG enrichment pathways of DAMs in (**A**) LeafCK vs. LeafS1, (**B**) LeafCK vs. LeafS2, (**C**) LeafCK vs. LeafS3, and (**D**) LeafCK vs. LeafS4. Abscissa represents the degree of enrichment; ordinate represents the enriched pathways; color of the point represents the *p*-value.

Table 3.1 Significant enrichment pathway of DAMs in the comparison groups.

Groups	KEGG pathway	Ko-ID	*p*-value	Corrected *p*-value	Percent (%)	Compounds (ID)
LeafCK vs. LeafS1	Linoleic acid metabolism	ko00591	3.69×10^{-4}	2.76×10^{-2}	11.54	C04056, C14827, C14826, C01595, C14835, C04717, C14762, C14825, C14767
	Fructose and mannose metabolism	ko00051	1.06×10^{-2}	0.79	5.13	C00794, C00392, C00111, C01096
	2-oxocarboxylic acid metabolism	ko01210	2.38×10^{-2}	1	14.1	C00141, C06010, C00166, C06006, C00025, C14463, C00036, C00082, C00158, C17213, C00078
LeafCK vs. LeafS2	Linoleic acid metabolism	ko00591	8.98×10^{-8}	4.31×10^{-6}	18.97	C14767, C14835, C14825, C04056, C07289, C14762, C04717, C06426, C01595, C14827, C14826
	Biosynthesis of unsaturated fatty acids	ko01040	3.38×10^{-3}	0.16	6.9	C01595, C06427, C16525, C06426
	Flavone and flavonol biosynthesis	ko00944	7.72×10^{-3}	0.37	15.52	C01750, C21833, C05903, C05625, C03951, C05623, C12630, C04608, C12667
	alpha-linolenic acid metabolism	ko00592	3.14×10^{-2}	1	6.9	C16342, C06427, C16346, C21923
	Fructose and mannose metabolism	ko00051	3.66×10^{-2}	1	5.17	C00392, C01096, C00794

Note: Percentage represents the proportion of metabolites annotated into this pathway accounted for total differential compounds.

Table 3.1 Significant enrichment pathway of DAMs in the comparison groups (Continued).

Groups	KEGG pathway	Ko-ID	p-value	Corrected p-value	Percent (%)	Compounds (ID)
LeafCK vs. LeafS3	Linoleic acid metabolism	ko00591	1.23×10^{-6}	8.63×10^{-5}	15.07	C04717, C04056, C14827, C07289, C01595, C14825, C14835, C14767, C14762, C06426, C14826
	Biosynthesis of unsaturated fatty acids	ko01040	4.03×10^{-4}	2.82×10^{-2}	6.85	C06426, C01595, C06427, C16525, C00249
	Fructose and mannose metabolism	ko00051	8.22×10^{-3}	0.58	5.48	C00111, C00794, C01096, C00392
	2-oxocarboxylic acid metabolism	ko01210	3.98×10^{-2}	1	13.7	C06010, C14463, C00141, C00078, C00166, C00158, C00322, C00077, C00311, C00036
LeafCK vs. LeafS4	Linoleic acid metabolism	ko00591	8.99×10^{-7}	5.03×10^{-5}	15.49	C14825, C14835, C06426, C04056, C01595, C04717, C14762, C14767, C14826, C07289, C14827
	Biosynthesis of unsaturated fatty acids	ko01040	7.38×10^{-3}	0.41	5.63	C06426, C06427, C01595, C16525
	alpha-linolenic acid metabolism	ko00592	1.15×10^{-2}	0.64	7.04	C16346, C06427, C21923, C16316, C16342
	Caffeine metabolism	ko00232	3.67×10^{-2}	1	5.63	C00385, C16357, C07130, C16353

Note: Percentage represents the proportion of metabolites annotated into this pathway accounted for total differential compounds.

3.3.5 Effects of tea leaves infected with *E. vexans* on the key chemical components and antioxidant activities

3.3.5.1 The content of total flavonoids, phenolics, amino acids, alkaloids, and terpenoids

Flavonoids, phenols, amino acids, alkaloids, and terpenoids are essential metabolites in tea plants with strong antioxidant properties. They influence the color or flavor of tea leaves and play an important role in plant stress resistance against diseases and pests. The contents of these substances in healthy and infected tea leaves are shown in **Figure 3.10**. The total flavonoids content increased gradually and reached the peak in the second stage of infection (LeafS2), which was 1.28 times of that in healthy leaves; subsequently, it rapidly decreased to a level that had no significant difference compared with healthy leaves in the third stage (LeafS3) ($p > 0.05$); afterwards, the content of total flavonoids increased again and was 17.19% higher than that of healthy leaves in the fourth stage (LeafS4) (**Figure 3.10A**). While the total phenolics content decreased to 87.09% of healthy leaves in the first stage of *E. vexans* infection, it subsequently accumulated rapidly in the second stage (LeafS2) and was 34.48 % higher than that of the LeafS1 stage; after that, the content of total phenolics gradually decreased with the gradual necrosis of lesions (**Figure 3.10B**). Similarly, the change trend of total alkaloids and terpenoids content was consistent with that of flavonoids content affected by *E. vexans* infection; that is, the contents of these two substances were the highest in the second infected stage, 1.29 and 1.19 times that of healthy leaves, respectively (**Figure 3.10D and E**). Additionally, the content of total amino acids increased slightly at the initial stage of infection and then decreased to 24.25 mg/g at the LeafS2 stage; afterwards, it rapidly increased in the third of *E. vexans* infection, with a concentration 10.97% higher than that of healthy leaves; in the final stage (LeafS4), it was reduced again to a level that was no significantly difference from the level of LeafS1 stage ($p > 0.05$) (**Figure 3.10C**).

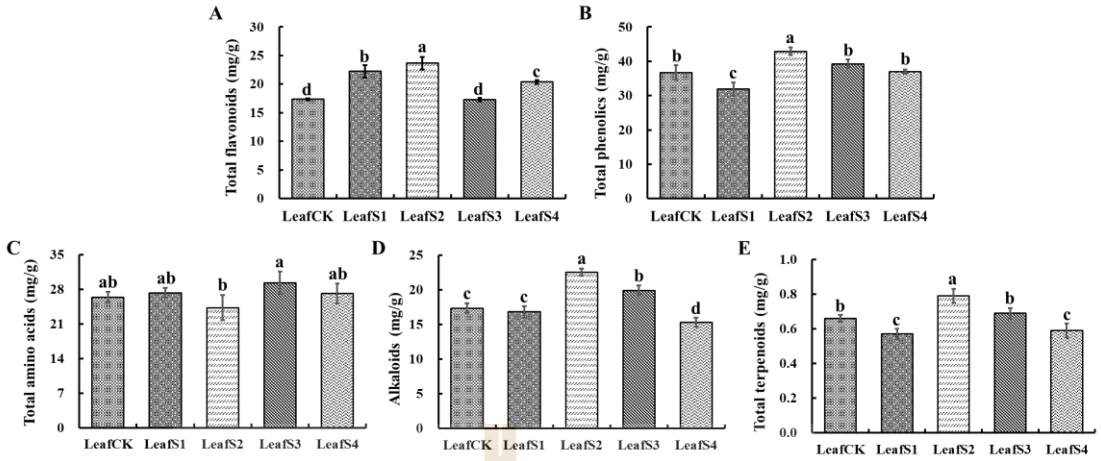

Figure 3.10 The content of chemical components of healthy leaves and four different infected leaves. (**A**) Total flavonoids content, (**B**) total phenolics content, (**C**) total amino acids content, (**D**) total alkaloids content, (**E**) total terpenoids content. Different letters above the bars indicate significant difference among the same data series ($p < 0.05$, n = 3). CK, healthy leaves; designated *E. vexans* infection stages: 1 (S1), 2 (S2), 3 (S3), and 4 (S4).

3.3.5.2 Antioxidant activities

The common assay methods, including DPPH radical scavenging activity, FRAP, and ABTS radical cation scavenging activity, were used to compare the antioxidant activity between healthy leaves and infected leaves at different stages. DPPH radical scavenging activity was observed to be the highest at approximately 2,182 µg Trolox/g in the second stage of infection (LeafS2), with the healthy leaves having the lowest activity, while DPPH values showed no significant difference at other stages of infection ($p > 0.05$) (**Figure 3.11A**). Similarly, FRAP and ABTS values were also found to be most abundant in the second stage of susceptibility with 3,000 ± 131 µmol Trolox/g and 2,263 ± 26 µmol Trolox/g, which were 3.15 % and 2.39 % higher than those of healthy leaves, respectively, with LeafS1 value being the lowest (**Figure 3.11B and C**). Meanwhile, the values of these two assays showed

a similar variation trend (decrease-increase-decrease). Overall, DPPH, ABTS, and FRAP values exhibited significant differences in antioxidant activity between healthy leaves and different degrees of susceptible leaves.

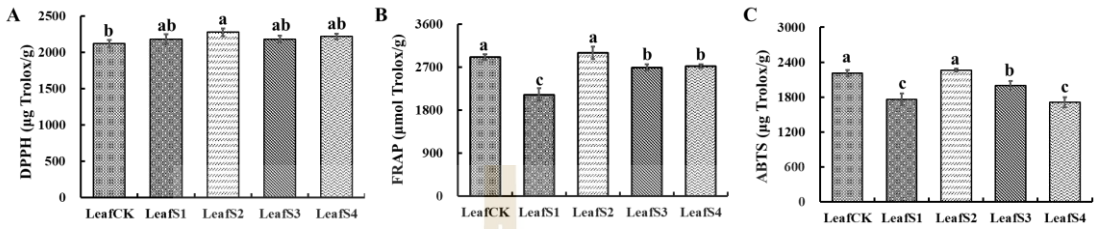

Figure 3.11 Antioxidant activities of healthy leaves and four different infected leaves. **(A)** 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, **(B)** ferric reducing antioxidant power (FRAP), **(C)** 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) radical cation scavenging activity. Different letters above the bars indicate significant difference among the same data series ($p < 0.05$, n = 3). CK, healthy leaves; designated *E. vexans* infection stages: 1 (S1), 2 (S2), 3 (S3), and 4 (S4).

3.4 Discussion

3.4.1 Metabolome analysis

Plant disease can trigger a series of reactions in plants from the regulation of gene expression and substance metabolism to production rate and yield variation. UPLC-MS/MS-based widely targeted metabolomics could be used to analyze the differential metabolites, KEGG pathways involved in plant–pathogen interaction and plant hormone signal transduction, and the alteration of plant endogenous hormones in plants infected with fungal pathogens (Wei et al., 2021). In this study, investigation of metabolites in healthy and infected leaves of Fuding Dabaicha provided important molecular information for exploring the relationship between fungal disease stress and host plants.

The defense responses of plants induced by pathogen infection significantly change the expression of disease resistance-related proteins, resulting in variations in the essential unit amino acids of protein composition (Martin et al., 2003). In the current research, *N*-monomethyl-*L*-arginine, arginine methyl ester, and (-) -jasmonyl-*L*-isoleucine were significantly up-regulated in the four comparisons of LeafCK vs. LeafS1, LeafCK vs. LeafS2, LeafCK vs. LeafS3, and LeafCK vs. LeafS4, indicating that the relative content of amino acids and derivatives accumulated to satisfy the growth requirement of the pathogen as the persistent infection, while the appearance of necrotic spots on leaves was accompanied by the decrease of amino acids and derivatives. This result was consistent with the findings of Shan et al. (2017), who reported that the increased content of amino acids was found in the infected leaves. In addition, the remarkable accumulation of other important primary metabolites involved in nucleotides and derivatives, organic acids, and lipids was similar to that of amino acids and derivatives, which has been suggested that pathogen progression required large amounts of primary metabolites to accelerate growth performance.

Phenolic compounds involved in flavonoids and phenolic acids, the most-mentioned secondary metabolites, may serve as passive or inductive barriers in response to microbial pathogens in plant–pathogen interaction, accumulating or altering significantly when plants are infected (Wang et al., 2016; Górniak et al., 2019). In our research, nine common flavonoids (e.g., kaempferol (3,5,7,4'-tetrahydroxyflavone), kaempferol-3-*O*-sophoroside-7-*O*-glucoside, phloretin, acacetin-7-*O*-glucuronide, acacetin-7-*O*-galactoside, and isovitexin-7-*O*-(6''-feruloyl) glucoside) and five common phenolic acids (e.g., 2,4,6-trihydroxybenzoic acid, antiarol, and 1-*O*-caffeoyl-(6-*O*-glucosyl)-*β*-*D*-glucose) were significantly up-regulated during the entire period (LeafS1 to LeafS4) of *E. vexans* infection (**Appendix Table 1**), which was consistent with the research reported by Xu et al. (2019), who found that disease resistance to *Botrytis cinerea* in grapes might be associated with dramatic elevation of flavonoids and phenolic acids. Similarly, the higher

accumulation of kaempferol glucosides and kaempferol triglycosides was observed in *E. vexans*-infected leaves (Mur et al., 2015). Salicylic acid, a well-known plant disease resistance hormone, induces the expression of defense-related genes in response to the cellular signaling of many pathogens (Koo et al., 2020), whose derivatives are also closely associated with plant disease resistance, such as 2,3-dihydroxybenzoic acid (Michael et al., 2010; Zhang et al., 2013). In our study, the significant accumulation of 2,3-dihydroxybenzoic acid might be related to the disease resistance of tea leaves infected with the pathogen. More importantly, proanthocyanidins are important antimicrobial compounds widely present in diverse plants (Wang et al., 2020). Proanthocyanidins were reported to significantly accumulate as an antimicrobial defense in black poplar (*Populus nigra* L.) stems caused by *Plectosphaerella populi* (Chhana et al., 2019), and Norway spruce [*Picea abies* (L.) *Karst*.] upon *Heterobasidion parviporum* infection (Marie et al., 2011). In the present study, galloylprocyanidin B4 and procyanidin C1 3'-*O*-gallate accumulate dramatically in susceptible leaves. Accordingly, it is well known that secondary metabolites such as flavonoids, phenolic acids, and anthocyanins are crucial antimicrobial and signaling molecules in plant defense systems (Steinkellner et al., 2007). Furthermore, the phenylpropane pathway is a biosynthetic pathway for flavonoids and phenolic acids, acting as cellular structures and signaling transduction molecules in plant development and defense (Dong and Lin, 2020; Yadav et al., 2020). Much research related to phenylpropane metabolism has been reported to enhance resistance of tomato against *Botrytis cinerea* (Li et al., 2021), olive against *Colletotrichum acutatum* (Gouvinhas et al., 2019), and wheat leaf against *Pyrenophora tritici – repentis* (Dorneles et al., 2018), consistent with the higher accumulation of phenylpropane pathway against *E. vexans* infection in the current study.

Flavonoid synthesis starts from flavonoid biosynthesis, including three downstream pathways: flavonoid and flavonol biosynthesis, isoflavone biosynthesis, and anthocyanin biosynthesis (Górniak et al., 2019). Of these, flavonoid biosynthesis,

as one of the most important downstream branches of phenylpropanoid metabolism, has been extensively reported to possess protective functions against biotic and abiotic stresses (Yadav et al., 2020). KEGG enrichment analysis illuminated that flavonoid metabolism involved in flavone and flavonol biosynthesis was significantly enriched in pairwise comparisons (**Figure 3.9, Appendix Figure 2**), especially in the middle of infection (LeafS2). Flavonoids are a class of natural compounds with the structure of 2-phenylchromone and are divided into the following categories: flavones, flavonols, flavanones, isoflavones, anthocyanidins, flavanols, chalcones, and dihydrochalcones (Shah and Smith, 2020). In our research, 347 flavonoids compounds were divided into seven subclasses, including flavones (77), flavonols (123), flavanols (24), flavonoid carbonoside (60), chalcones (14), flavanones (37), and flavanonols (12), most of which were significantly accumulated in susceptible leaves caused by *E. vexans* infection (**Figure 3.8**). These results found in our research were consistent with the previous study reported by Gill et al. (2017), who reported that the accumulation or alteration of the isoflavonoid pathway was closely associated with *Medicago sativa* L. defense against *Fusarium oxysporum* infection. Therefore, flavonoid metabolism involved in flavone and flavonol biosynthesis, especially flavonoid compounds, may be responsible for the resistance of Fuding Dabaicha to *E. vexans* infection.

3.4.2 Chemical components and antioxidant activities

Several chemical components, including flavonoids, phenolics, alkaloids, free amino acids, and terpenoids, play important roles in plant resistance to pathogen invasion and are also strongly associated with the formation of the unique flavor of tea (Pokharel et al., 2021).

The fresh and sweet substances in tea are mainly derived from amino acids, which are essentially primary metabolites and contribute significantly to flavor and other qualities of plants (Zhang et al., 2017). Our results showed that the change trend of total free amino acid content was also similar to that of amino acid

metabolites, indicating that amino acids, as the primary nutrient substrate for pathogens, played a direct role in causing damage to plant pathogens and contributed to the formation of disease resistance mechanisms in crops. On the other hand, the decrease of total free amino acid content in tea leaves might be to promote the synthesis of soluble protein required for immune defense in the second stage of susceptibility (LeafS2), potentially resulting in the reduction of freshness and sweetness of the manufactured tea. Afterwards, the serious damage of tea leaf cells was accompanied by protein hydrolysis reaction, which promoted the significant accumulation of total free amino acids at the middle and later periods of infection. These findings were similar to previous work on tomato wilt disease (*Verticillium alboatrum*), wherein the concentration of amino acids increased substantially in infected plants relative to the healthy treatment (Dixon, 2021).

Tea alkaloids are a type of organic compound rich in nitrogen heterocyclic structure in *C. Sinensis*, mainly including caffeine, theobromine, and theophylline, among which caffeine contains the highest concentration, accounting for 2 to 4 % of tea leaves (Jiang et al., 2019). Alkaloids are believed to be the primary bioactive compounds owing to their numerous biological properties, such as antimicrobial, anti-inflammatory, and antioxidant (Chen et al., 2011; Nibir et al., 2017). In particular, caffeine primarily stored in vacuoles of spongy tissue cells in tea leaves is considered an active substance in bio-defense against diseases and pests (Kim and Sano, 2008). In the present study, total alkaloids content exhibited a slight decrease, followed by a sustained increase to the highest level, which might be attributed to pathogen infection activating the tea plants' disease resistance mechanism. Afterwards, the descent in total alkaloid content during the fourth stage of *E. vexans* infection might be attributed to the increased severity of pathogen-induced damage to sponge tissue cells, which affects alkaloid synthesis and storage.

Terpenoids are a class of natural products with various structures and participate in multiple plant bioprocesses. It is believed that their primary function is

to provide chemical defense against a variety of biotic or abiotic stresses, especially in plant defense against fungal pathogens (He et al., 2022). A previous study has been reported that the content of furanoterpenoid, a natural terpenoid phytoalexin, accumulated significantly in tissues infected with either the sweet potato or taro strains of *Ceratocystis fimbriata* (Yasuda and Kojima, 2014). Additionally, another report showed that the levels of WAF-1, a labdane-type diterpene that belongs to terpenoids, accumulated rapidly in the leaves of tobacco plants following infection with *Tobacco mosaic virus* (TMV), indicating that diterpene can sever as an endogenous signal responsible for the defense mechanism in tobacco plants against TMV infection (Shigemi et al., 2003). After being infected with pathogens, there was a significant variation in total terpenoids content, characterized by rapid accumulation, particularly during the mid-stage of susceptibility. This suggested that the biosynthesis pathway of many terpenoid volatile substances might change following the infection of tea plants, resulting in an increase of their contents in response to pathogen invasion.

Phenolics, a class of aromatic compounds synthesized by plants, are typically employed as a defense mechanism against various stresses (He et al., 2022). The total phenolics content decreased at the early stage of tea leaf infection with the pathogen, suggesting that the pathogen required nutrients absorbed from tea plants for their growth. In the meantime, the pathogen infection destroyed the sponge tissue cells, resulting in the oxidation of phenolics initially stored in vacuoles into other compounds and a subsequent decrease in their content. Then, the total phenolics content significantly accumulated against further pathogen infection. This trend of change was consistent with the overall pattern observed in total terpenoids.

Flavonoids with 2-phenylflavone structure are the most described natural compounds in many studies, playing an important role in plant growth, development, flowering, and fruiting, as well as in antimicrobial and plant-pathogen interactions (Dixon et al., 2002). However, the change trend of total flavonoids

content was slightly different from that of other secondary metabolites, such as phenolics, terpenoids, and alkaloids. The total flavonoids content accumulated gradually with the aggravation of infection at the second and third stages, which indicated that the defense response of flavonoids to pathogens occurred earlier than that of the other three types of compounds, possibly owing to its stronger resistance action to disease. This is consistent with the previous reported by Miranda et al. (2007) and Carlsen et al. (2008) that flavonoids content might accumulate or flavonoid compounds could alter in response to pathogen infection.

As for antioxidant activity involved in FRAP assay and ABTS radical cation scavenging activity, the results of these two methodologies were found to correlate positively with the concentration in total terpenoids (y = 31.614x + 0.0054, R^2 = 0.9996), alkaloids (y = 0.0388x + 0.0044, R^2 = 0.9995), and phenolics (y = 0.2563x + 0.0186, R^2 = 0.9974). The values of DPPH radical scavenging activity were observed to be most correlated with the total flavonoids content (y = 2.2152x - 0.0298, R^2 = 0.9932). Overall, the antioxidant activity of the second susceptible stage (LeafS2) was higher than that of healthy leaves and other susceptible stages in both free and bound states.

3.5 Conclusion

In this research, widely targeted metabolome analysis was performed to investigate the expression of metabolites in Fuding Dabaicha caused by *E. vexans* infection. In total, 1,166 metabolites were identified from the healthy and susceptible leaves, including flavonoids, phenolic acids, lipids, amino acids and derivatives, organic acids, alkaloids, nucleotides and derivatives, lignans and coumarins, terpenoids, tannins, and others. Among these, 77 common DAMs were screened from the four comparisons of LeafCK vs. LeafS1, LeafCK vs. LeafS2, LeafCK vs. LeafS3, and LeafCK vs. LeafS4 mainly involved in flavonoids, phenolic acids, and tannins related to disease resistance. Among these, 73 compounds significantly

accumulated across the stages of infection, especially in the mid-stage (LeafS2 and LeafS3). In addition, the research also found several metabolic pathways associated with disease resistance, such as flavone and flavonol biosynthesis and phenylpropane pathway. Furthermore, the total content of flavonoids, phenolics, amino acids, alkaloids, and terpenoids altered significantly in four different infection periods, especially the LeafS2 stage, in which the concentration, except for amino acids, accumulated the most. The leaves affected by *E. vexans* infection at the second stage had the relatively highest antioxidant activity. This information provides comprehensive insights into Fuding Dabaicha's resistance to *E. vexans* infection.

3.6 References

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CHAPTER IV

TRANSCRIPTOMICS AND PHYTOHORMONE METABOLOMICS PROVIDE COMPREHENSIVE INSIGHTS INTO THE RESPONSE MECHANISM OF TEA LEAVES AGAINST BLISTER BLIGHT

ABSTRACT

Blister blight, caused by obligate biotrophic pathogen *E. vexans*, is a prevalent foliar disease in tea plants that results in significant losses in both yield and quality. To further understand the response mechanism of tea plants against *E. vexans* infection, transcriptomics and targeted hormone metabonomics were performed to investigate the gene expression and phytohormone signaling networks. Transcriptome analysis revealed that the most of differentially expressed genes (DEGs) were highly enriched in the pathways of photosynthesis-antenna proteins, plant hormone signal transduction, plant-pathogen interaction, MAPK signaling pathway-plant, and phenylpropanoid biosynthesis. The majority of disease resistance (R) genes (e.g., CDPK, PIK1, and PRS4) were activated in response to pathogen invasion in the fungal PAMP related pathway. The activation of infection-induced genes corresponds to transcription factors such as WRKY, MYB, bHLH, and AP2/ERF. SA acted as a crucial role in defense activation of tea immunity against *E. vexans* infection, the substances (SA and SAG) of which were positively correlated with the expression levels of disease-resistant genes (NPR1, TGA, and PR1) in the SA signaling pathway. The biosynthesis of defense-associated hormone metabolites such as JA, ETH, and BRs might be involved in associated with the defense response of tea plants to *E. vexans* infection. The correlation analysis of DEGs and hormone metabolites exhibited that JA and JA-ILE were positively regulated by the DEGs (e.g., BSK, ERF1/2, ETR, SAUR, and DELLA), suggesting JA- and BR-, ETH-, AUX-signaling might synergistically activate the expression of some defense-related genes. This

finding was further validated through quantitative real-time PCR, which displayed the involvement of defense-related gene expressions in the response of tea plants to the infection caused by *E. vexans*. Collectively, this study provides comprehensive insights into the response mechanism of tea defense against blister blight disease to act as a valuable resource for understanding plant immunity against diverse pathogen infection and other biotic stresses in tea plants.

Keywords: *Camellia sinensis*; tea; *Exobasidium vexans*; blister blight; transcriptome; gene expression; plant hormone

4.1 Introduction

Tea [*Camellia sinensis* (L.) O. Kuntze], one of the oldest known non-alcoholic beverages processed from fresh leaves, is an important woody cash species widely distributed in Asia, Africa, and South America (Wangnow et al., 2018). It is frequently consumed by people around the world due to its beneficial components involved in polyphenols, caffeine, theanine, flavanones, and medicinal ingredients for the treatment of various diseases (Wei et al., 2021; Zhou et al., 2021). However, tea plants are frequently damaged by various plant diseases, especially foliar pathogens (Ramkumar et al., 2021). Blight blister disease caused by *E. vexans* Massee, an obligate biotrophic fungus, occurs on tender shoots of tea plants, seriously threatening the yield and quality of harvestable tea. Tea plants normally suffer from blister blight disease under the favorable conditions involved in wet and cool weather with higher relative humidity (> 80%), moderate temperature (15-25°C), and prolonged surface moisture (Kerr and Rodrigo, 1967), which occurs frequently where tea plants are cultivated in tea growing regions, such as India, Sri Lanka, China, Japan, and Indonesia (Barthakur 2011; Sen et al., 2020). Currently, the main control strategy for blister blight is the application of chemical reagents, but tea plants possibly suffer from problems of fungicide residues. Along with increasing emphasis on food safety and environmental protection, the sustainable governance of fungal diseases has become the primary instructive direction to control plant diseases. Therefore, a thorough exploration of blister blight-defense mechanisms will contribute to the development of resistant breeding, leading to better economic and environmental sustainability in tea cultivation. However, there has been little systematic research on the response mechanism of tea plants defense against blister blight disease, resulting in inappropriate cultivation practices of tea and a scarcity of resistant varieties.

Plant infection triggers a complex cascade of responses that encompass gene expression, metabolism, and physiology to contain the pathogen and counter the damage inflicted by the infection (Mansfeld et al., 2017). Gene expression profiles in

response to pathogen infection among plants, particularly those lacking a reference genome, are commonly performed by RNA-Seq-based comparative transcriptome analysis (Sun et al., 2019). The utilization of this approach has been proven to be significant for the identification of genes which play a crucial role in plant-pathogen interactions in plant crops involved in wheat against *Blumeria graminis* f. sp. *Tritici* (Velho et al., 2022), rice (*Oryza sativa*) against *Xanthomonas oryzae* pv. *oryzae* (Im et al., 2019), and cotton (*Gossypium hirsutum*) against *Verticillium dahlia* (Hu et al., 2020). Currently, there is limited understanding regarding the molecular mechanisms that regulate defense responses in tea plants. Additionally, only a few of genes associated with resistance to blister blight disease has been identified in tea plants. The cDNA-AFLP and SSH techniques evidenced that the certain defense-related new genes were screened and identified from tender tea leaves infected by *E. vevans*; simultaneously, it was found that proteins related to energy metabolism, transportation, protein modification, cell wall defense, oxidative stress response, and signal transduction in tea plants were significantly associated with resistance to blister blight disease (Bhorali et al., 2012). Transcription analysis revealed that a total of 149 genes involved in the immune response of tea against blister blight disease, including transcription factors, defense related enzymes, R genes, and multidrug resistant transporters, with most genes up-regulated; meanwhile, tea plants can activate nucleotide binding leucine rich repeat (NB-LRR) mediated effector-triggered immunity (ETI) and trigger salicylic acid (SA) signal pathway mediated by up-regulation of R genes to induce the production of secondary metabolites and programmed cell death (PCD), thus resisting the infection of *E. vexans* pathogen (Jayaswall et al., 2016). The transcription factor CsWRKY14 derived from tea leaves was involved in SA signaling pathway and activated the expression levels of pathogen-related genes induced by SA to mediate the resistance of tea plants against blister blight disease (Liu et al., 2021) Consequently, it is particularly important to investigate the immunity mechanisms of tea in response to blister blight infection and screen the key defense-related genes.

Plants possess diverse height-regulated metabolic networks including phytohormone-mediated metabolism, whose vital roles in the regulation of the signaling networks for plants response to various pathogens, biotic and abiotic stresses (Rajendra and Jones, 2009). It is known that three important plant hormones, salicylic acid (SA), jasmonic acid (JA), and ethylene (ETH), act as a major role in regulating plant defense responses against pathogen invasions. Generally, SA is an indispensable endogenous signaling molecule in plant immunity, which is usually involved in activating defense responses against biotrophic and hemibiotrophic pathogens (Shigenaga and Argueso, 2016). On the contrary, JA and ETH are typically related to defense against necrotic pathogens, among which JA can stimulate the production of secondary metabolites associated with primary defense and the expression of some pathogenesis-related (PR) proteins, as well as establish induced systemic resistance (ISR) (Campos et al., 2014). Additionally, brassinosteroids (BRs) are involved in protection of plants from pathogen infection, such as enhancing the resistance of tobacco against *Tobacco mosaic virus* (TMV) and rice against *Xanthomonas oryzae* (Nakashita et al., 2003; Deng et al., 2016). As very few studies have revealed the relationship between plant hormones and the response of tea to plant diseases, despite previous attempts to investigate them. For example, SA was reported to function as a crucial role in the response of tea against anthracnose disease induced by *Colletotrichum* (Shi et al., 2019). Therefore, it would be meaningful to investigate the molecular function of hormones in tea plants defense response against blister blight disease.

In the present study, transcriptomics and widely targeted hormone metabonomics were performed to investigate the differences in gene expression and hormone content of tea plants involved in healthy and various stages of infected leaves. Moreover, an analysis was performed to explore the correlation between differentially expressed genes (DEGs) and phytohormones with a better understanding of response mechanism of tea plant defense against *E. vexans* infection. Besides that, quantitative real-time PCR (qRT-PCR) was employed to

validate the accuracy of data obtained from transcriptomics and phytohormone metabolomics. Therefore, this study could provide valuable reference information regarding gene expression and signaling networks related to the immune response of tea plants against *E. vexans* infection, which will be expected to advance the breeding of resistant cultivars.

4.2 Materials and methods

4.2.1 Cultivation conditions and collection of plant materials

Plant samples from tea cultivar namely Fuding Dabaicha with 15 years of age, growing in the tea plantations of Maojian town, Duyun city, China (1,738 m altitude; 107°36′E, 26°18′N) under the natural conditions with annual average temperature of 16°C and precipitation of 1200 mm, were collected from healthy leaves that did not appear any *E. vexans* infestation and infected leaves corresponding to symptoms of incipient stage with translucent spot formation (Stage 1), disease spots with typical sporulating lesions (Stage 2), blister spots depression (Stage 3), and sample of blisters causing necrotic spots on leaves (Stage 4), which were marked as LeafCK, LeafS1, LeafS2, LeafS3, and LeafS4 (**Figure 4.1**). The samples were subsequently snap-frozen in liquid nitrogen and transferred to -80°C until further used for analysis of plant hormone metabolome and transcriptome.

Figure 4.1 Various stages of blister blight disease on tea (*Camellia sinensis*) leaves. Healthy leaves (CK) and tea leaves infected with tea blister blight (*E. vexans*) at designated infection stages 1 (S1), 2 (S2), 3 (S3), and 4 (S4).

4.2.2 Transcriptome sequencing and data analysis

Plant samples were taken about 1.0 cm away from the diseased spot (infected leaves) or the same site (healthy leaves). Three biological replicates were assembled for each treatment, and each replicate consisted of five mixed subsamples from tea leaves at various degrees of infection.

Around 100 mg of tea sample was used to extract the total RNA with RNAplant plus Kit (Tiangen Biotechnology Co., Ltd, Beijing, China) in accordance with the manufacturer's protocol, after which the concentration and purity of the extracted RNA was measured using NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE), as well as RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 1 μg of the isolated RNA from each sample was used as input material to construct cDNA libraries by using NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, USA) according to the manufacturer's recommendations and index codes were added to attribute sequences to each sample. Subsequently, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA) for

the selection of the cDNA fragments with a length priority of 240 bp. Then, 3 µl of USER Enzyme (NEB, USA) was used with size-selected and adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C before PCR. Further, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. Raw reads of FASTQ format were firstly processed by FASTQC software for quality control. In this step, clean reads were obtained by removing reads containing adapters with Cutadapt (Version 1.15); besides that, Q20, Q30, GC-content and sequence duplication level of the clean reads were calculated. All the downstream analyses were based on clean data with high quality. Only reads with a perfect match or one mismatch were further analyzed and annotated based on the reference genome with Hisat2 tools soft. Gene function was annotated based on the following databases: Nr (NCBI non-redundant protein sequences, ftp://ftp.ncbi.nih.gov/blast/db/); COG (Clusters of Orthologous Groups of proteins, http://www.ncbi.nlm.nih.gov/COG/); KOG (Clusters of Protein homology, http://www.ncbi.nlm.nih.gov/KOG/); Swiss-Prot (A manually annotated and reviewed protein sequence database, http://www.uniprot.org/); Pfam (Homologous protein family, http://pfam.xfam.org/); KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/); and GO (Gene Ontology, http://www.geneontology.org/).

Gene expression levels were quantified as fragments per kilobase of transcript per million fragments mapped reads (FPKM). The DEGs was analyzed using the DESeq R package (1.10.1) with false discovery rate (FDR)-adjusted p-value < 0.01. GO enrichment analysis of the DEGs was performed using the GOseq R packages, which can adjust the gene length deviation in DEGs, and the significantly enriched GO terms were defined as p-value < 0.01 and q-value < 0.05. The statistical enrichment of DEGs in KEGG pathways was performed by KO-BAS software.

4.2.3 Determination of phytohormone metabolites

The plant hormones including auxin, cytokinins (CKs), abscisic acid (ABA), jasmonates (JAs), SA, gibberellins (GAs), ETH, and strigolactones (SLs) from healthy and infected leaves were detected in this research. The sample extracts were

determined using an UPLC-ESI-MS/MS system (UPLC, ExionLC™ AD, https://sciex.com.cn/, accessed on 18 June 2022; MS, Applied Biosystems 6500 Triple Quadrupole, https://sciex.com.cn/, accessed on 18 June 2022) equipped with column of Waters ACQUITY UPLC HSS T3 C18 (100 mm × 2.1 mm, 1.8 μm). The solvent system consisted of water with 0.04% acetic acid (A) and acetonitrile with 0.04% acetic acid (B). The gradient program for pump B started at 5% (0-1 min), then increased to 95% (1-8 min), continued to maintain at 95% (8-9 min), and finally ramped back to 5% (9.1-12 min). The flow rate was set to 0.35 mL/min, temperature was adjusted to 40℃ and injection volume was set as 2 μl. Mass spectrometry was acquired in both positive and negative modes using electrospray ionization performed by Analyst 1.6.3 software (AB Sciex). All phytohormones were analyzed in c and quantified using Multiquant 3.0.3 software (Sciex).

4.2.4 Standard curve and absolute quantification

Standard solution of plant hormones was prepared into various concentrations: 0.01 ng/ml, 0.05 ng/ml, 0.1 ng/ml, 0.5 ng/ml, 1 ng/ml, 5 ng/ml, 10 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml, and 500 ng/ml, and the mass spectrum peak intensity data of the quantitative signal corresponding to each concentration standard were then obtained. The ratio of integral peak area of all samples detected was substituted into the linear equation of the standard curve for calculation, and finally the absolute content data of hormones in the actual samples were acquired.

Hormone content in the sample (ng/g) = $c \times V \times 10^3/m$

In the formula, "c" represents the concentration value (ng/mL) obtained by substituting the ratio of hormone integral peak area in the samples into the standard curve; "V" represents the volume (uL) of the solution used for redissolution; and "m" represents the quality (g) of the weighted sample.

4.2.5 Validation of gene expression by qRT-PCR

Samples were the same as those of transcriptome data (LeafCK, LeafS1, LeafS2, LeafS3, and LeafS4) with three biological replicates for each treatment, which were performed to validate the reliability and accuracy of RNA-Seq in this study by qRT-PCR. cDNA was synthesized using MonScript™ RTIII All-in-One Mix with dsDNase reverse transcription kit (Monad Biotech Co., Ltd., Wuhan, China). qRT-PCR analysis was performed on a LightCycler 96 PCR detection system (Roche, California, USA) by using QuantiNova SYBR Green PCR kit (QIAGEN, German) according to the manufacturers' instruction. The conditions and primer details for qRT-PCR were recorded in the **Appendix Table 3-5**. Relative expression levels of target genes were calculated by the quantitative method of $2^{-\Delta\Delta Ct}$ using β-Actin genes as internal control for standardization.

4.2.6 Statistical analysis

The data of plant hormone contents and qRT-PCR were expressed as mean ± standard deviation (SD). All data were subjected to one-way analysis of variance (ANOVA) by SPSS Statistics 22.0 (SPSS Inc., Chicago, USA) and EXCEL Version 2010 (Microsoft, USA), and p-value < 0.01 was accepted as statistical significance.

4.3 Results and discussion

4.3.1 Transcriptome sequencing and functional annotation of unigenes

The fifteen leaf samples involved in healthy leaves and four successive stages of infected leaves (LeafCK, LeafS1, LeafS2, LeafS3, and LeafS4, with three biological replicates for each treatment) were subjected to transcriptome sequencing analyses to explore the molecule mechanism of tea plants responsive to *E. vexans* infection. A total of approximately 481,772,991 bp raw reads were generated, and around 458,831,420 bp clean reads were obtained with an average error rate of 0.02% after eliminating the adaptors and low-quality reads. Q20 and Q30 values (reads with average quality scores > 20 or 30) were obtained for more than 95.93%

and 93.14%, respectively, and GC content ranged from 44.26% to 44.74%, with an average ratio of 44.54% (**Appendix Table 6**). In addition, the proportion of total mapped reads ranged from 62.84% to 86.92%, and an average percentage of 74.48% of clean reads were uniquely mapped against the assembled genome (**Appendix Table 7**). These results showed that the sequencing data was of relatively higher quality and reliability, and satisfied the demands for subsequent assembly and analysis. BLAST software was used to align the excavated unigenes with the common databases for functional annotations, of which a total of 45,133 unigenes were annotated into COG, GO, KEGG, KOG, Pfam, Swiss-Prot, eggnog, and NR databases, with 11,519, 35,010, 28,212, 21,699, 30,383, 28,022, 34,158, and 45,008, respectively (**Appendix Table 8**). Additionally, the biological replicate correlation analysis of DEGs levels among samples indicated that high consensus of samples within the group insured the reliability for further DEGs analysis (**Appendix Figure 3**). Principal components analysis (PCA) plot of gene expression profiling from 15 samples showed that biological replicates of each treatment gathered together and samples between groups were remarkably separated from each other, with the variation of 88.2% and 5.9% accounted for the first two principal component analysis (PC1 and PC2) (**Figure 4.2A**), suggesting that the pronounced variations in gene expression levels among tea plants between healthy and varying degrees of infected leaves.

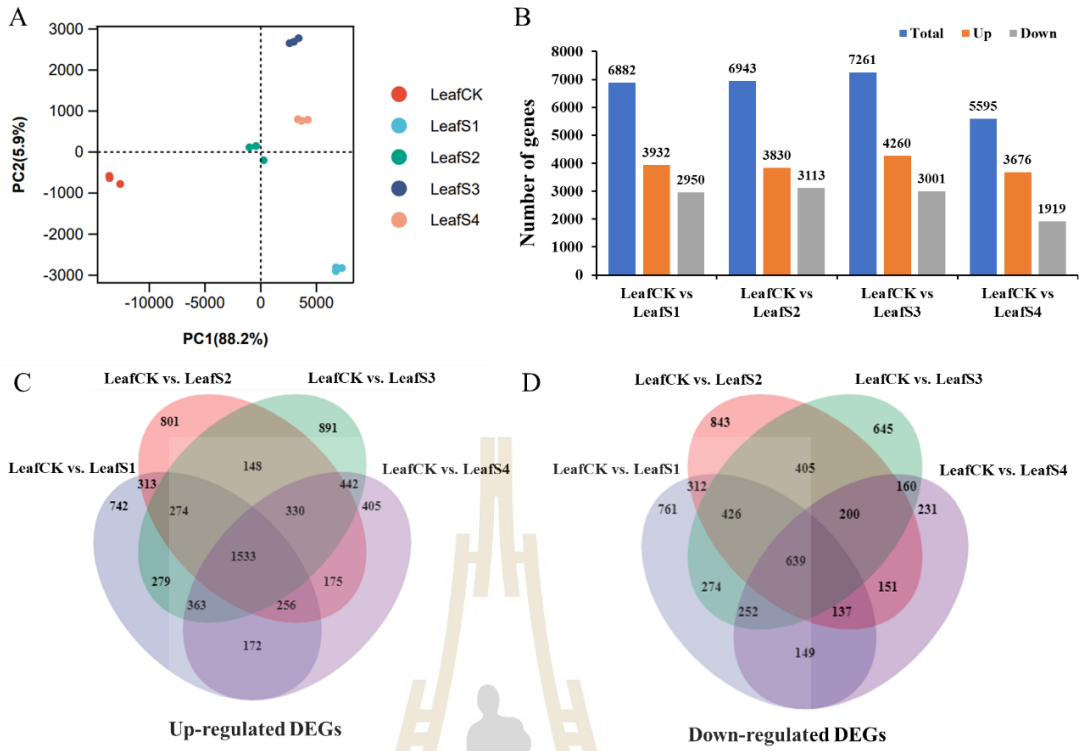

Figure 4.2 Analysis of DEGs in the four comparison groups. (**A**) PCA analysis of the expression of unigenes for LeafCK vs. LeafS1, LeafCK vs. LeafS2, LeafCK vs. LeafS3, and LeafCK vs. LeafS4. (**B**) Bar diagram of the total, up- and down-regulated genes from pairwise comparisons. (**C**) Venn diagram for up-regulated DEGs from the four comparisons. (**D**) Venn diagram for down-regulated DEGs from the four comparisons.

4.3.2 Identification of differentially expressed genes

The DEGs from four different stages were identified by fragments per kilobase of transcript per million fragments mapped (FPKM) values and screened based on set criteria (Fold Change ≥ 2 and FDR < 0.01). A total of 6,882, 6,943, 7,261, and 5,595 DEGs were obtained in the four comparisons of LeafCK vs. LeafS1, LeafCK vs. LeafS2, LeafCK vs. LeafS3, and LeafCK vs. LeafS4, respectively, with 3,932, 3,830, 4,260, and 3,676 up-regulation, and 2,950, 3,113, 3,001, and 1,919 down-regulation (**Figure 4.2B, Appendix Figure 4**), which indicated that the gene expression levels of

tea plants altered significantly between healthy and different infected leaves. Numerous DEGs were identified in this study, which might be ascribed to the complicate genetic background. Previous reports on transcriptome analysis of *E. vexans* infection in tea also exhibited that over ten thousand DEGs have been enriched in different cultivars at four successive stages (Jayaswall et al., 2016). Venn diagram showed that 1,533 common up-regulated and 639 common down-regulated DEGs were shared during the four stages (**Figure 4.2C, D**). Additionally, 742 up-regulated DEGs were uniquely observed in LeafCK vs. LeafS1, while the number of DEGs gradually accumulated as the symptoms of blister spots aggravated during the mid-stages (LeafCK vs. LeafS2 and LeafCK vs. LeafS3), and 405 up-regulated DEGs, unique to LeafCK vs. LeafS4, were found to decrease (**Figure 4.2C**). Besides that, the number of down-regulated DEGs exhibited a similar trend to that of up-regulated DEGs across the four stages, and the largest number of DEGs were obtained in LeafS4 (**Figure 4.2D**). Furthermore, we found that the number of up-regulated DEGs were highly more than that of down-regulated DEGs, indicating that much more genes might contribute to the positive regulation of tea plant responsive to *E. vexans*.

4.3.3 Functional annotation and enrichment analysis

Totals of 4,866, 4,944, 5,241, and 4,045 DEGs obtained from the four comparisons of LeafCK vs. LeafS1, LeafCK vs. LeafS2, LeafCK vs. LeafS3, and LeafCK vs. LeafS4 were annotated into 55 GO terms with three main categories involved in biological processes (BPs), molecular functions (MFs), and cellular components (CCs) (**Appendix Figure 5**). Specifically, GO term classification revealed that 21 terms were associated with BPs, out of which “metabolic process”, “cellular process”, and “single-organism process” were the most abundant GO terms; 16 terms were related to MFs, among which “binding” and “catalytic activity” were the main GO terms; 18 terms connected with “membrane” and “membrane part” were the richest GO terms. Additionally, GO enrichment terms with top 20 enriched categories showed that DEGs at the early stage (LeafCK vs. LeafS1) were enriched in the terms of “chloroplast thylakoid membrane”, “photosystem II”, “photosynthesis, light

harvesting", "photosynthesis", and "oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen" correlated with photosynthesis and oxidoreduction reaction. Whereas GO terms of "defense response", "response to abiotic stimulus", "isoprenoid biosynthetic process", "iron ion binding", and "terpene synthase activity" were closely related to disease stress and metabolic regulation, especially the biosynthesis of terpenoids and isoprenoids, which were found to be enriched in the second stage (LeafCK vs. LeafS2). On the other hand, the DEGs grouped into the GO-enriched terms of "toxin metabolic process", "glutathione metabolic process", "response to toxic substance", "integral component of membrane", "protein serine/threonine kinase activity", and "protein kinase activity" associated with protein processing and transport and metabolic reaction of toxic substances were observed to be enriched in the third stage (LeafCK vs. LeafS3). In addition to the previous three stages involved in photosynthesis and oxidoreduction reaction, the DEGs at the final stage (LeafCK vs. LeafS4) were enriched in the GO terms of "protein kinase activity", "transcription factor activity, sequence-specific DNA binding", and "ATP binding" connected with regulation of protein molecules, especially TF expression (**Appendix Table 9**). These results indicated that the differences at various stages after tea plants infected with *E. vexans* were mainly manifested in carbohydrate metabolism and disease stress response.

Furthermore, a total of 2,222, 2,231, 2,361, and 1,858 DEGs acquired from the four comparisons of LeafCK vs. LeafS1, LeafCK vs. LeafS2, LeafCK vs. LeafS3 and LeafCK vs. LeafS4 were assigned to 132, 132, 131, and 129 KEGG pathways, respectively. Among these, the most enriched pathways included "photosynthesis-antenna proteins", "plant hormone signal transduction", "plant-pathogen interaction", "MAPK signaling pathway-plant", and "phenylpropanoid biosynthesis" (**Appendix Figure 6**). According to the top 20 significantly enriched pathways (both up-regulation and down-regulation) of each pairwise comparison, eight common KEGG pathways involved in "photosynthesis-antenna proteins", "MAPK signaling

pathway-plant", "plant hormone signal transduction", "phenylpropanoid biosynthesis", "starch and sucrose metabolism", "carotenoid biosynthesis", "glucosinolate biosynthesis", and "plant-pathogen interaction" were the most classified into the four stages, with "plant hormone signal transduction" and "MAPK signaling pathway-plant" being the most highly enriched pathways (**Figure 4.3**). In addition to the eight abovementioned pathways, five enriched pathways including "flavonoid biosynthesis", "isoflavonoid biosynthesis", "flavone and flavonol biosynthesis", "diterpenoid biosynthesis", and "sesquiterpenoid and triterpenoid biosynthesis" were uniquely obtained from the middle and final stages (LeafCK vs. LeafS2, LeafCK vs. LeafS3 and LeafCK vs. LeafS4) (**Figure 4.3B, C, D, Appendix Table 10**). Thereby, we conjectured that these DEGs significantly enriched in the pathways were probably correlated with the resistance of tea plants against *E. vexans*.

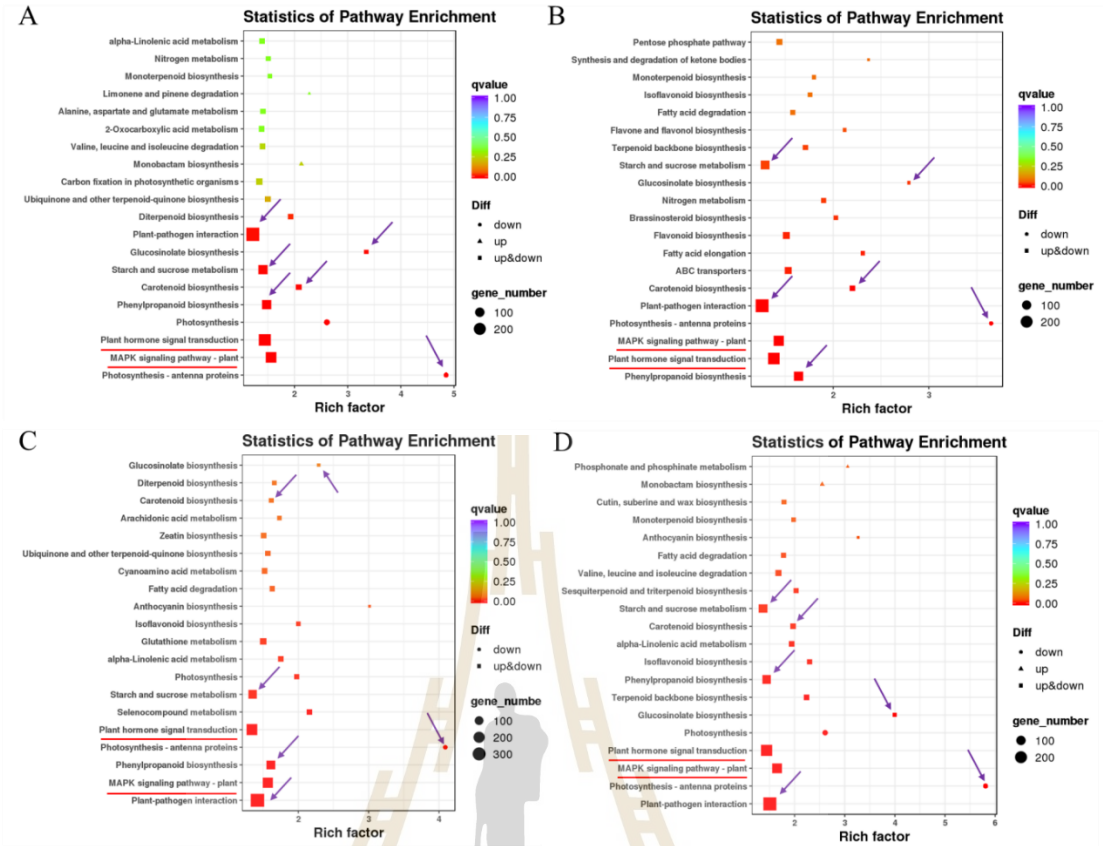

Figure 4.3 Top 20 enriched KEGG pathways of differentially expressed genes in (**A**) LeafCK vs. LeafS1, (**B**) LeafCK vs. LeafS2, (**C**) LeafCK vs. LeafS3, and (**D**) LeafCK vs. LeafS4. The red lines highlight the significant enrichment pathways for the four comparisons. The purple arrows highlight the possible pathways related with the resistance of tea plants against *E. vexans*.

4.3.4 DEGs related to plant-pathogen interaction

"Plant-pathogen interaction" was observed to be the most significantly enriched metabolic pathway, especially the fungal PAMP (pathogen-associated molecular pattern) related pathway (**Figure 4.4**). In general, defense genes, hypertensive response (HR), and PCD can be produced by PTI or ETI (Jones and Dangl, 2006). Calcium serves as a decisive role in the PTI, leading to the three correlative regulatory pathways. Among them, the upstream six CDPK (calcium-

dependent protein kinase, e.g., evm.TU.Cha09g010390 and evm.TU.Cha13g004170) and two RBOH (respiratory burst oxidase homologues, evm.TU.Cha14g008510 and evm.TU.Cha03g007990) were significantly up-regulated in the reactive oxygen species (ROS) related pathway during the *E. vexans* infection, especially in the late stages. The production of ROS acts as a signaling molecule to induce HR and cell wall reinforcement (Torres et al., 2002). Calcium-independent protein kinase St CDPK5 of potato was demonstrated to directly regulate ROS production through inducing the phosphorylation of Rboh (Kobayashi et al., 2007). Therefore, tea plants infected with *E. vexans* led to an increase in CDPK activity, which induced a significant accumulation of the downstream NADPH oxidase homologous gene RBOH to regulate ROS to inhibit the growth of pathogen. Additionally, the expression of calmodulin may result in stomatal closure. The upstream CNGC (cyclic nucleotide-gated channel, *Camellia_sinensis*_newGene_33791) was remarkably down-regulated during the transfer of calcium in LeafS1 and LeafS2. Whereas, majority of downstream CaM/CMLs (crassulacean acid metabolism/calmodulin-like proteins) genes exhibited the significant up-regulated expressions at the early and final stages, with only two DEGs (evm.TU.Cha02g002850 and evm.TU.Cha03g016780) and NOS (nitric oxide synthase, evm.TU.Cha01g007270) down-regulated by the first stage, which indicated that changes in the expression levels of these genes might be associated with stomatal closure. Furthermore, the high expression of WRKY-dependent TFs induces the synthesis of downstream PR proteins (Tang et al., 2013). In the current study, the most DEGs encoding WRKY 25/33 and WRKY 29 gradually accumulated during the whole infection, whereas only the expression of two FRK1 and one PR1 were strongly down-regulated at the early stage, suggesting that the elevated accumulation of WRKY TFs and PRs was positively correlated with the induction of defense-related genes (**Figure 4.4**). Besides that, specific disease R genes can recognize the effectors of PTI and ETI immunized by the pathogens (Jones and Dangl, 2006). After exposure to pathogen effectors, the expression of the majority of R genes associated with RPM1, RIN4, PRS2, PBS1, and HSP90 containing NB-LRR

(nucleotide binding leucine rich repeat) in tea leaf cells was notably up-regulated rather than down-regulated, which significantly activated ETI, as well as initiated HR, PCD, and defense signal expansion (Moffett, 2009). While that of other DEGs encoding PIK1 and PRS4 exhibited an increase trend at four stages (**Appendix Figure 7**). Thus, the dramatic accumulation of these R genes in plant-pathogen interactions might contribute to resist the invasion of pathogen.

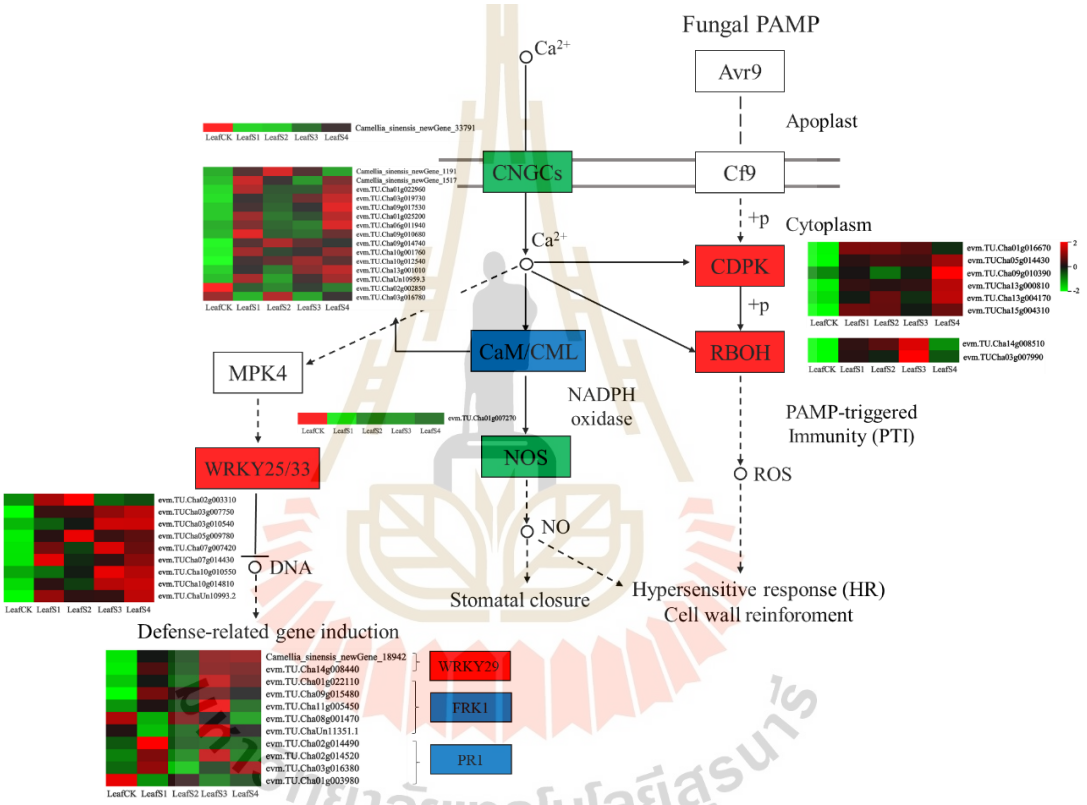

Figure 4.4 DEGs associated with plant-pathogen interaction pathway. The maximum/minimum $\log_2^{FPKM}$ value was set to ± 2.0 (red for up-regulation, green for down-regulation). Each horizontal row represents a gene with its ID, and the vertical columns represent five stages.

4.3.5 Transcription factors related to disease resistance genes

Transcription factor families involved in WRKY, MYB, bHLH, AP2/ERF, bZIP (basic leucine zipper domain), and NAC (N-acetyl cysteine) function as important roles in the regulation of plant defense responses and defense gene expression (Vidhyasekaran, 2016). A total of 309 TFs from 21 families were identified among the DEGs obtained, of which MYB, bHLH, AP2/ERF, and WRKY families were the most abundant, accounting for more than 81 % of all TF families (**Figure 4.5**). MYB gene is a family of TF with the largest number of genes in plants, which is involved in the regulation of flavonoid metabolism pathway, hormone signaling and stress responses, as well as plant growth and development such as cell differentiation and morphological construction (Ma et al., 2022). Importantly, the most represented TFs were observed in the MYB family, with 76 DEGs. The majority of these genes showed up-regulated expression in the four different stages of infection, with 43, 35, 38, and 43 TFs being up-regulated in each respective stage. Conversely, only 6, 13, 9, and 10 TFs exhibited down-regulated expression from the early stage to the final stage (**Figure 4.6A**). This result indicated that MYB TFs might mediate plant defense responses against *E. vexans* infection, which was consistent with its involvement in the resistance of wheat against *Bipolaris sorokiniana* (Zhang et al., 2012), apple against *Alternaria alternate* (Zhu et al., 2017), and cotton against *Verticillium dahliae* (Zhu et al., 2022). Additionally, bHLH, which is well-known the second-largest TF after MYB family in plants (Bano et al., 2021), was found to exhibit the relatively abundant differential expression of TFs with 68 DEGs. Among these, the majority of 39, 41, 28, and 28 up-regulated genes were markedly expressed in Stage 1 to Stage 4, respectively (**Figure 4.6B**). The resistance of tobacco to *Powdery mildew* was reported to be closely correlated with bHLH (Guo et al., 2020). Besides that, bHLH genes were demonstrated to play an important role in regulating the defense response of potato against the common scab (Tai et al., 2013). Therefore, the significant accumulation of bHLH in the present study might be associated with the formation of resistance against *E. vexans*. Furthermore, AP2/ERF and WRKY families

were also highly expressed across the whole infected stage, with 60 and 47 TFs, respectively, which indicated that these two TF families might contribute to the defense responses against blister blight disease in tea plants (**Figure 4.6C, D**). WRKY TFs form a large family of regulatory proteins known to act as a crucial role in the regulation of plant immunity (Pandey and Somssich, 2009), whereas AP2/ERF family affects defense responses to pathogens and environmental stimuli (Girardi et al., 2013). Thus, it was presumed that AP2/ERF and WRKY TFs might positively dominate the resistance of tea against *E. vexans*. Collectively, the expression of DEGs within all TF families showed a significant up-regulation compared to down-regulation, suggesting that the notably increased expression of TFs was speculated to offer important insights into the regulatory mechanisms of tea plants in response to *E. vexans* infection.

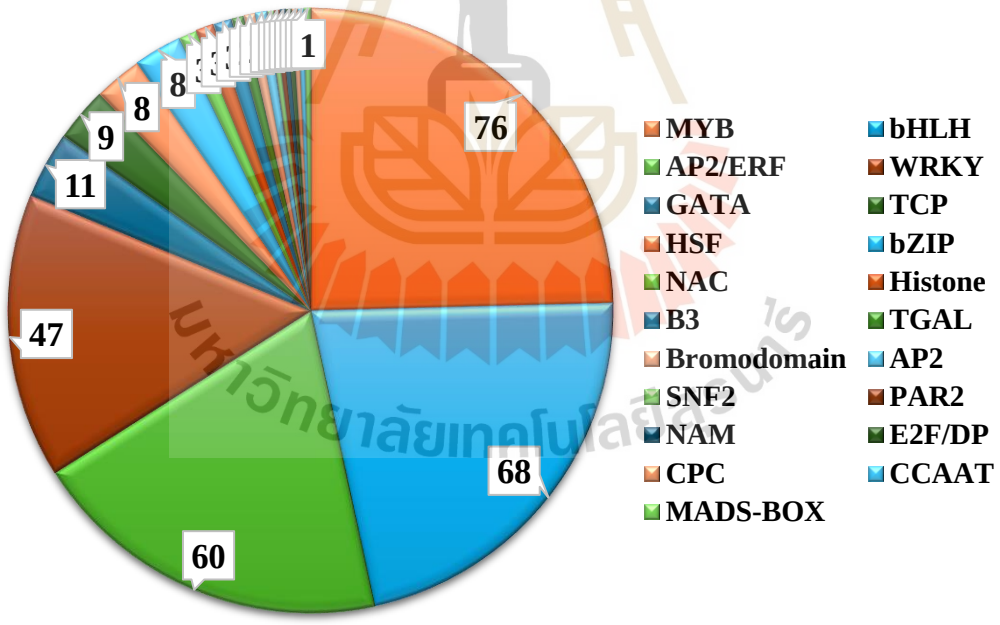

Figure 4.5 Number of the differentially expressed genes (DEGs) in different transcription factor (TF) families.

Figure 4.6 Expression of DEGs in different transcription factor (TF) families. (**A**) MYB, (**B**) bHLH, (**C**) AP2/ERF, (**D**) WRKY. The maximum/minimum $\log_2^{FPKM}$ value was set to ± 2.0 (red for up-regulation, green for down-regulation). Each horizontal row represents a gene with its ID, and the vertical columns represent five stages.

Figure 4.6 Expression of DEGs in different transcription factor (TF) families. (**A**) MYB, (**B**) bHLH, (**C**) AP2/ERF, (**D**) WRKY. The maximum/minimum $\log_2^{FPKM}$ value was set to ± 2.0 (red for up-regulation, green for down-regulation). Each horizontal row represents a gene with its ID, and the vertical columns represent five stages (Continued).

4.3.6 Analysis of DEGs involved in phytohormone signal transduction

Within the “phytohormone signal transduction” pathway, a total of 227 DEGs were found to be significantly enriched in the response of tea leaves to *E. vexans* infection, including SA, JA, ETH, BR, auxin (AUX), cytokinine (CK), gibberellin (GA), and abscisic acid (ABA). Plant hormones associated with stress response and disease resistance are regarded as the crucial regulators of plant-pathogen interactions, particularly SA, JA, ETH, and BR (Pieterse et al., 2009).

SA is thought to play an important role in plant stress responses against diverse biotrophic pathogens and the induction of systemic acquired resistance (SAR) (Grant and Lamb, 2006). NPR1 (non-expressors of pathogenesis-related genes 1) serves as a vital regulator within SA signaling pathway, interacting with TGA (TGACG motif-binding factor) to activate SA-responsive PR genes and regulate SA-dependent plant defense responses (Dong, 2004). In addition, the accumulation of PR1 protein with antifungal activity enhances the plant immunity of host plants in extracellular spaces, such as vacuoles or cell walls (Hammerschmidt, 2009). The DEGs in the SA signaling pathway involved in two NPR1 genes (evm.TU.Cha10g001750 and evm.TU.Cha13g001840), two TGA genes (evm.TU.Cha06g000980 and evm.TU.Cha10g003980), and five PR1 genes (e.g., evm.TU.Cha02g014490 and anevm.TU.ChaUn26553.1) were observed to be significantly up-regulated, while only one PR1 gene (evm.TU.Cha01g003980) was down-regulated across the four susceptible stages (**Figure 4.7**). Therefore, the remarkably up-regulated expression of DEGs (NPR1 and TGA) in SA signaling pathway ultimately promoted the generation of PR, which indicated that tea plants responding to *E. vexans* infection was more inclined to biotrophs.

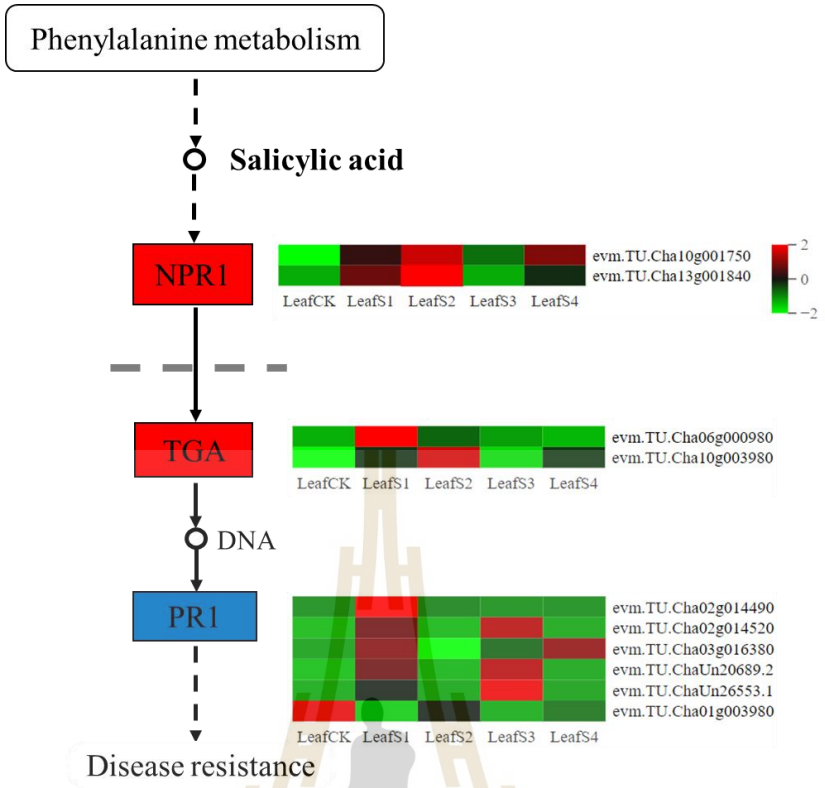

Figure 4.7 DEGs involved in salicylic acid signaling pathway. Heat map represents the expression values of DEGs associated with hormone metabolism at five groups (red for up-regulation, green for down-regulation).

JA plays crucial roles in various processes involved in plant life cycle and stress resistance, especially in plant defense response to microbial pathogens (Derksen et al., 2013). JAZ (jasmonate ZIM-domain) serves as a vital regulator of the signaling pathway, which can affect gene transcription and transmit JA signaling from the receptor to the cellular nuclear (Chini et al., 2007). In the JA signaling pathway of this study, all six up-regulated JAZ genes (e.g., evm.TU.Cha03g000380 and evm.TU.Cha06g020130) were identified during the four different stages of infection, with particular prominence observed in the first two stages. Additionally, MYC2 (myelocytomatosis) has been shown to inhibit JA mediated expression of pathogen responsive genes in *Arabidopsis* (Dombrecht et al., 2007). Ten significantly up-regulated (e.g., evm.TU.Cha02g000600 and evm.TU.Cha03g000890) and only one

down-regulated (evm.TU.ChaUn6810.2) MYC2 genes were detected in the JA signaling pathway (**Figure 4.8**), which indicated that MYC2 might activate the expression of JA-responsive genes following *E. vexans* infection in tea plants.

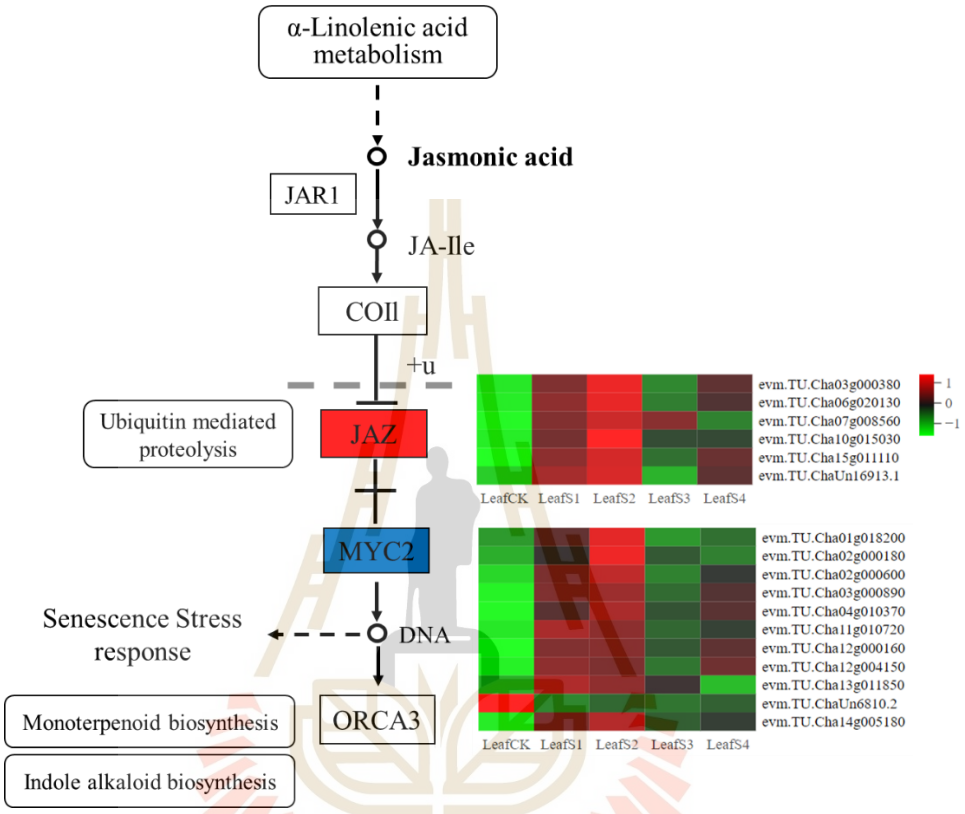

Figure 4.8 DEGs involved in jasmonic acid signaling pathway. Heat map represents the expression values of DEGs associated with hormone metabolism at five groups (red for up-regulation, green for down-regulation).

ETH is involved in various plant physiological processes, including seed germination, plant growth, initiation of plant senescence, stimulation of fruit maturation, and induction of resistance in plant-pathogen interactions (Geraats Bart et al., 2003). In the ETH signaling pathway, almost all 19 DEGs involved in ETR (ethylene receptor), CTR1 (constitutive triple response mutant 1), EBF1/2 (ethylene insensitive 3-binding F-box protein 1/2), and ERF1/2 (ethylene response factor 1/2)

exhibited significant up-regulation in their expression levels, whereas only one SIMKK (evm.TU.Cha13g002280) showed down-regulated. These findings suggested a positive response of the ETH signal pathway to *E. vexans* infection in tea plants (**Figure 4.9**).

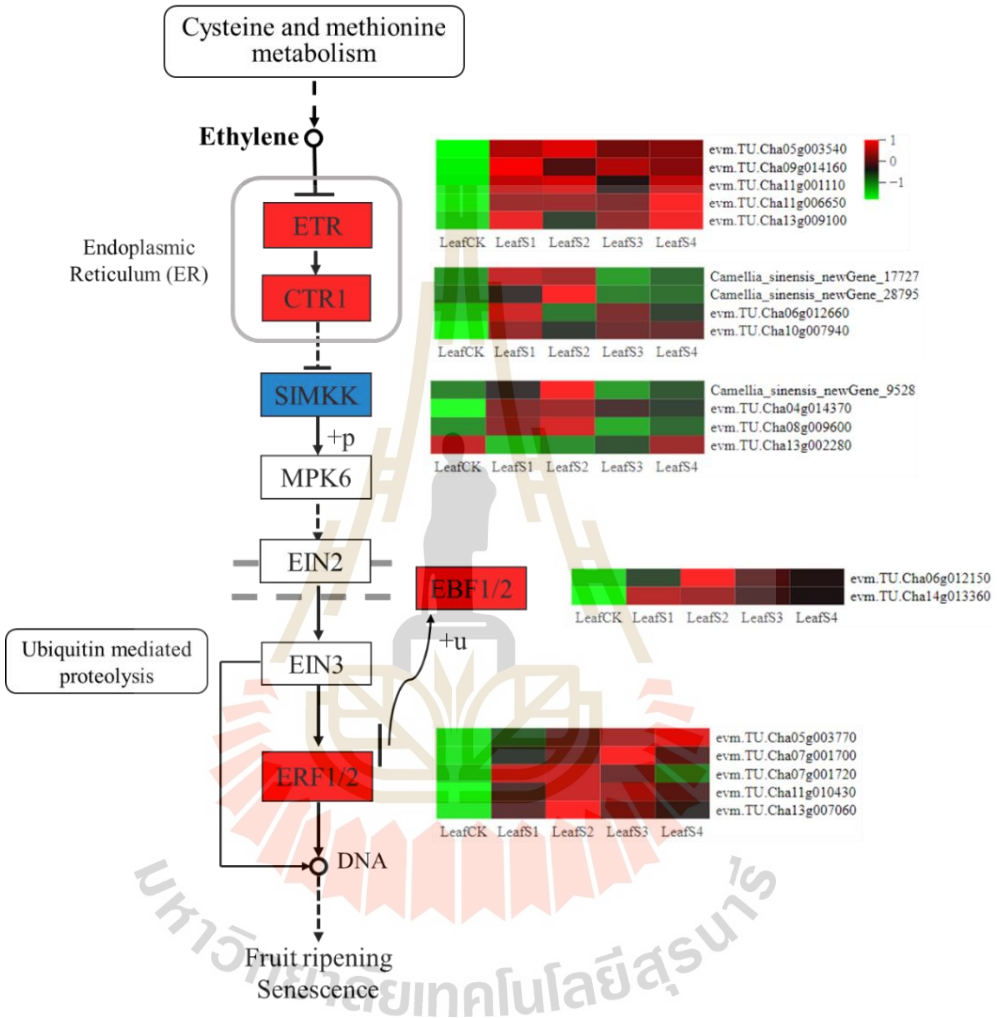

Figure 4.9 DEGs involved in ethylene signaling pathway. Heat map represents the expression values of DEGs related to hormone metabolism at five groups (red for up-regulation, green for down-regulation).

The BR signaling pathway involved in cell division and cell elongation was more complex with numerous DEGs exhibiting abnormal changes in expression. For example, the majority of DEGs such as 17 BRI1 (brassinosteroid insensitive 1), 17 BAK1 (BRI1-associated receptor kinase 1), BKI1 (BRI1 kinase inhibitor 1), BSK (brassinosteroid-signaling kinase), BZR1/2 (brassinozole-resistant l/2), and CYCD3 genes were strongly up-regulated across all infection stages of tea plants, whereas seven BAK1, 24 BRI1, and TCH4 genes were markedly down-regulated only during the early stage of *E. vexans* infection (**Figure 4.10**). Additionally, emerging evidence elucidated that BRs possessed a complicate positive effect on innate immunity and regulation of plant defense responses against various pathogens (Cheon et al., 2013). BAK1, an important regulator of PAMP (pathogen-associated molecular pattern) signaling, interacts with flagellin receptor to modulate both plant defense and development (Shan et al., 2008). Several DEGs related to BR signaling pathway were significantly up-regulated following fungal infection in our results, indicating that BRs might participate in regulating the response to *E. vexans* in tea.

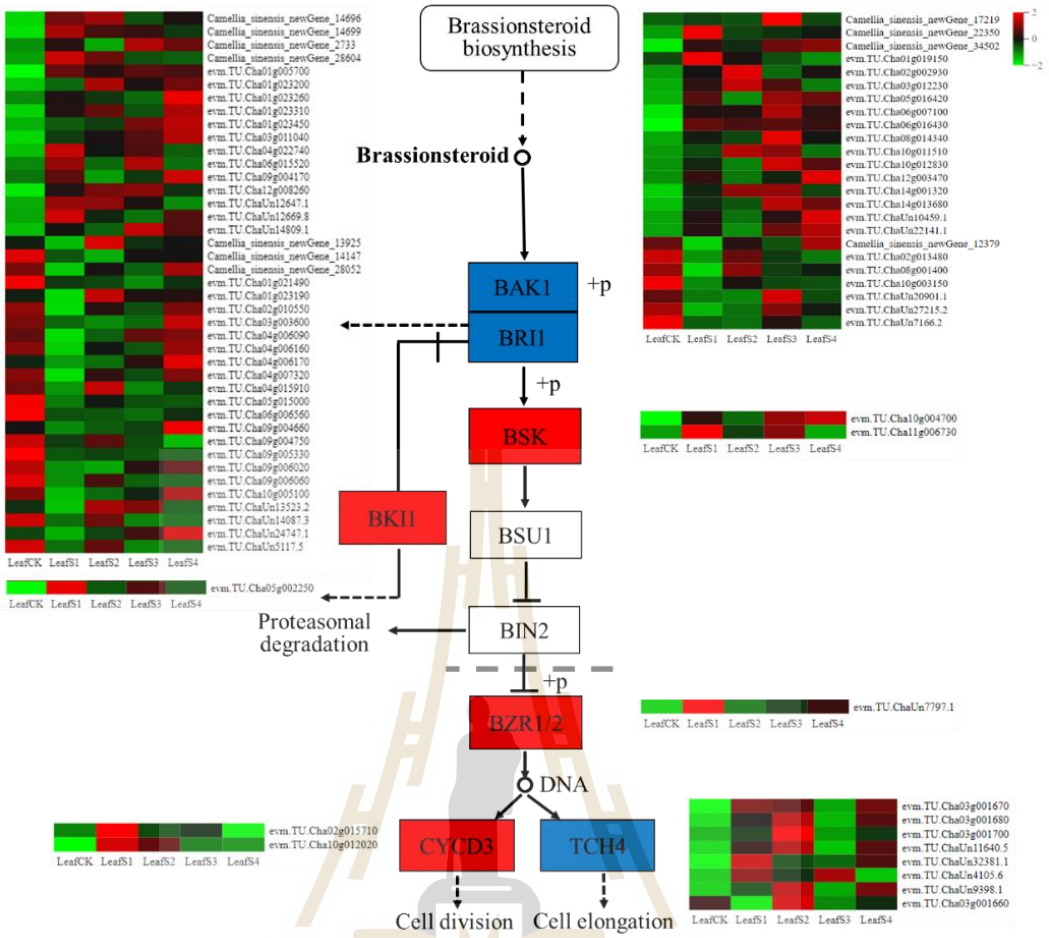

Figure 4.10 DEGs involved in brassionsteroid signaling pathway. Heat map represents the expression values of DEGs associated with hormone metabolism at five groups (red for up-regulation, green for down-regulation).

The similar profiles of gene expression occurred for AUX, a generally acknowledged plant hormone that is conducive to cell enlargement and plant growth. Meanwhile, AUX acts as a key regulatory factor in plant-microbe interactions (Kunkel and Brooks, 2002). The DEGs associated with six AUX/IAA (e.g., evm.TU.Cha03g006520), four GH3 (e.g., evm.TU.Cha05g009180), five SAUR (e small auxin-up RNA), and one ARF gene (evm.TU.Cha04g001260) were found to be up-regulated, whereas ARF (*Camellia_sinensis*_newGene_32872), AUX/IAA (evm.TU.ChaUn7157.1), two TIR1 genes (*Camellia_sinensis*_newGene_18999 and

evm.TU.ChaUn11742.7), GH3 (evm.TU.Cha08g011050), and five SAUR genes (e.g., evm.TU.Cha02g000150 and evm.TU.Cha06g001260) exhibited a significant downward trend during the process of infection. However, minor DEGs related to AUX1, AUX/IAA, and ARF genes were remarkably up-regulated or down-regulated only in the early stage of *E. vexans* infection (**Figure 4.11**). Among these, AUX-responsive GH3 genes have been proved to play crucial roles in plant defense responses in rice and *Arabidopsis* (Ding et al., 2008). Thereby, majority DEGs associated with AUX signaling pathway were strongly expressed by fungal infection, which were speculated to promote disease susceptibility and inhibition of AUX signaling could enhance plant resistance.

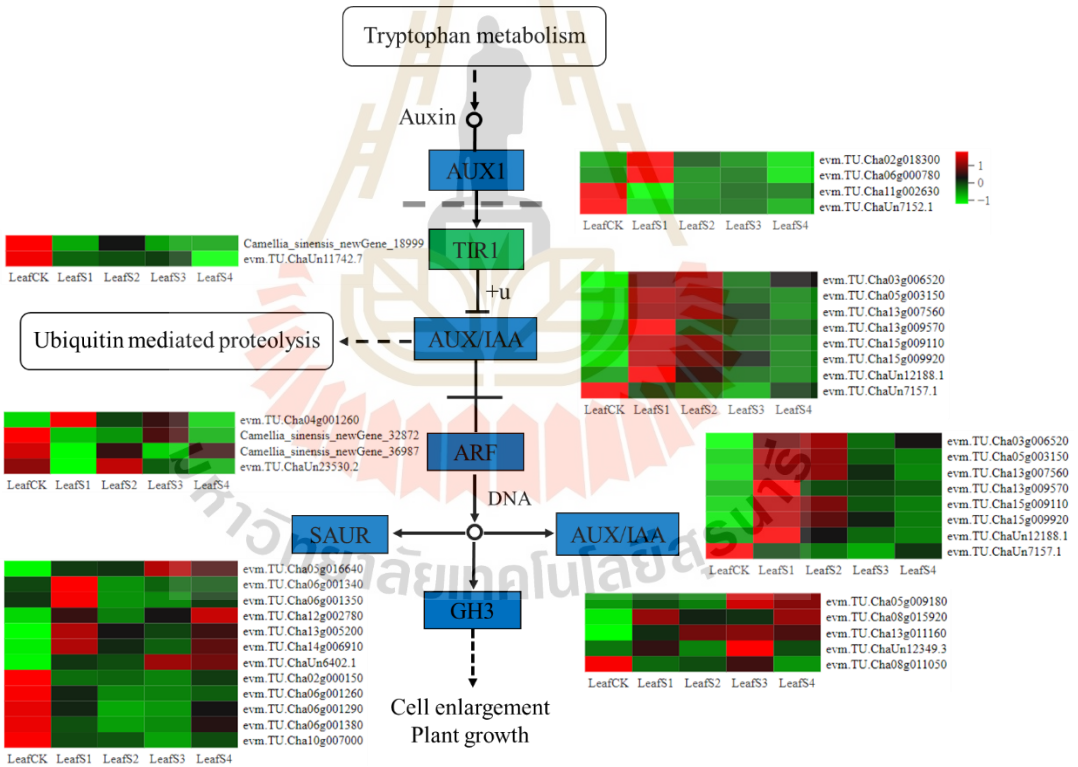

Figure 4.11 DEGs involved in auxin signaling pathway. Heat map represents the expression values of DEGs associated with hormone metabolism at five groups (red for up-regulation, green for down-regulation).

CK is involved in diverse processes including vascular differentiation, chloroplast biogenesis, seed growth, root branch, leaf senescence, and stress tolerance (Müller and Sheen, 2007; Gundesli et al., 2020). Interestingly, CK was reported to regulate plant defense responses against clubroot disease caused by *Plasmodiophora brassicae* and leaf infected with *Rhodococcus fascians* in *Arabidopsis* (Siemens et al., 2006; Depuydt et al., 2008). Several DEGs involved in CK biosynthesis and signaling pathway were observed to be differentially expressed, most of which associated with B-ARR genes (e.g., evm.TU.Cha02g016550) and A-ARR genes (e.g., evm.TU.Cha01g006240) were significantly up-regulated, whereas four of them such as evm.TU.Cha10g010110, evm.TU.Cha06g010480, evm.TU.Cha15g009870, and *Camellia_sinensis*_newGene_33933 were only observed at the early infected stage. However, only five DEGs related to CRE1, AHP, B-ARR, and A-ARR were down-regulated at each infected stage (**Figure 4.12**). These results were speculated that DEGs involved in CK signaling pathway might influence the plant defense response against *E. vexans* infection.

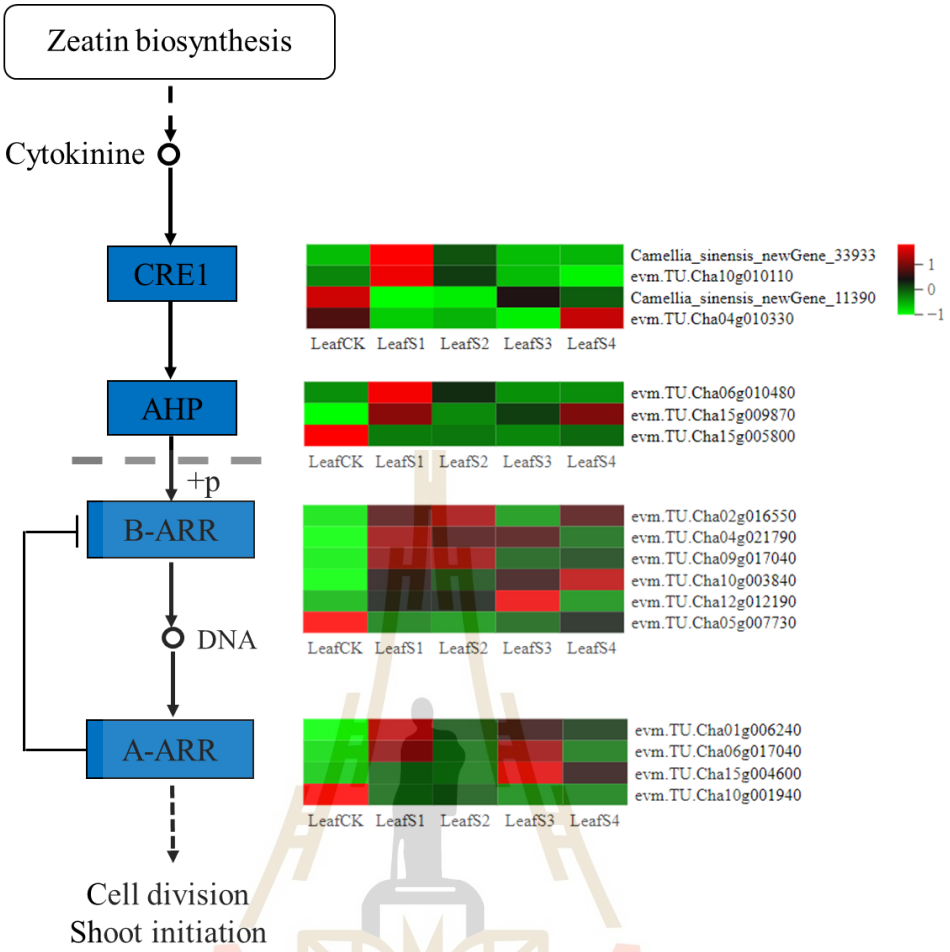

Figure 4.12 DEGs involved in cytokinine signaling pathway. Heat map represents the expression values of DEGs associated with hormone metabolism at five groups (red for up-regulation, green for down-regulation).

GA is supposed to interact with host plants as a signaling factor in fungi or bacteria (MacMillan, 2001). For the GA signaling pathway in the current study, the majority of DEGs were found to be involved in GID1 (gibberellin insensitive dwarf 1), DELLA, and TF, with remarkably up-regulated expression levels. Additionally, four DEGs associated with GID1 (evm.TU.Cha02g008940) and DELLA (evm.TU.Cha01g004370, evm.TU.Cha01g022030, and evm.TU.Cha08g011530) were significantly down-regulated during the four different stages of *E. vexans* infection (**Figure 4.13**). The up-regulated expression of majority DELLA proteins was observed

in our study, which was consistent with the research reported by Navarro et al who indicated that GA promoted resistance to biotrophs and susceptibility to nectrotrophs (Navarro et al., 2008).

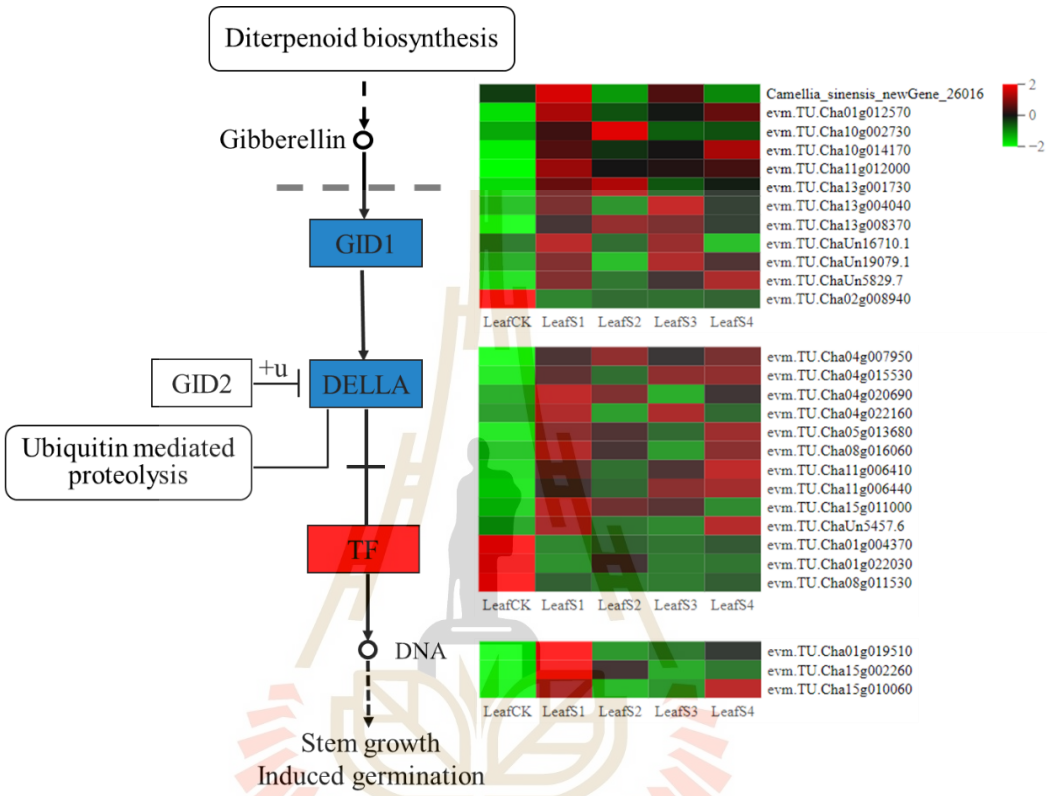

Figure 4.13 DEGs involved in gibberellin signaling pathway. Heat map represents the expression values of DEGs associated with hormone metabolism at five groups (red for up-regulation, green for down-regulation).

Additionally, majority of markedly up-regulated DEGs enriched in ABA signaling pathway were associated with PP2C (e.g., evm.TU.Cha01g009500), SnRK2 (evm.TU.Cha14g005800), and ABF (e.g., evm.TU.Cha02g016090), whereas only two DEGs involved in PYR/PYL (evm.TU.Cha10g003740) and ABA (evm.TU.Cha11g003400) were significantly down-regulated during the infection (**Figure 4.14**). ABA acts as a negative regulator in plant defense against diverse biotrophs and nectrotrophs

(Asselbergh et al., 2008), whereas the role of which seems to be more complicated. However, our findings suggested that ABA appeared to be a positive regulator of plant defense response to *E. vexans* infection, which might depend on the type of pathogen or synergistic role with other hormones (Ton et al., 2009).

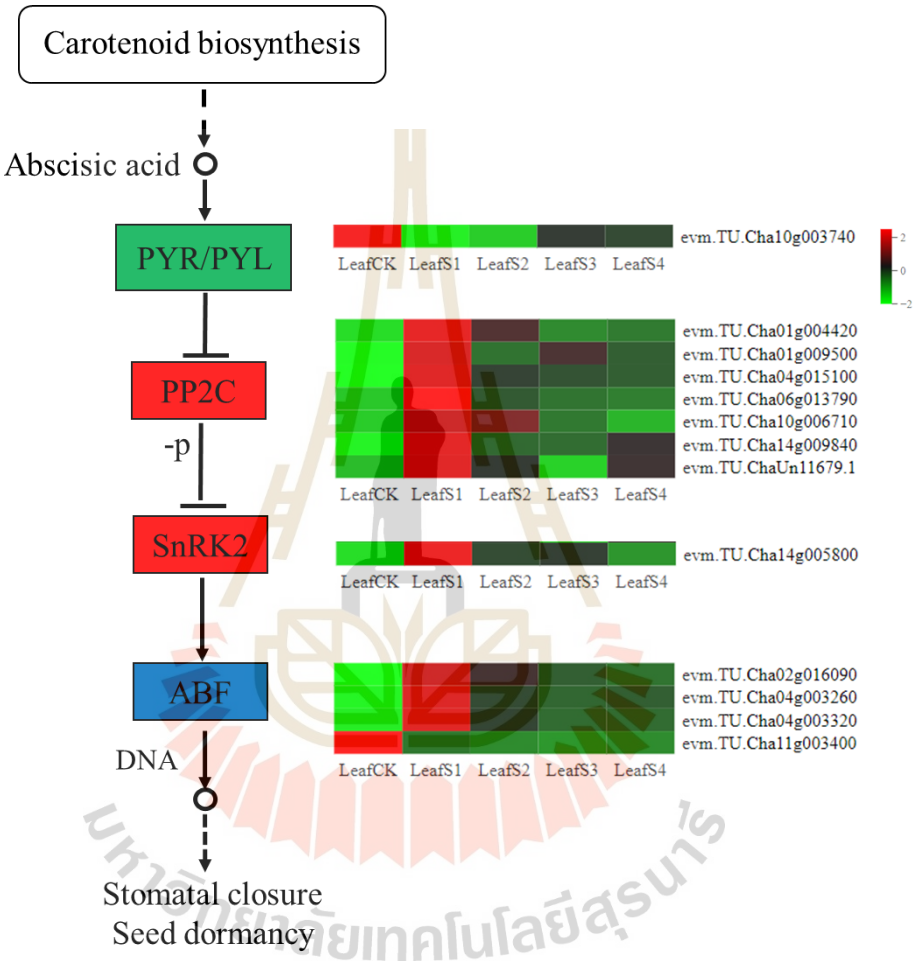

Figure 4.14 DEGs involved in abscisic acid signaling pathway. Heat map represents the expression values of DEGs associated with hormone metabolism at five groups (red for up-regulation, green for down-regulation).

4.3.7 Phytohormone metabolome analysis of tea plants response to *E. vexans* infection

To further elucidate the regulatory effects of phytohormones on the defense response of tea plants infected with *E. vexans*, the contents of several important endogenous hormones involved in JAs (jasmonates), SAs, ETH, SLs (strigolactones), GAs, and ABAs were performed by LC-MS/MS analysis. The nine substances of JAs including JA, MEJA, H2JA, JA-ILE, OPDA, JA-Phe, JA-Val, OPC-4, and OPC-6 were shown in **Table 4.1**. The most abundant JA was detected in both healthy and susceptible leaves, the content of which gradually increased by 1.1-fold to 1.8-fold as the aggravation of *E. vexans* infection. Additionally, the concentrations of JAs-associated hormones involved in MEJA, H2JA, JA-ILE, OPDA, JA-Phe, JA-Val, and OPC-4 exhibited a similar trend with JA, that is, the levels of these hormones in infected leaves were significantly higher than those in healthy leaves. However, the content of OPC-6 was only detected in LeafS1 and LeafS3, which were 27.01 ± 3.24 ng/g and 22.69 ng/g ± 3.71 ng/g, respectively. Consistent with the previous research, JA was demonstrated to act as an important role in plant defense response to pathogens (Derksen et al., 2013). Thus, plants infected with pathogens were accompanied by an increase of JAs, which was presumed that tea plants attacked by *E. vexans* might activate phytohormone signaling networks involved in strong JA pathway, subsequently induced the production of JA-dependent defense substances (MEJA, JA-ILE, JA-Phe, *etc.*) and the accumulation of plant resistance to fungal pathogen.

Table 4.1 The contents of jasmonates (JAs) at different stages of *E. vexans* infection.

Substances	Content (ng/g)				
	LeafCK	LeafS1	LeafS2	LeafS3	LeafS4
JA	161.31±14.67b	341.42±7.11a	343.48±4.52a	379.00±115.97a	444.54±54.18a
MEJA	8.64±0.37d	14.19±0.52a	12.51±0.43b	10.90±0.49c	8.63±0.57d
H2JA	0.29±0.05b	0.46±0.07a	0.51±0.05a	0.52±0.06a	0.61±0.12a
JA-ILE	11.15±0.86c	31.12±3.12b	30.66±0.73b	37.56±7.07b	57.84±11.15a
JA-Phe	0.16±0.02d	0.26±0.03c	0.43±0.03b	0.46±0.05b	0.72±0.08a
OPDA	35.79±1.66d	80.57±2.91a	47.18±1.27c	47.15±4.89c	65.71±6.25b
JA-Val	0.81±0.04c	4.09±0.10b	3.49±0.23b	4.20±0.57b	7.76±1.07a
OPC-4	10.85±0.86e	27.03±3.90b	16.92±0.17d	22.06±1.18c	42.96±2.58a
OPC-6	-	27.01±3.24a	-	22.69±3.71b	-

Note: Different lower-case letters beside the values indicate significant difference among the groups ($p < 0.05$). JA, jasmonic acid; MEJA, methyl jasmonate; H2JA, dihydrojasmonic acid; JA-ILE, jasmonoyl-L-isoleucine; OPDA, cis (+)-12-oxophytodienoic acid; JA-Phe, N-[(-)-jasmonoyl]-(l)-phenalanine; JA-Val, N-[(-)-jasmonoyl]-(L)-valine; OPC-4, 3-oxo-2-(2-(Z)-pentenyl) cyclopentane-1-butyric acid; OPC-6, 3-oxo-2-(2-(Z)-pentenyl)cyclopentane-1-hexanoic acid.

SAs substances serve as an important endogenous signal molecule capable of activating plant disease resistance and defense response (Rajendra and Jones, 2009), which exhibited relatively high levels of these identified hormones. Among these, SA reached the most abundant concentration at the early stage of *E. vexans* infection, with its content showing a pattern of initial decline followed by an increase in the late stage of infection; while SA content in healthy leaves was significantly higher than that in LeafS2 ($p < 0.05$). In addition, the content of SAG found in the healthy leaves was the lowest among all the treatments, exhibiting the same trend with the changes in SAs during the whole infection period (**Figure 4.15A**).

These results suggested that SA might be involved in the process of tea defense response to *E. vexans* infection, which was consistent with a report demonstrating the crucial role of SA in activating tea immunity against *Colletotrichums* (Shi et al., 2019).

SLs are known to be categorized as a new class of plant hormones that derived from carotenoids, which are involved in plant defense responses to abiotic and biotic stresses (Mostofa et al., 2018; Siamak and Parisa, 2020). Among SLs compounds, ST with the lowest level observed in healthy leaves showed no significant difference compared to the susceptible leaves in LeafS2 ($p > 0.05$); whereas the content of ST reached the highest value in LeafS4. Regarding 5DS, another class of SLs phytohormone, the content in susceptible leaves was generally higher than that in healthy leaves except for LeafS2, with the change trend being consistent with that of ST (**Figure 4.15B**). Accordingly, it was speculated that SLs might operate a positive role in plant immunity and contribute to plant defense response during the *E. vexans* infection in tea plants.

Furthermore, the highest contents of ABA and ABA-GE were 35% and 96% higher in LeafS1 compared with the healthy leaves, respectively; while the concentration of ABAs observed in healthy leaves was not significantly different from that in LeafS2 and LeafS4 ($p > 0.05$) (**Figure 4.15C**). Thus, we speculated that the accumulation of ABAs in the early stage of infection might be associated with the defense response of tea plants to *E. vexans* infection, whereas the decrease of which in the middle and late stages were possibly a physiological performance of infection severity.

Additionally, ACC, a direct precursor of ETH biosynthesis, was found to be relatively high in healthy leaves and late stages of infection, with no significant difference observed ($p > 0.05$); while the relative level of ACC was the lowest in LeafS1 and LeafS2, the content of which gradually accumulated with the aggravation of infection (**Figure 4.15D**). These findings indicated that ETH- signaling might act as a

positive regulator to activate expression of defense-related genes following *E. vexans* infection, which was consistent with a previous report exhibited that the introduction of ACC synthase gene *OsACS2* into rice displayed relatively strong resistance against *Magnaporthe oryzae* (Helliwell et al., 2013). Emerging evidence indicated that GAs was also involved in stress responses. GA_3 was reported to act as a negative role in disease resistance to both *Xoo* and *M. oryzae* pathogens in rice (Yang et al., 2008). Interestingly, GA_3 was only detected in LeafS1, with the content value of 0.83 ± 0.15 ng/g (**Figure 4.15D**), suggesting that GA_3 might be involved in the response of tea plants to *E. vexans*, but only in the early stage of pathogen infection. However, the reason for this appearance remains unclear and should require investigation in the future.

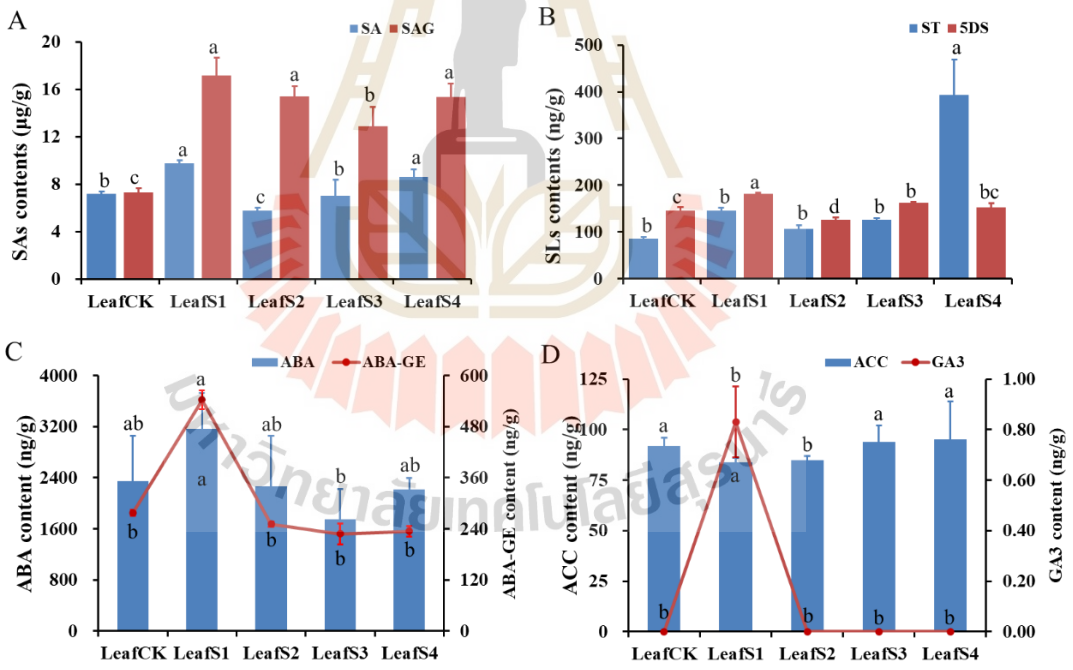

Figure 4.15 The content changes of plant hormones in (**A**) Salicylic acids (SA), (**B**) Strigolactones (SLs), (**C**) Abscisic acids (ABAs), and (**D**) Ethylene (ETH) and gibberellins (GAs) at different stages of *E. vexans* infection. Different letters above the bars or lines represent significant difference between each other ($p < 0.05$).

4.3.8 Correlation analysis of differentially expressed genes and phytohormones

Correlation analysis of DEGs and DAMs annotated in “plant hormone signal transduction” pathway showed close consistency with highly significant differences (p-value < 0.01). The results indicated that a total of 44 DEGs strongly related to two plant hormones with a Pearson correlation coefficient > 0.8 were screened during the whole stage of infection, of which 42 DEGs at the final stage were identified within this pathway, with the least observed at the second stage (**Figure 4.16**). Among them, the two accumulated DAMs involved in JA and JA-ILE were positively regulated by 13 common DEGs, such as the genes encoding BAK1 (*Camellia_sinensis*_newGene_34502 and evm.TU.Cha06g016430), BRI1 (evm.TU.Cha01g023450 and evm.TU.Cha03g011040), ERF1/2 (evm.TU.Cha05g003770), ETR (evm.TU.Cha11g006650), SAUR (evm.TU.Cha12g002780), GID1 (evm.TU.Cha10g014170 and evm.TU.ChaUn5829.7), BSK (brassinosteroid signal kinase, evm.TU.Cha10g004700), and DALLE (evm.TU.Cha11g006410 and evm.TU.Cha11g006440), while they were also negatively regulated by the only two DEGs, such as BRI1 (evm.TU.Cha09g005330) and A-ARR (Type A response regulators, evm.TU.Cha10g001940). Notably, more DEGs positively regulated DAMs than those that negatively regulated them. Numerous evidence suggested that JA were associated with plant defense responses against necrotrophic pathogens (De Vleesschauwer et al., 2013), whereas it might also act as a crucial role in plant immunity against biotrophic pathogens in our study. JA-ILe is considered as a bioactive JA molecule perceived by plants (Thines et al., 2007). The high expression of two important JA-mediated signaling molecules such as JA and JA-ILe was positively regulated by DEGs encoding PTI factor BAK1, BRI1, and BSK of BR-dependent signaling pathway, ERF1/2 and ETR of ETH-dependent signaling pathway, SAUR of AUX-dependent signaling pathway, and DELLA of GA-dependent signaling pathway, which indicated that JA- and BR-, ETH-, AUX-, GA-signaling might synergistically activate the expression of some defense-related genes in tea plants

infected with *E. vexans* (Glazebrook, 2005; Llorente et al., 2008). However, JA and JA-ILe were negatively regulated by the gene encoding A-ARR of CK-mediated signaling, which was speculated that JA- and CK-signaling might operate an antagonistic role during the infection of tea plants.

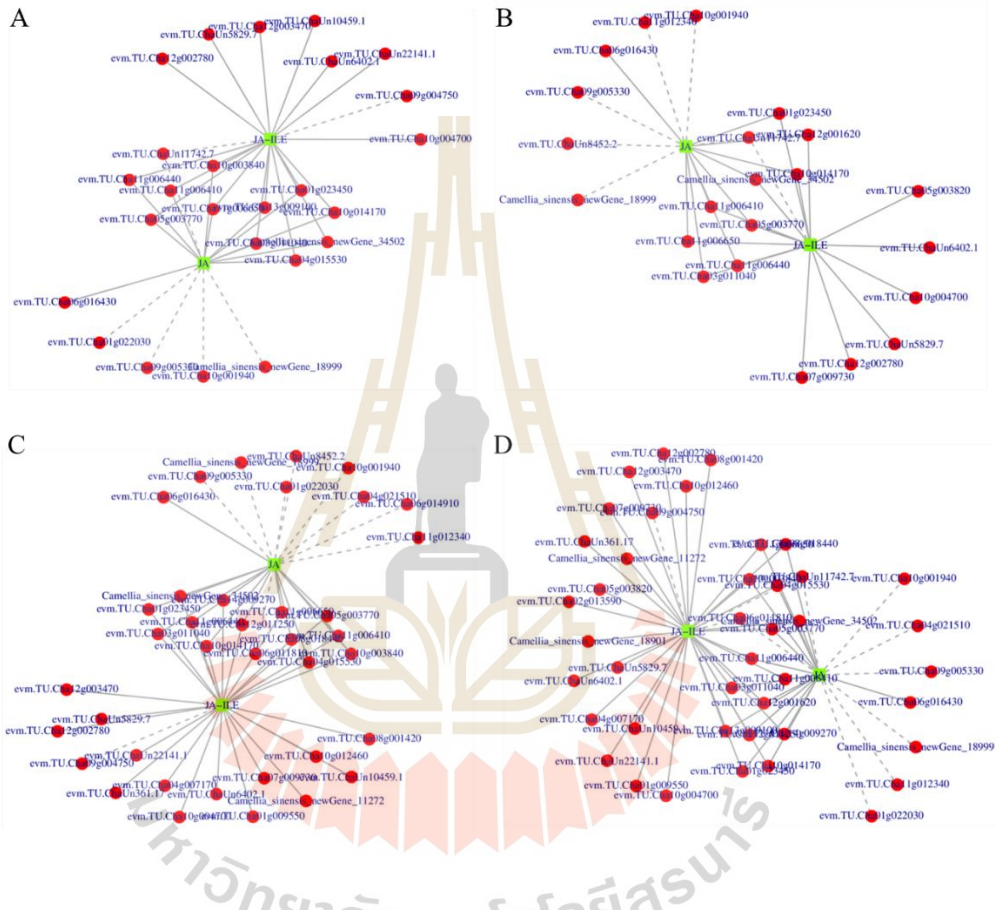

Figure 4.16 Network analysis of DEGs and DAMs involved in phytohormone signal transduction pathways of tea infected with *E. vexans*. (**A**) LeafCK vs. LeafS1, (**B**) LeafCK vs. LeafS2, (**C**) LeafCK vs. LeafS3, and (**D**) LeafCK vs. LeafS4. Red circles represent genes, and green circles represent metabolites. Solid and dotted lines indicate positive and negative correlations, respectively, as calculated by a Pearson correlation coefficient > 0.8 or < −0.8, respectively.

4.3.9 qRT-PCR validation of differentially expressed genes

Six targeted genes (both five up-regulated and one down-regulated) across the whole stage of *E. vexans* infection were selected from the RNA-Seq data for qRT-PCR analysis to verify the expression patterns. Among these, expression levels of DEGs involved in AP2 (evm.TU.Cha13g007060), bZIP (evm.TU.Cha02g016090), WRKY6 (evm.TU.Cha07g014430), MYB (*Camellia_sinensis*_newGene_41861), RPM1 (evm.TU.Cha04g000480), and RPS2 (evm.TU.ChaUn15033.3), as determined by qRT-PCR analysis, were observed to be closely consistent with RNA-Seq data, exhibiting the similar up- or down-regulated expression patterns (**Figure 4.17**). Thereby, these results indicated that the two analysis methods for RNA-Seq and qRT-PCR might provide stable and reliable information for genes to reveal the molecular mechanism of tea plants response to *E. vexans* infection.

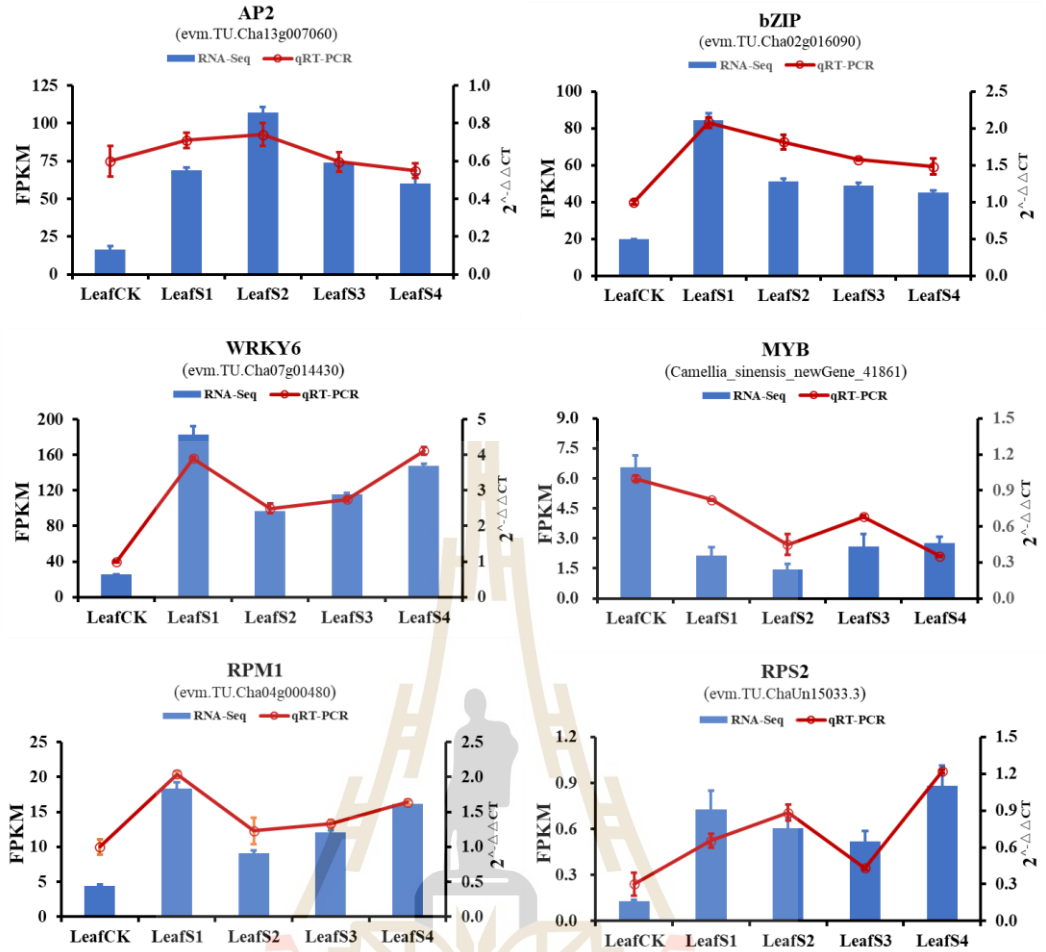

Figure 4.17 qRT-PCR validation of DEGs associated with disease resistance genes. The six targeted genes included AP2, bZIP, WAKY6, MYB, RPM2, and RPS. The error bars represent ± SE of three replicates ($p \leq 0.05$).

4.4 Conclusion

In summary, this study presents, for the first time, a comprehensive analysis of the immune response expression in tea plants following *E. vexans* infection at the transcriptional and hormone metabolic levels. The transcriptome analysis revealed that most disease-resistant genes were activated in response to pathogen invasion, particularly those involved in "plant hormone signal transduction" and "plant-pathogen interaction", exhibiting up-regulated expression. Additionally, SA acted as a crucial role in defense activation of tea immunity against *E. vexans* infection,

confirming that tea responding to pathogen infestation was more inclined to biotrophs. Equally notable was that JA- and BR-, ETH-, AUX-signaling might synergistically activate the expression of some defense-related genes after *E. vexans* infection, the mechanism of which requires further verification. Overall, this study provides powerful guidance for understanding of the immune response mechanism involved in tea plants defense against blister blight.

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CHAPTER V

EVALUATION MEDHODS OF APPLYING ENHANCERS TO CONTROL TEA BLISTER BLIGHT DISEASE

ABSTRACT

Blister blight disease, caused by an obligate biotrophic fungal pathogen *E. vexans*, poses a severe threat to both the yield and quality of tea. Given that the application of chemical fungicides on tea leaves has significantly increased the health hazards associated with tea consumption, substantial efforts are currently being made to manage these pathogens by enhancing the inherent natural defense mechanisms of tea plants against invading pathogens. This study was aimed to investigate the potential of salicylic acid (SA) and tea saponin (TS) in inducing resistance in tea plants against blister blight. Foliar spraying of SA and TS significantly reduced the disease severity of blister blight under greenhouse conditions, with the most effective performances observed at concentrations of 0.5 mg/mL and 6.0 mg/mL, respectively. In field trials, the spray application of SA in combination with TS resulted in a disease control effect of 49.89%. Additionally, combination of SA and TS had the potential to enhance the disease resistance of tea plants by increasing the activity of defense enzymes involved in polyphenol oxidase (PPO), peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), phenylalanine ammonia-lyase (PAL), β-1,3-glucanase, and chitinase. These results suggested that the combination of SA and TS could serve as a potential enhancer in the integrated control management in tea cultivation, and TS can also be used as a synergist combined with other fungicides to resist the invasion of plant pathogens.

Keywords: Tea; *Exobasidium vexans*; blister blight disease; defense enzymes; salicylic acid; tea saponin

5.1 Introduction

Tea, which is derived from the delicate leaves of the *Camellia sinensis* plant, ranks among the most widely consumed beverages globally (Li et al., 2018). However, the production and quality of tea are directly or indirectly affected by several abiotic and biotic factors. The most devastating biotic factor is blister blight disease infected with fungus *E. vexans* (Pandey et al., 2021). This type of fungal pathogen typically has a negative impact on the tender buds and leaves, resulting in a reduction of 20-50% in the yield of harvested new shoots, which directly hampers the economic growth of tea-producing countries due to its substantial export value (Sowndhararajan et al., 2013). In recent decades, the variations in climate and irrational interventions for field management have affected the susceptibility of plants to this pathogen, and the emergence and dissemination of disease. A variety of chemical fungicides with various modes of action are employed to control the diverse foliar pathogens in tea plants. Despite these fungicides have demonstrated effectiveness, concerns persist regarding potential phytotoxicity and pesticide residues, in addition to their impact on environmental pollution and human health hazards (Saravanakumar et al., 2006; Ajay et al., 2010). Hence, it is imperative to investigate eco-friendly methods for disease management, which can help achieve the sustainability objectives of tea cultivation without imposing significant health risks on consumers.

To date, some non-chemical strategies have been attempted to explore their ability to induce defense responses in the systems of tea plants. For example, the field application of chitosan has been found to effectively reduce the incidence of tea blister blight disease, as well as enhance the activity of relevant defense enzymes and the total phenol content in comparison to untreated plants (Chandra et al., 2017). The application of 0.2% isobavachalcone (IBC), a new model of plant-derived fungicide, in combination with chitosan has been reported to demonstrate a potential controlling effect on the severity of blister blight disease, with a protection efficacy of 61.72% (Yang et al., 2023). The spray application of calcium chloride

($CaCl_2$) at 1% provided disease protection of 71% against blister blight (Chandra et al., 2014). Additionally, various biotic and abiotic enhancers (Sowndhararajan et al., 2013; Adhikari and Rai, 2022), and plant growth-promoting rhizobacteria (PGPR) bioformulas (*Pseudomonas fluorescens* Pf1) (Saravanakumar et al., 2006) have been successfully employed to induce resistance under field trials against blister blight disease in tea plants.

Plants have intrinsic mechanisms to resist pathogen infections, which can be either constitutive or systemically induced (Ram et al., 2022). Specific defense mechanisms are activated in plants following pathogen infection, leading to the induction of systemic acquired resistance (SAR). The SAR mechanism relies on salicylic acid (SA) as a signaling molecule and is correlated with the production of defense enzymes and pathogenesis-related (PR) proteins (Patil and Nandi, 2022). In addition to pathogens, various certain compounds applied to plants simulate the host-pathogen interaction to trigger SAR (Esther et al., 2023). These compounds are commonly referred to as SAR enhancers. SA is recognized as a defense-associated phytohormone that plays a crucial role in regulating plant immunity (Koo et al., 2020). Besides that, SA as an exogenous enhancer has been proven to induce SAR in various crop plants against invasion of different pathogens, including *Botrytis cinerea* in tomato (Li et al., 2016), *Phytophthora palmivora* in rubber tree (Deenamo et al., 2018), bacterial wilt in chilli (Chandrasekhar et al., 2017), and *Fusarium* wilt in chickpea (Saikia et al., 2003).

Tea saponin is a pentacyclic triterpenoid compound extracted from the seeds of various plants in the *Camellia* genus of the *Theaceae* family, including *C. sinensis*, *C. oleifera*, *C. cuspidata*, and *C. sasanqua* (Yu et al., 2022). Tea saponin, as a natural surfactant, exhibits excellent properties and possesses various beneficial bioactivities, including anticancer (Jia et al., 2017), anti-tumor (Jia et al., 2017), antioxidant (Lin et al., 2018), anti-inflammatory (Zhang et al., 2022), and antibacterial (Zhao et al., 2020), which has the potential for extensive applications in daily chemical products, pharmaceuticals, and the food industry. Additionally, tea

saponin, known for its saponin's hemolysis, ichthyootoxin, antibacterial/fungal, and insecticidal properties, has been used as a bio-agent, such as fungicides, insecticides, acaricides, and biopesticide synergist, in agricultural production (Shudh et al., 2018; Cui et al., 2019; Luo et al., 2021). However, tea saponin, as a plant-based fungicide and synergist, has not yet been reported to be applied in combination with chemical enhancer SA against fungal diseases in tea plants. In the present study, an attempt was made to investigate the effects of foliar application of SA and TS, along with their combination at greenhouse conditions and field trials in controlling blister blight disease.

5.2 Materials and methods

5.2.1 Pre-experiment on screening enhancer products on tea blister blight disease under greenhouse conditions

The healthy two-year-old tea seedlings, cultivar 'Fuding Dabaicha', were used for this experiment under greenhouse conditions to screen the appropriate dosage of enhancers. The experiment involved 10 plants in each treatment, which were maintained at 24-26°C and a relative humidity of 80-90%. Basidiospores of *E. vexans* obtained from naturally infected blister lesions at the tea plantation in Guiding, China (26°N, 107°E; altitude 1738m), were mixed with sterilized water and adjusted to suspension at 10^6 spores/mL. The enhancers containing salicylic acid (SA) at the concentrations of 0.05, 0.20, 0.35, 0.50, and 0.65 mg/mL and tea saponin (TS) solutions at the concentrations of 1.5, 3.0, 4.5, 6.0, and 7.5 mg/mL were sprayed twice continuously onto both surfaces of the leaves at an interval of five days using a manual sprayer, with the water-treated plants serving as controls. After three days of spraying, the leaves of plants were inoculated with spore suspension in all treatments. Five days after inoculation, disease severity of plants from each treatment was evaluated using the method described by Ajay et al. (2010) with some modification (**Table 5.1**) and the disease reduction (DR) of plants was calculated according to the following formula:

$$DS\ (\%) = \frac{\text{Score for each group leaves} \times \text{Number of leaves per infection level}}{\text{The number of leaves assessed} \times 4} \times 100$$

$$DR\ (\%) = \frac{\text{DS of negative control - DS of enhancers}}{\text{DS of negative control}} \times 100$$

Table 5.1 Blister blight severity assessment.

Score	Description
0	No translucent spots/blister/necrotic spots (No disease) on leaves
1	One to two disease spots appear on the leaves
2	Three to five disease spots appear on the leaves
3	More than five disease spots appear on the leaves
4	Twig/stem is infected/die back/leaf fall/stem breakage

5.2.2 Evaluation of enhancer products on tea blister blight disease under field conditions

The field trial was conducted in the tea plantation of Guiding, China, from September to October 2023. The tea plantation is located at an average elevation of 1387 m and experiences a climate with an average annual temperature of 14.3°C, relative humidity of 80-90%, and an annual rainfall of 1080 mm. The 15-year-old tea bushes of 'Fuding Dabaicha' were used to assess the efficacy of the enhancers involved in SA and TA or their combination against blister blight disease caused by *E. vexans* (**Table 5.2**). The experiment was laid out in a randomized complete block design (RCBD) with five treatments and repeated four times with each consisting of 20 bushes. The enhancers were sprayed twice continuously onto both surfaces of the leaves at an interval of seven days using a tank sprayer, with the water-treated bushes serving as controls. The foliar spraying was performed once on the fourteenth day after the second application. During the 7th, 14th, 21st, and 28th days following the final spray application, the fifty shoots (two leaves and a bud) of the same age with the uniform size were randomly collected from each treatment and evaluated for the presence of blister spots. The disease was scored by the

method of Saravanakumar et al. (2006) on six-point scale (where 0 = no infection, 1 = lesions cover < 1% leaf area, 3 = lesions cover < 10% leaf area, 5 = lesions cover < 25 % leaf area, 7 = lesions cover < 50 % leaf area, 9 = lesions cover ≥ 50% leaf area), percentage disease index (PDI) and disease control effect were assessed using the following formula:

$$\text{PDI}\ (\%) = \frac{\text{Sum of all numerical scoring}}{\text{Total number of leaves} \times \text{Maximum score}} \times 100 \quad (1)$$

$$\text{Control effect}\ (\%) = \frac{\text{PDI of negative control - PDI of enhancers}}{\text{PDI of negative control}} \times 100 \quad (2)$$

Table 5.2 Test concentrations for each agent in the diseased plant plot.

Treatments	Concentration	Note
SA	0.5 mg/mL	Optimum concentration SA (salicylic acid)
TS	6.0 mg/mL	TS (tea saponin)
IBC	600 × (dilution time)	Commercial bioagent
SA+TS	0.5 mg/mL+6.0 mg/mL	A ratio of 1:1
CK	-	-

5.2.3 Detection of defense enzymes

5.2.3.1 Extraction of crude enzyme

The first leaves were collected from the treated and control plants following the completion of field experiment to determine the activities of defense-related enzymes, including peroxidase (POD), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), β-1,3 glucanase, and catalase (CAT), superoxide dismutase (SOD), and chitinase. Approximately 0.1 g of fresh leaf tissues were homogenized with liquid nitrogen and subsequently extracted with 1 mL of buffer containing 0.05 mol/L of 1% polyvinylpyrrolidone (PH 7.8) and 20 µl of 1 mM phenylmethane

sulphonyl fluoride. The extract was then centrifuged for 10 min at 12,000 × g and 4°C to collect supernatants as the crude enzyme extract. The experiment was repeated three times.

5.2.3.2 POD detection

POD enzyme activity was measured as described by Chandra et al. (2017) with some modifications. The reaction mixture was prepared by combining 150 µl of substrate containing 70 µl of 0.2 M phosphate buffer (pH 6.0), 40 µl of guaiacol, and 40 µl of 0.3% H_2O_2 with 50 µl of enzyme extract in a water bath at 37°C for 5 min. A buffer solution of the same volume was used as the control instead of the enzyme extract. The absorbance was measured at the wavelength of 470 nm. One unit of enzyme activity (U) was calculated as a 0.005 increase in OD value per gram of sample per minute.

5.2.3.3 PPO detection

PPO enzyme activity was determined as the method of Chandra et al. (2017) with slight modifications. The reaction mixture contained 0.1 mol/L citric acid phosphate buffer (pH 5.6), proline solution (10%, w/v), and catechol solution (1%, w/v) in a ratio of 10:2:3, along with 200 µl of enzyme extract. The mixture without catechol was set as the control. After incubating for 30 min in a water bath at 37°C, 1.2 ml of 6 mol/L urea was added to terminate the reaction. The absorbance was recorded at the wavelength of 420 nm. One unit of enzyme activity (U) was calculated as a 0.01 increase in OD value per gram of sample per minute.

5.2.3.4 PAL detection

PAL enzyme activity was estimated by following the method of Ajay and Baby, (2010). The reaction mixture was prepared by mixing 5 µl of enzyme extract, 145 µl of 1.1 M sodium borate buffer (pH 8.0) and 40 µl of 0.1 M *L*-phenylalanine, and then was incubated for 30 min in a water bath at 30°C. The enzyme solution was replaced with buffer of the same volume as the control. The absorbance was adjusted at the wavelength of 290 nm. One unit of enzyme activity (U) was

calculated as a 0.01 increase in OD value per gram of sample per minute.

5.2.3.5 β-1,3 glucanase detection

β-1,3 glucanase activity was determined according to the method described by Chandra et al. (2014) with some modifications. The reaction mixture was prepared by mixing 30 µl of crude enzyme extract and 30 µl of laminarin (2%, w/v), and was then incubated at 40°C for 10 min. The reaction was terminated by the addition of 190 µl of dinitrosalicylic acid, followed by heating in a boiling water bath for 5 min. The resulting solution, which exhibited a distinct color, was diluted with distilled water to a volume of 4.5 ml with subsequent vortexes. The absorbance was recorded at the wavelength of 540 nm. One unit of enzyme activity (U) was quantified as the amount of enzyme needed to liberate 1 mg of the reduction of glucose per hour.

5.2.3.6 CAT detection

CAT enzyme activity was measured by following the method of Jha and Mohamed, (2023) with slight modifications. About 10 µl of enzyme extract was added to 190 µl of the reaction solution containing 0.15 M phosphate buffer (pH 7.0) and 30% (w/v) H_2O_2. The absorbance was measured at the wavelength of 240 nm. An enzyme activity unit (U) was defined as a 0.01 decrease in OD value per gram of sample per minute. One unit of enzyme activity (U) was defined as the catalysis of the degradation of 1 µmol H_2O_2 per gram of sample per minute.

5.2.3.7 SOD detection

SOD enzyme activity was evaluated as described by Prochazkova et al. (2001). The reaction mixture was prepared by mixing 20 µl of enzyme extract, 120 µl of phosphate buffer (50 mM, pH 7.8), 20 µl of methionine (13 mM), 20 µl of nitroblue tetrazolium chloride (25 mM), EDTA (0.1 mM), and 20 µl of sodium carbonate (50 mM), followed by heating in a water bath at 37°C for 30 min. The absorbance was measured at 450 nm, and one unit of enzyme activity (U) was calculated as the concentration of SOD inhibiting the reduction of nitroblue tetrazolium by 50%.

5.2.3.8 Chitinase detection

Chitinase activity was assayed by following the method of Prakongkha et al. (2013). The 100-µl reaction mixture containing 100 µl of crude enzyme extract, 20 µl of colloidal chitin (0.1%, w/v), and 80 µl of sodium acetate buffer (0.05 M, pH 5.0), was incubated at 37°C for 3 h. The absorbance value was recorded at 580 nm, and the standard curve was calculated by preparing from *N*-acetyl glucosamine (GlcNAc). One unit of enzyme activity (U) was defined as the amount of chitinase decomposing chitin in grams of tissue to produce 1 mmol of GlcNAc per hour.

5.2.3.9 Statistical analysis

All the parameters determined to evaluate the efficiency of the enhancers against tea blister blight disease caused by *E. vexans* were subjected to One-way ANOVA analysis and were followed by Duncan multiple range test with a significant level of $p < 0.05$ for mean comparisons using SPSS software version 23.0 (IBM, USA).

5.3 Results

5.3.1 The effects of enhancers on tea blister blight disease under greenhouse conditions

The present study was to screen the optimal concentration of SA and TS for controlling blister blight disease under greenhouse conditions. The plants treated with enhancers exhibited a lower disease severity compared to untreated ones on inoculation (**Figure 5.1A**). Among these, plants had the lowest level of disease severity after treatment with SA at 0.5 mg/mL and TS at 6.0 mg/mL, respectively. For disease reduction, SA treatments were significantly superior to TS (**Figure 5.1B**). Briefly, SA treatments provided the protective effects of 12.12%, 20.01%, 28.33%, 41.43%, and 36.02%, respectively, for the five gradient concentrations of 0.05 mg/mL, 0.20 mg/mL, 0.35 mg/mL, 0.50 mg/mL, and 0.65 mg/mL. Among these, SA at 0.50 mg/mL achieved the highest performance. Similarly, concentrations of TS at 1.5 mg/mL, 3.0 mg/mL, 4.5 mg/mL, 6.0 mg/mL, and 7.5 mg/mL reduced disease infection by 9.71%, 25.66%, 22.04%, 35.64%, and 31.82%, respectively, compared to

CK group. Notably, the most significant reduction rate was observed with TS at 6.0 mg/mL.

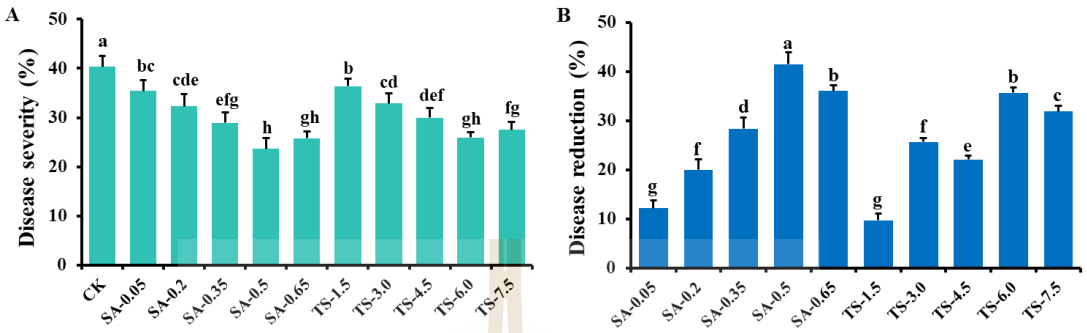

Figure 5.1 The effects of enhancers at different concentration on tea blister blight disease under greenhouse conditions. (**A**) Disease severity, (**B**) Disease reduction. Salicylic acid (SA) and tea saponin (TS) at different concentrations were compared with the untreated plant (CK group). Vertical bars indicate standard errors of the means. Different letters indicate significant differences at $p < 0.05$.

5.3.2 The effects of enhancers for controlling tea blister blight under field conditions

The field experiment was performed based on the pre-experiment to evaluate the efficiency of SA and TS (0.50 mg/mL and 6.0 mg/mL) in combination against tea blister blight disease. The initial average percent disease index (PDI) for tea blister blight disease was 6.33 in September 2023. The application of enhancers (SA, TS, IBC, and SA+TS) significantly reduced the PDI of tea plants compared to the untreated groups (**Figure 5.2A**). The efficiency of foliar application of IBC, a commercial bio-agent, was the most effective compared to the separate or combined use of SA and TS, reaching a control effect of 59.61% on the 14th day (**Figure 5.2B**). SA treatment could provide a higher protection more than 34.5% during the entire application period. Although the control effect of foliar application

for TS treatment was lower than that of other treatments, it was only 34.8% on the 28th day. However, the combination of SA with TS enhanced the control effect by 49.89%, which was 19.06% and 30.25% higher than the individual application of SA and TS, respectively. Additionally, the protection treated with these enhancers was superior on the 14th day over all other days.

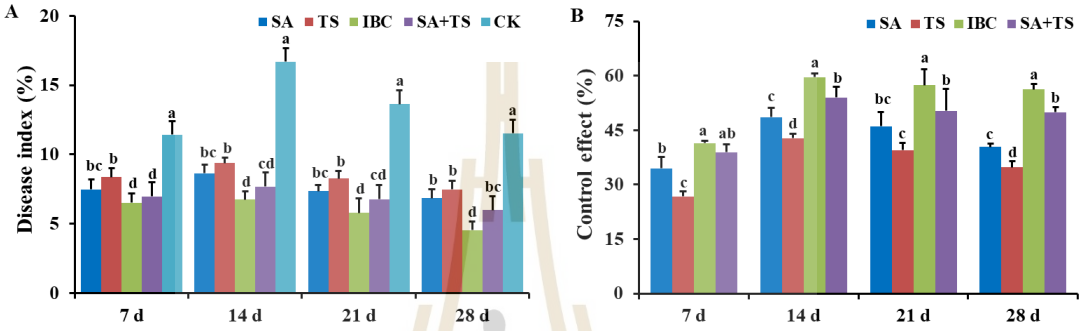

Figure 5.2 The effects of different enhancers on tea blister blight disease under field conditions. (**A**) Percent disease index, (**B**) Control effect. SA at 0.50 mg/mL, TS at 6.0 mg/mL, combination of SA with TS, and commercial agent IBC (0.2% isobavachalcone) at 600× (dilution times) were compared with the CK group. Vertical bars indicate standard errors of the means. Different letters indicate significant differences at $p < 0.05$.

5.3.3 Evaluation of defense enzymes

The foliar application of enhancers including SA, TS, combination of SA with TS, and IBC resulted in the accumulated activity of defense enzymes involved in PPO, POD, CAT, SOD, PAL, β-1,3-glucanase, and chitinase in tea leaves compared to untreated plants (**Figure 5.3**). The highest accumulation of these seven enzymes was observed in the tea leaves treated with IBC (positive bio-agent), increased by 0.95-fold for PPO, 1.25-fold by POD, 2.19-fold by CAT, 1.39-fold by SOD, 0.39-fold by PAL, 0.31-fold by β-1,3-glucanase, and 4.29-fold by chitinase in comparison to untreated group. This was followed by the combination of SA and TS, with 0.47-fold, 0.79-fold,

0.81-fold, 1.08-fold, 0.15-fold, 0.3-fold, and 1.47-fold increases in PPO, POD, CAT, SOD, PAL, β-1,3-glucanase, and chitinase in tea leaves compared to untreated plants.

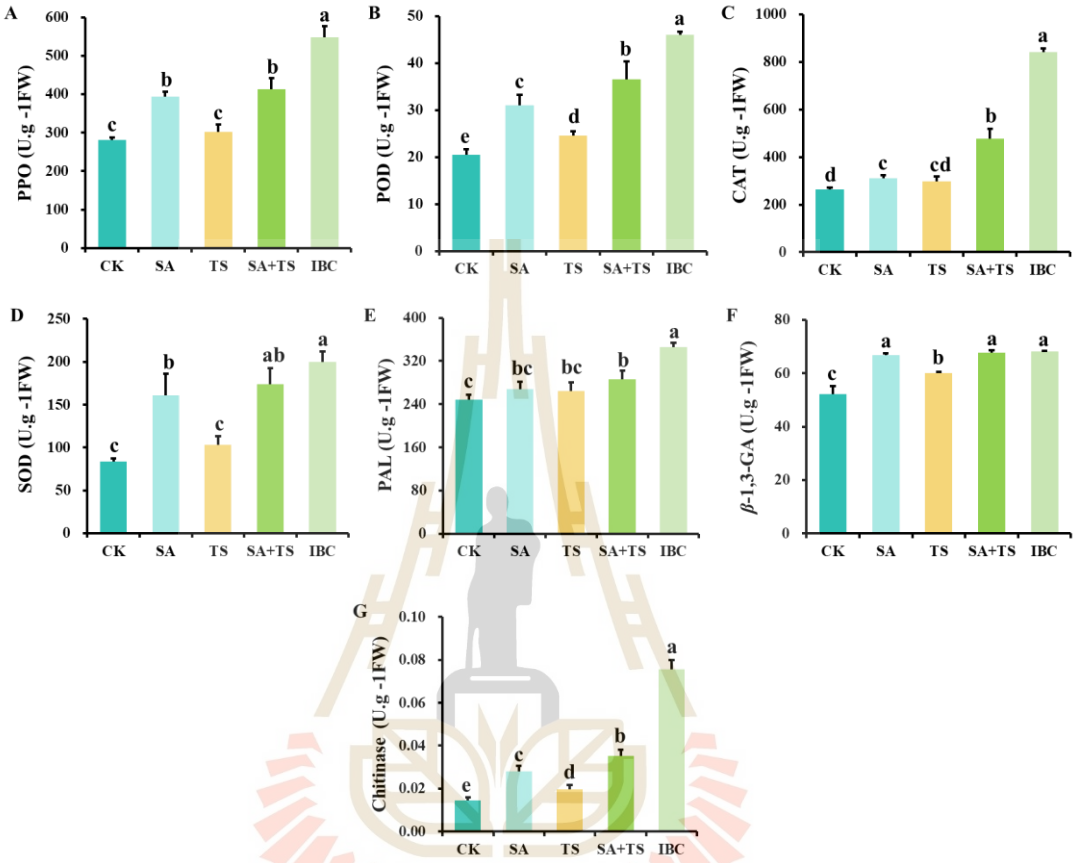

Figure 5.3 Defense enzyme activity on tea blister blight disease under field conditions. (**A**) PPO activity, (**B**) POD activity, (**C**) CAT activity, (**D**) SOD activity, (**E**) PAL activity, (**F**) β-1,3-GA activity, (**G**) Chitinase activity. Different letters indicate significant differences at $p < 0.05$.

5.4 Discussion

Until now, the application of enhancers to protect plants has emerged as a superior alternative widely adopted in crop protection compared to the extensive use of chemical fungicides. Plants possess inherent chemical and mechanical barriers along with defense systems that require specific stimuli for activation against the array of pathogens (Montesano et al., 2003). Enhancers are proficient at triggering diverse plant defense mechanisms, leading to a cascade of responses that include reinforcement of the cell wall through lignin deposition, an oxidative burst resulting in hypersensitive responses, synthesis of antimicrobial compounds, and activation of defense genes. The current study presented evidence supporting the effectiveness of foliar application of enhancers (SA and TS) in protecting to tea plants against blister blight infection in both greenhouse and field trial, and the efficacy of these enhancers is dependent on the activation of tea-induced defense-related enzymes.

Following its discovery in 1979 as an enhancer that triggers resistance in tobacco plants against *Tobacco mosaic virus* (TMV), numerous studies indicates that SA serves as a crucial plant hormone involved in resistance to diverse pathogens (Koo et al., 2020). Exogenous application of SA could activate systemic acquired resistance (SAR) in a variety of plants against *Fusarium oxysporum* of watermelon (Zhu et al., 2022), *Colletotrichum capsici* of chilli (Geat et al., 2015), *Alternaria alternate* of peanut (Chitra et al., 2008), *Magnaporthe grisea* of rice (Xie et al., 2011). Under the field trials in this study, SA treatment provided a higher protection with 40.38% by the 28th day, which is relatively satisfactory. TS is a type of pentacyclic triterpenoid saponin mixture that is commonly found in the tissues of various *Camellia* species, which possesses diverse beneficial properties including antioxidant (Lin et al., 2018), anti-inflammatory (Zhang et al., 2022), and antibacterial (Zhao et al., 2020). Additionally, TS has been demonstrated to exhibit significant inhibitory effects on fungal diseases in the field and the growth of fungi in vitro. The minimum inhibitory concentrations of tea saponin were found to be 0.078, 0.156, and 0.156 mg/mL for inhibiting the infection of *Candida albicans*, *Saccharomyces cerevisiae*,

and *Penicillium*, respectively (Yu et al., 2022). Notably, TS, as a new type of plant-derived synergist and complexing agent, has a significant synergistic effect on insecticidal (Cui et al., 2012; Lv et al., 2015) and fungicidal activities (Hao et al., 2010; Chen et al., 2014). In this study, the enhancers were applied twice consecutively to the surface of infected leaves at a seven-day interval. Although the efficacy of foliar application in treating TS was inferior to that of other interventions, it reached only 34.8%. Nevertheless, the combined use of SA with TS significantly improved the control efficacy by 49.89%, surpassing the individual application of SA or TS by 19.06% and 30.25%, respectively. Notably, this combined efficacy was exceeded only by foliar spraying of botanic fungicide isobavachalcone (IBC). The evidence clearly indicated that the combined application of TS with other antimicrobial agents can enhance the suppression of blister blight disease under field conditions.

The application of enhancers stimulates the activity of defense enzymes to enhance plants' resistance to diseases. This study represented our first investigation into the roles of defense-related enzymes, including PPO, POD, CAT, SOD, PAL, β-1,3-glucanase, and chitinase, in the induction process of resistance against tea blister blight mediated by combination of SA and TS. POD and PPO catalyze the final step in the biosynthesis of lignin and other oxidative phenols, with POD participating in generation or regulation of active oxygen species (ROS) that potentially serve as significant roles in reducing pathogen virulence and transmission (Hammerschmidt et al., 1982). PAL is an enzyme that plays a crucial role in the phenylpropanoid pathway, which is involved in the biosynthesis of various compounds such as flavonoids, lignin, and phytoalexins in plants (Bowles, 1990). Hence, the accumulation of PAL activity may be a contributing factor to the decrease in disease incidence. The defense enzymes β-1,3-glucanase and chitinase classified as pathogenesis-related protein (PR) play crucial roles in hydrolyzing β-1,3-glucan and chitin, the prominent components of fungal cell walls (Zhang et al., 2016, 2019). The induction levels of POD, PPO, PAL, β-1,3-glucanase, and chitinase in tea plants treated with SA, especially combined use of SA and TS, were higher than those of

the untreated plants, which was similar to the findings of Ajay and Baby (2010), Chandra et al. (2014, 2014, 2017), and Osei et al. (2021) where tea leaves and potato exhibited higher POD, PPO, PAL, β-1,3-glucanase, and chitinase enzymes activity upon treatment with several enhancers such as $CaCl_2$, IBC, chitosan, SA, and ASM. CAT serves as the primary antioxidant enzyme in the ROS scavenging system of plants during exposure to abiotic stress. It can effectively reduce the hydrogen peroxide (H_2O_2) levels generated within plant cells, thereby alleviating the oxidative stress damage to plant and maintaining cellular stability (Soydam-Aydın et al., 2015). SOD is an important enzyme that plays a crucial role in scavenging superoxide radicals and protecting cells from oxidative damage, which is essential in combating the pathogens of fungal diseases (Liu et al., 2019). It was clear from our findings that the application of SA+TS to tea plants could produce significantly higher amount of CAT and SOD activity compared to untreated plants. Similarly, these results were consistent with the induction of CAT and SOD against *Verticillium dahliae* in pepper when treated with arbuscular mycorrhizal fungi (AMF) and SA (Furkan et al., 2022) along with *Ralstonia solanacearum* in peanut upon treatment with silica nanoparticles (SiO2 NPs) (Deng et al., 2024). Meantime, the present results demonstrated that the variation in disease severity among treatments was associated with the enhancers' capability to stimulate defense enzymes.

5.5 Conclusion

In conclusion, the foliar spraying of enhancers (SA, TS, and SA+TS) can stimulate resistance in tea plants against blister blight disease. Although the effectiveness of foliar application for TS was found to be comparatively lower, the combination of SA with TS could enhance the control efficacy of blister blight disease by almost 50% under filed trial. Additionally, plants treated with SA+TS exhibited the higher activity of defense enzymes compared to untreated plants. Therefore, the combined use of SA and TS could potentially serve as an alternative to traditional fungicides for effectively controlling blister blight disease in tea plants.

Furthermore, TS can also be integrated as a synergist into fungicides or bio-agents to achieve better disease control of tea or other crop plants.

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CHAPTER VI
OVERALL DISCUSSION AND CONCLUSION

6.1 Overall discussion

Firstly, alterations in metabolites, chemical components, and antioxidant activity in tea plants infected with *E. vexans* were analyzed using UPLC-MS/MS for a comprehensive metabolomic and chemicals assessment (**Chapter III**). Subsequently, transcriptomics and targeted hormone metabonomics were employed to delve deeper into the mechanism by which tea plants respond to *E. vexans* infection (**Chapter IV**). Lastly, the efficacy of foliar spraying enhancers (SA+TS) was assessed for managing blister blight on tea plants (**Chapter V**).

In Chapter III, a total of 1,166 metabolites were identified from the healthy and susceptible leaves, including flavonoids, phenolic acids, lipids, amino acids and derivatives, organic acids, alkaloids, nucleotides and derivatives, lignans and coumarins, terpenoids, tannins, and other compounds. Among these, flavonoids were the predominant compounds, accounting for 29.8% of the total metabolites, followed by phenolic acids at 15.6%. Phenolic compounds, including flavonoids and phenolic acids, can serve as passive or inducible barriers in plant-pathogen interactions to resist microbial pathogens (Wang et al., 2016; Górniak et al., 2019). They typically accumulate or change significantly upon plant infection. In our research, significantly up-regulated levels of nine common flavonoids (e.g., kaempferol (3,5,7,4'-tetrahydroxyflavone), kaempferol-3-*O*-sophoroside-7-*O*-glucoside, phloretin, acacetin-7-*O*-glucuronide, acacetin-7-*O*-galactoside, and isovitexin-7-*O*-(6''-feruloyl) glucoside) and five common phenolic acids (e.g., 2,4,6-trihydroxybenzoic acid, antiarol, and 1-*O*-caffeoyl-(6-*O*-glucosyl)-*β*-*D*-glucose) were observed in *E. vexans*-infected leaves, which was consistent with the finding described by Xu et al. (2019) who showed that disease resistance to *Botrytis cinerea* in grapes might be

related to significant accumulation of flavonoids and phenolic acids. This was similar to another study reported by Mur et al. (2015) who exhibited that the higher levels of kaempferol glucosides and kaempferol triglycosides were observed in *E. vexans*-infected leaves. Salicylic acid (SA) and their derivatives, such as 2,3-dihydroxybenzoic acid, were reported to act as important molecular signaling in response to pathogen infection (Michael et al., 2010; Zhang et al., 2013; Koo et al., 2020). Our current study demonstrated that the significant accumulation of 2,3-dihydroxybenzoic acid might be related to resistance of tea leaves to *E. vexan* pathogen. Proanthocyanidins significantly accumulated in black poplar (*Populus nigra* L.) stems against *Plectosphaerella populi* (Chhana et al., 2019), and Norway spruce [*Picea abies* (L.) *Karst*.] against *Heterobasidion parviporum* (Marie et al., 2011). Similarly, galloylprocyanidin B4 and procyanidin C1 3'-*O*-gallate dramatically accumulated in susceptible leaves in our study. Additionally, the phenylpropane pathway serves as a biosynthetic pathway for flavonoids and phenolic acids, playing essential roles in plant defense as cell structure and signal transduction molecules (Dong and Lin, 2020; Yadav et al., 2020). The phenylpropane pathway discovered in our study showed higher accumulation in tea plants against *E. vexans* infection, which was consistent with the results of Dorneles et al. (2018), Gouvinhas et al. (2019), and Li et al. (2021) where the phenylpropane metabolism enhanced the resistance of tomato to *Botrytis cinerea*, olive to *Colletotrichum acutatum*, and wheat leaf to *Pyrenophora tritici-repentis*. What's more, flavonoid biosynthesis is one of the most crucial downstream branches of phenylpropane metabolism, widely recognized for its protective effects against biotic and abiotic stresses (Yadav et al., 2020). KEGG pathways analysis showed that flavone and flavonol biosynthesis, flavonoid biosynthesis, and isoflavonoid biosynthesis associated with flavonoid metabolism were significantly enriched in pairwise comparisons, particularly flavone and flavonol biosynthesis showing notable enrichment in LeafS2. Therefore, flavonoid metabolism involved in flavone and flavonol biosynthesis may be responsible for the resistance of tea plants against *E. vexans* infection.

In addition, quantitative analysis was conducted on several compounds including flavonoids, phenolics, alkaloids, free amino acids, and terpenoids in our study. These compounds serve as crucial roles in plant resistance against pathogen invasion and are also closely correlated with the development of the distinct flavor of tea (Pokharel et al., 2021). The fresh and sweet substances present in tea predominantly derive from amino acids, which are essentially primary metabolites that make significant contributions to the flavor and other characteristics of plants (Zhang et al., 2017). Our study demonstrated that the trend of total free amino acids content was similar to the trend of amino acid metabolites, suggesting that amino acids, as the primary nutritional substrate for pathogens, played a direct role in causing damage to plant pathogens and contribute to the formation of the crop's disease resistance mechanisms. Meanwhile, the reduction of total free amino acids in tea plants might stimulate the production of soluble proteins essential for immune defense during the second stage of infection, subsequently leading to a decline in the freshness and sweetness of processed tea. After that, the severe damage to host cells was accompanied by protein hydrolysis reactions, which promoted the significant accumulation of total free amino acids in the later stages of infection. These findings were consistent with the study of Dixon (2021) where tomato plants infected with *Verticillium* exhibited higher concentrations of total amino acids compared to the healthy plants. Alkaloids are considered primary bioactive compounds due to their diverse biological properties, including antibacterial, anti-inflammatory, and antioxidant activities (Chen et al., 2011; Nibir et al., 2017). Caffeine, a type of alkaloid, is predominantly stored in the vacuoles of the sponge tissue cells in tea leaves, and is considered to be a bioactive substance for biological defense against pests and diseases (Kim and Sano, 2008). In this study, the total alkaloids slightly decreased, then continued to rise to the highest level, which might be attributed to the pathogen infection activating the tea plant's resistance mechanism. Subsequently, a decrease in total alkaloids in the fourth stage of *E. vexans* infection might be due to the increasing severity of pathogen-induced

damage to sponge tissue cells, thereby affecting alkaloid synthesis and storage. The primary function of terpenoids is to provide chemical defense against various biotic or abiotic stresses, particularly in plant defense against fungal pathogens (He et al., 2022). Furanoterpenoid, a natural terpenoid phytoalexin, was reported to significantly accumulate in tissues infected with either the sweet potato or taro strains of *Ceratocystis fimbriata* (Yasuda and Kojima, 2014). The significant accumulation of WAF-1, a labdane-type diterpene of terpenoids, was observed in tobacco leaves following *Tobacco mosaic virus* (TMV) infection, suggesting that diterpene can sever as an endogenous signal responsible for the defense mechanism in tobacco plants against TMV infection (Shigemi et al., 2003). In current study, total terpenoids rapidly accumulated in *E. vexan*-infected leaves, indicating that various terpenoid volatile compounds possibly increase in content with the infection of tea plants to resist the invasion of pathogens. Phenolics, as important secondary aromatic metabolites, are synthesized through the pathways of shikimate/phenylpropanoid or polyketide acetate/malonate, serving as defense molecules against various biotic and abiotic stresses (Kumar et al., 2023). In our study, the trend of change in total phenolics was consistent with the overall pattern observed in total terpenoids and alkaloids, which indicated that the presence of pathogens could induce the host to produce phenolics, and alterations in total phenolics content were accompanied by the development of the disease. Flavonoids serves as important roles in antimicrobial and plant-pathogen interactions (Dixon et al., 2002). The accumulation of total flavonoids increased gradually during the mid-stages of infection as the severity of disease progress in this study, which was consistent with the reports of Miranda et al. (2007) and Carlsen et al. (2008) where flavonoids content might accumulate or flavonoid compounds could alter in response to pathogen infection.

Besides that, the contents of several phytochemicals were reported to positively correlate with antioxidant activity in diverse plants (Ayoub et al., 2016; Bhambhani et al., 2021; Carlos et al., 2023). Regarding the antioxidant activity

including ABTS and FRAP assays, it was observed that the findings of these two methods were positively correlated with the concentrations of (y = 31.614x + 0.0054, R^2 = 0.9996), alkaloids (y = 0.0388x + 0.0044, R^2 = 0.9995), and phenolics (y = 0.2563x + 0.0186, R^2 = 0.9974). The DPPH free radical scavenging activity demonstrated the strongest correlation with total flavonoids content (y = 2.2152x - 0.0298, R^2 = 0.9932). Overall, it could be deduced that the antioxidant activity of the second susceptible stage (LeafS2) was higher than that of healthy leaves and other susceptible stages in both free and bound states. Therefore, these findings provide comprehensive insights into the effects on the metabolite changes, chemical components, and antioxidant activities following *E. vexans* infection.

In Chapter IV, a total of 6,882, 6,943, 7,261, and 5,595 differentially expressed genes (DEGs) were obtained from four pairwise comparisons, which suggested that gene expression levels in tea plant altered significantly following *E. vexans* infection. Additionally, the number of up-regulated DEGs were highly more than that of down-regulated DEGs, indicating that much more genes might contribute to the positive regulation of tea plants in response to *E. vexans*.

Numerous DEGs were highly expressed in several pathways, particularly in plant hormone signal transduction and plant-pathogen interactions. Within the 'phytohormone signal transduction' pathway, a total of 227 DEGs significantly enriched in the response of tea leaves to *E. vexans* infection, including salicylic acid (SA), jasmonic acid (JA), and ethylene (ETH), brassinosteroids (BR), auxin (AUX), cytokinine (CK), gibberellin (GA), and abscisic acid (ABA). Plant hormones serve as the crucial regulators of plant-pathogen interactions in response to various stresses (Pieterse et al., 2009). SA is a crucial signaling molecule in plant responses to stress caused by various biotrophic pathogens and in the activation of systemic acquired resistance (SAR) (Grant and Lamb, 2006). NPR1 (non-expressors of pathogenesis-related genes 1) functions as a critical regulator in the SA signaling pathway, which interacts with TGA (TGACG motif-binding factor) to trigger SA-responsive PR genes and regulate SA-dependent defense responses (Dong, 2004). Additionally, the

accumulation of PR1 protein with antifungal activity enhances the plant's immunity in the extracellular space (such as vacuoles or cell walls) of the host plant (Hammerschmidt, 2009). Several DEGs (NPR1, TGA, and PR1) were up-regulated in SA signaling pathway in response to blister blight infection, indicating that tea plants infected with *E. vexans* exhibited a higher preference towards biotrophs. Meanwhile, the concentration of SAs substances (SA and SAG) significantly accumulated in *E. vexans*-infected leaves, which was consistent with the report of Shi et al. (2019) where SA served as a crucial role in triggering tea immunity against *Colletotrichums*. JA and ETH are involved in the plant's defense responses against necrotrophs (David et al., 2013). Our findings showed that most DEGs were highly up-regulated in the JA and ETH signaling pathways, suggesting that JA and ETH might also play crucial roles in local immunity against biotrophic pathogens. JA-dependent defense substances (e.g., JA, MEJA, JA-ILE, JA-Phe, and OPDA) were significantly accumulated in infected plants to resist fungal pathogen in our study. BRs play complex and beneficial roles in plant innate immunity by enhancing resistance of tobacco against *Tobacco mosaic virus* (TMV) and rice against *Xanthomonas oryzae* infection (Nakashita et al., 2003). Several DEGs associated with BR signaling pathway are also involved in the regulation of plant defense response. For example, BAK1 (BRI1-associated receptor kinase 1), an important regulator of PAMP (pathogen-associated molecular pattern) signaling, interacts with flagellin receptor to modulate both plant defense and development (Shan et al., 2008). Several DEGs such as BAK1, BRI1, BZR1/2, and BSK involved in BR signaling pathway were strongly up-regulated following fungal infection in our findings, indicating that BRs might participate in regulating the response to *E. vexans* in tea plants. In addition to involvement in the regulation of plant growth and development, these phytohormones like AUX, CK, GA, and ABA also serve as significant regulator of defense responses within plant-pathogen interactions (MacMillan, 2001; Kunkel and Brooks, 2002; Asselbergh et al., 2008; Depuydt et al., 2008). Most of DEGs encoding AUX/IAA, GH3, and ARF in AUX-mediated pathway, A-ARR and B-ARR in CK-mediated pathway, GID1, DELLA, and TF in GA-mediated

pathway along with PPC2, SnRK2, and ABF in ABA-mediated pathway were strongly activated by *E. vexans* infection. Furthermore, several endogenous hormones including SLs (strigolactones), GAs, and ABAs were subjected to quantitative analysis. Similarly, the overall levels of these hormones were observed to increase during the *E. vexans* infection, indicating that SLs, GAs, and ABAs are crucial signaling molecules in response to blister blight infection.

Phytohormone signaling pathway does not function in isolation but is intricately linked with a complicated regulatory network that interplay with developmental processes and diverse defense signaling pathways (Rajendra and Jones, 2009). Plants are subject to the combined action of multiple plant hormones in resisting pathogen invasion, which may either synergize or antagonize each other. The significant accumulation of two JA-mediated signaling molecules, JA and JA-ILe, were positively influenced by DEGs encoding several key proteins, including BAK1, BRI1, and BSK of BR-dependent signaling pathway; ERF1/2 and ETR of ETH-dependent signaling pathway; SAUR of AUX-dependent signaling pathway; and DELLA of the GA-dependent signaling pathway, whereas negatively regulated by DEGs encoding A-ARR of CK-mediated signaling. These indicated that JA signaling pathway might work synergistically with the BR, ETH, AUX, and GA signaling pathways to enhance the expression of defense-related genes in tea plants following *E. vexans* infection, whereas function antagonistically with CK signaling pathway. A potential mechanism of the defense dependent on JA and ETH involves their collaborative action in inducing numerous defense-related genes (Norman-Setterblad et al., 2000). JA responses were synergized by ETH to enhance the resistance of *Arabidopsis* to powdery mildew (Ellis and Turner, 2001).

As for plant-pathogen interaction pathway, we specifically analyzed the DEGs associated with fungal PAMP (pathogen-associated molecular pattern) pathway. Hypersensitive response (HR) and programmed cell death (PCD) can be triggered via PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006). Calcium ion (Ca^{2+}) acts as a pivotal role in the plant innate immune system

like PTI, influencing the activation of three regulatory pathways. Within the reactive oxygen species (ROS)-associated pathway, the upstream RBOH (respiratory burst oxidase homologues) and CDPK (calcium-dependent protein kinase) were significantly up-regulated following *E. vexans* infection. The production of ROS serves as a signaling molecule that triggers hypersensitive response (HR) and reinforces the cell wall (Torres et al., 2002). Calcium-independent protein kinase St CDPK5 has been reported to directly regulate the generation of ROS by inducing the Rboh phosphorylation (Kobayashi et al., 2007). Second pathway is associated with calmodulin (CaM). Barley (*mlo* mutation) defense against powdery mildew was modulated by Ca^{2+}-dependent interaction with CaM and resistance gene MOL (Kim et al., 2002). The up-regulated expression of CaM/CML could enhance the resistance of tea plants to *E. vexans* infection though reinforcing cell wall and inducing stomatal closure. Another one is associated with WRKY 25/33, the enhanced level of which stimulated significant accumulation of downstream defense-related protein encoding PR1.

Within this pathosystem, *E. vexans* infection was correlated with the expression of transcription factors (TFs) from various families, such as WRKY, MYB, bHLH, and AP2/ERF. These TFs act as important roles in the regulation of plant defense responses and defense gene expression (Vidhyasekaran, 2016). MYB as the largest family of TFs in plants is involved in regulating key bioprocesses including flavonoid metabolism, hormone signaling, stress responses, and pivotal aspects of plant growth and development (Ma et al., 2022). MYB families have been reported to participate in the resistance of wheat to *Bipolaris sorokiniana* (Zhang et al., 2012) and cotton to *Verticillium dahliae* (Zhu et al., 2022). bHLH is recognized as the second-largest group of TFs in plants (Bano et al., 2021), following the MYB family, which has been demonstrated to act as an important role in regulating the defense response of tobacco to powdery mildew (Guo et al., 2020). WRKY TFs form a large family of regulatory proteins known to act as a crucial role in the regulation of plant immunity (Pandey and Somssich, 2009), whereas AP2/ERF family affects defense responses to

pathogens and environmental stimuli (Girardi et al., 2013). Most of these prominent TF families were significantly activated in response to disease infection caused by tea blister blight. Collectively, integrated analysis of transcriptome and phytohormone acts as a valuable resource for identifying plant immunity of tea against blister blight.

In Chapter V, the optimal concentrations of SA (0.05, 0.20, 0.35, 0.50, and 0.65 mg/mL) and TS (1.5, 3.0, 4.5, 6.0, and 7.5 mg/mL) were systematically selected to effectively manage blister blight under controlled greenhouse conditions. The results showed that SA at 0.50 mg/mL and TS at 6.0 mg/mL reduced disease infection by 41.43% and 35.64%, respectively. Therefore, the field experiment was performed following a pre-experiment to assess the effectiveness of SA and TS at concentrations of 0.50 mg/mL and 6.0 mg/mL, respectively, in combination against blister blight. The results demonstrated that the combined use of SA and TS exhibited a significant inhibitory effect on blister blight and enhanced the activity of defense enzymes.

Plants possess innate chemical and mechanical barriers, as well as defense systems that are activated in response to specific stimuli to combat various pathogens (Montesano et al., 2003). Enhancers can activate different patterns within the plant defense system, which lead to the initiation of multiple defense responses. These include strengthening the cell wall through the deposition of lignin, inducing an oxidative burst that triggers a hypersensitive reaction, synthesizing antimicrobial compounds, and activating defense genes. Systemic acquired resistance (SAR) is a heightened level of disease resistance in plants that arises from infection by a necrotizing pathogen or the application of enhancers (Gao et al., 2015). The primary function of SAR is to protect plant organs from pathogenic infections that are spatially distant from the site where the enhancer was applied (Vallad and Goodman, 2004). Several chemical enhancers that trigger SAR have been identified, with SA being a prominent component, which accumulates in both infected and uninfected leaves, playing a critical role in the induction of SAR (Gaffney et al., 1993). SA is a defense-related phytohormone that serves as a crucial role in enhancing

resistance against various microbial pathogens (Kunkel and Brooks, 2002; Vlot et al., 2009; Lim, 2023). The endogenous level of SA within the plants has been well-confirmed to positively correlate with the resistance response to biotrophic and hemibiotrophic pathogens (Glazebrook, 2005). Additionally, the application of exogenous SA had the potential to activate SAR to enhance the resistance of watermelon against *Fusarium oxysporum* (Zhu et al., 2022), chilli against *Colletotrichum capsiciv* (Geat et al., 2015), peanut against *Alternaria alternate* (Chitra et al., 2008), and rice against *Magnaporthe grisea* (Xie et al., 2011). In the present study, the highest level of protection achieved by 48.57% on the 14th day with the use of SA at a concentration of 0.50 mg/mL under the field conditions, which is considered satisfactory. Whereas the foliar application of SA at 100, 250, and 500 ppm in highly susceptible 'Assam' tea bushes where the control effects of blister blight was relatively low (Ajay and Baby, 2010) compared to 'Fuding Dabaicha' variety treated with SA at 0.50 mg/mL used in this field trials. TS is a mixture of pentacyclic triterpenoid compound extracted from the seeds of the *Camellia* species (Yu et al., 2022). It exhibits multiple beneficial properties (Jia et al., 2017; Zhao et al., 2020; Zhang et al., 2022), particularly in agriculture, where it is utilized as a plant-based agents or bio-synergists due to its antibacterial/fungal and insecticidal capabilities (Hao et al., 2010; Chen et al., 2014). In this study, the synergistic application of SA and TS resulted in a substantial enhancement in control efficacy, displaying an increase of 49.89%, which surpassed the efficacy achieved through the separate application of SA and TS by 19.06% and 30.25%, respectively. This was followed by the application of IBC.

The analysis of defense-related enzymes has provided further insights into the interaction between blister blight disease and host tea plants following the exogenous application of SA and TS. In this study, the combined use of SA with TS under field trials exhibited an increase in defense enzyme activity. Chemical, abiotic, and plant-derived enhancers have been demonstrated to activate systemic resistance in host, resulting in a decrease in plant diseases (Nandakumar et al., 2001).

The accumulation of enzyme activities, such as POD, PPO, PAL, SOD, CAT, β-1,3-glucanase, and chitinase, have been proven to be effective in controlling various fungal diseases (Chen et al., 2000; Nandakumar et al., 2001; Saravanakumar et al., 2006; Lanubile et al., 2014). POD and PPO catalyze the final step in the lignin biosynthesis and other oxidative phenols (Hammerschmidt et al., 1982). Additionally, POD, known as a pathogenesis-related protein (PR-2), involvement in the generation or regulation of reactive oxygen species (ROS) may play a crucial role in reducing the virulence and dissemination of pathogens. Higher levels of PPO and POD was observed in tea plants treated with combination of SA and TS compared to untreated plants, which was consistent with the results of Chandra et al. (2014), Jogaiah et al. (2020), and Moosa et al. (2022) where sweet oranges, tea leaves, and cucumbers exhibited higher activities of PPO and POD following treatment with chemical or abiotic enhancers. PAL is a pivotal defense enzyme in the phenylpropanoid pathway, responsible for the biosynthesis of various compounds including flavonoids, lignin, and phytoalexins in plants (Bowles, 1990). Phenolic compounds and lignin enhance the strength of plant cell walls, thereby increasing the resistance of host plants to cell wall-degrading enzymes and toxins produced by pathogens, and serving as a mechanical barrier against pathogen penetration through the cell wall (Nicholson and Hammerschmidt, 1992). The defense enzymes β-1,3-glucanase (PR-2) and chitinases (PR3, PR4, PR8, PR11) function by hydrolyzing β-1,3-glucan and chitin, the prominent constituents of fungal cell walls (Zhang et al., 2019). Jia et al. (2023) and Gholamnezhad (2019) demonstrated that infected plants treated with *Bacillus velezensis* and plant extracts produced significantly higher amount of PAL, β-1,3-glucanase, and chitinases compared to untreated plants. CAT and SOD are significant antioxidant enzymes that play crucial roles in scavenging superoxide radicals and protecting cells from oxidative damage, which are essential in combating the pathogens of fungal diseases (Soydam-Aydın et al., 2015; Liu et al., 2019). A previous study showed that SOD and CAT activities in peanut plants upon treatment with antagonistic strain *B. velezensis* increased significantly by 1.32 and

1.57 folds compared to those in infected leaves (Jia et al., 2023), which was similar to our study showing that CAT and SOD activities in tea plants treated with combined use of SA and TS increased by 0.81 and 1.08 folds, respectively, compared to those untreated. Suveditha et al. (2022) and Li et al. (2023) confirmed that the resistance of pepper against *Chilli Veinal Mottle Virus* and kiwifruit against *Botrytis cinerea* by application of enhaners like IAA and SA was accompanied by enhanced activities of CAT and SOD. These findings indicated that exogenous application of SA in combination with TS served as a beneficial role in enhancing resistance to blister blight.

6.2 Conclusion

The significant changes occurred in antimicrobial metabolites (flavonoids and phenolic acids), biochemical components (flavonoids, phenolics, alkaloids, terpenoids and amino acids), and antioxidant activity during the process of blister blight infection. Additionally, defense-related genes (CDPK, RBOH, CaM/CMLs, FRK1, and PR1), transcription factors (WRKY, MYB, bHLH, and AP2/ERF), phytohormone signaling molecules (SA, JA, ETH, and BR) along with endogenous SAs, JAs, and SLs played crucial roles in defense activation of tea immunity to *E. vexans*. Furthermore, exogenous TS was employed as a synergist in combination with SA to improve the control efficacy of blister blight by enhancing the activity of defense enzymes. Hence, this research would have significant scientific and practical implications for addressing the actual challenges faced by tea production.

6.3 References

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APPENDICES

APPENDIX A

Appendix Table 1 Common up- and down-regulated metabolites (flavonoids) exhibited in four comparisons.

Compounds	LeafCK vs. LeafS1				LeafCK vs. LeafS2				LeafCK vs. LeafS3				LeafCK vs. LeafS4			
	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T
6-Methylflavone	1.28	1.45E-02	9.63	↓	1.26	6.73E-03	10.27	↓	1.28	8.91E-03	10.5	↓	1.30	1.51E-02	10.2	↓
Phloretin	1.22	1.25E-03	1.39	↓	1.21	1.33E-03	1.47	↓	1.23	1.39E-03	1.40	↓	1.29	1.91E-03	2.92	↓
Kaempferol(3,5,7,4'-Tetrahydroxy flavone)	1.27	6.76E-03	1.56	↓	1.25	5.81E-03	1.38	↓	1.28	6.86E-05	2.23	↓	1.30	4.18E-04	3.74	↓
Dihydroquercetin	1.26	4.25E-03	1.19	↓	1.25	5.66E-05	1.26	↓	1.27	1.02E-03	1.28	↓	1.29	4.71E-05	1.34	↓
Acacetin-7-O-galactoside	1.23	4.57E-03	1.11	↓	1.25	8.21E-04	1.80	↓	1.27	1.56E-05	2.33	↓	1.28	3.02E-04	1.41	↓
Acacetin-7-O-glucuronide	1.22	2.78E-03	1.72	↓	1.15	8.92E-03	1.26	↓	1.18	7.32E-03	1.19	↓	1.22	3.52E-03	1.45	↓
Acacetin-7-O-rutinoside	1.27	7.75E-03	6.08	↓	1.24	3.34E-04	3.58	↓	1.28	5.82E-02	-8.4	↑	1.30	5.82E-02	-8.4	↑
Isovitexin-7-O-(6''-feruloyl)glucoside	1.21	1.15E-03	2.12	↓	1.21	4.18E-02	3.28	↓	1.26	8.45E-03	4.12	↓	1.29	1.60E-04	4.55	↓
Kaempferol-3-O-sophoroside-7-O-glucoside	1.26	2.33E-04	1.29	↓	1.25	1.31E-05	1.81	↓	1.25	3.68E-02	1.86	↓	1.29	8.60E-03	2.31	↓

Appendix Table 1 Common up- and down-regulated metabolites (phenolic acids and terpenoids) exhibited in comparisons (Continued).

Compounds	LeafCK vs. LeafS1				LeafCK vs. LeafS2				LeafCK vs. LeafS3				LeafCK vs. LeafS4			
	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T
2,3-Dihydroxybenzoic Acid*	1.24	8.83E-04	1.04	↓	1.22	4.86E-03	1.16	↓	1.27	1.09E-04	1.95	↓	1.30	6.21E-05	2.45	↓
2,4,6-Trihydroxybenzoic Acid	1.27	1.17E-05	2.22	↓	1.24	3.35E-04	1.50	↓	1.25	3.27E-03	1.56	↓	1.26	4.76E-03	1.20	↓
Antiarol	1.28	1.29E-03	10.35	↓	1.25	5.61E-02	10.11	↓	1.28	3.60E-02	10.96	↓	1.30	8.64E-03	11.66	↓
Camaldulenic acid	1.28	4.50E-03	9.72	↓	1.26	2.11E-02	11.87	↓	1.28	6.20E-03	14.92	↓	1.30	7.06E-03	14.28	↓
3,11-Dioxo-19α-hydroxyurs-12-en-28-oic acid	1.27	6.03E-02	8.32	↓	1.25	6.04E-02	10.29	↓	1.28	2.34E-02	11.34	↓	1.30	6.51E-03	12.71	↓
Cyclohexanondientriterpenic acid	1.27	4.13E-04	4.27	↓	1.26	3.36E-03	5.23	↓	1.28	1.40E-03	5.84	↓	1.30	9.75E-05	5.63	↓
1-O-Caffeoyl-(6-O-glucosyl)-β-D-glucose	1.25	1.35E-02	1.26	↓	1.18	6.05E-02	1.47	↓	1.28	9.37E-04	1.87	↓	1.28	1.61E-02	1.59	↓
1-O-Caffeoyl-4,6-di-O-galloyl-β-D-glucose	1.27	6.53E-02	12.65	↓	1.26	5.40E-02	11.89	↓	1.28	1.36E-02	12.71	↓	1.30	1.62E-02	12.86	↓

Appendix Table 1 Common up- and down-regulated metabolites (alkaloids, tannins, lignans and coumarins, nucleotides and derivatives) exhibited in four comparisons (Continued).

Compounds	LeafCK vs. LeafS1				LeafCK vs. LeafS2				LeafCK vs. LeafS3				LeafCK vs. LeafS4			
	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T
O-Acetyl-L-carnitine	1.28	1.67E-02	18.15	↓	1.26	1.92E-04	18.72	↓	1.28	2.60E-02	18.49	↓	1.30	5.90E-03	18.67	↓
Isobutyryl carnitine	1.28	3.21E-02	11.76	↓	1.26	1.12E-02	11.40	↓	1.28	1.34E-02	11.56	↓	1.30	1.27E-02	12.24	↓
Nicotianamine	1.20	3.77E-02	-1.93	↑	1.11	8.67E-02	-1.02	↑	1.21	5.99E-02	-1.47	↑	1.14	6.13E-02	-1.19	↑
1-Hydroxypinoresinol-4'-O-Glucoside	1.24	8.21E-03	-2.34	↑	1.26	8.58E-03	-13.71	↑	1.24	6.64E-03	-2.13	↑	1.26	1.14E-02	-1.47	↑
Pinoresinol-4-O-(6''-acetyl)glucoside	1.28	2.45E-02	10.57	↓	1.26	3.74E-02	11.03	↓	1.28	2.23E-02	11.95	↓	1.30	4.16E-03	12.61	↓
Galloylprocyanidin B4	1.27	4.22E-03	1.84	↓	1.25	2.67E-03	1.98	↓	1.27	8.43E-03	2.14	↓	1.29	1.12E-03	1.67	↓
Procyanidin C1 3'-O-gallate	1.28	1.72E-03	3.44	↓	1.26	8.24E-05	4.29	↓	1.28	4.46E-05	4.03	↓	1.30	4.98E-03	3.72	↓
7-Methylxanthine*	1.22	7.68E-04	1.81	↓	1.28	3.32E-05	1.53	↓	1.21	9.40E-03	1.10	↓	1.23	2.61E-03	1.08	↓
2-Deoxyribose-1-phosphate	1.22	8.07E-03	2.40	↓	1.27	1.06E-02	2.52	↓	1.22	5.59E-03	2.11	↓	1.17	7.46E-02	1.67	↓
Ribosyladenosine	1.22	1.54E-04	1.84	↓	1.27	5.85E-05	2.55	↓	1,23	4.15E-03	3.50	↓	1.23	2.22E-03	3.81	↓

Appendix Table 1 Common up- and down-regulated metabolites (organic acids and amino acids) exhibited in comparisons (Continued).

Compounds	LeafCK vs. LeafS1				LeafCK vs. LeafS2				LeafCK vs. LeafS3				LeafCK vs. LeafS4			
	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T
3-Methyl-2-Oxobutanoic acid	1.22	1.45E-06	2.16	↓	1.27	7.08E-04	2.44	↓	1.23	5.34E-04	2.71	↓	1.23	9.03E-04	2.16	↓
4-Oxopentanoicacid	1.22	1.19E-03	11.57	↓	1.28	5.35E-04	11.94	↓	1.23	1.77E-02	11.94	↓	1.24	5.48E-03	11.48	↓
Trans-4-Hydroxy-L-proline	1.21	5.89E-03	-2.28	↑	1.22	2.21E-03	-1.74	↑	1.11	8.21E-03	-1.24	↑	1.20	7.92E-03	-1.33	↑
2-Methylglutaric acid	1.22	4.11E-03	14.45	↓	1.28	2.19E-03	14.80	↓	1.23	6.69E-04	14.91	↓	1.24	1.23E-02	15.08	↓
Adipic acid	1.22	2.10E-02	14.54	↓	1.28	3.65E-03	15.30	↓	1.23	7.17E-04	15.47	↓	1.24	4.50E-03	15.38	↓
3-Hydroxy-3-Methyl-2-oxopentanoic acid	1.22	5.72E-02	11.38	↓	1.28	6.77E-03	11.62	↓	1.23	4.36E-03	11.81	↓	1.24	1.72E-02	11.32	↓
8-Aminooctanoic acid	1.22	1.88E-02	10.96	↓	1.28	2.50E-02	10.19	↓	1.23	6.42E-04	10.48	↓	1.24	1.17E-02	9.93	↓
Pimelic acid	1.10	2.96E-02	1.27	↓	1.17	9.89E-03	1.30	↓	1.18	3.07E-03	1.74	↓	1.20	5.81E-03	2.17	↓
2-Propylsuccinic acid	1.21	1.78E-02	1.97	↓	1.22	5.15E-02	1.60	↓	1.22	4.06E-03	1.85	↓	1.23	4.55E-03	2.36	↓
Suberic acid	1.22	8.24E-03	10.80	↓	1.28	3.66E-02	11.22	↓	1.23	5.49E-02	10.19	↓	1.24	7.47E-03	11.39	↓
N-Monomethyl-L-arginine	1.20	1.52E-03	1.36	↓	1.23	4.69E-03	1.26	↓	1.20	4.07E-03	1.68	↓	1.20	1.13E-02	1.56	↓
Arginine methyl ester	1.19	1.88E-03	1.45	↓	1.24	2.52E-03	1.46	↓	1.21	6.49E-04	1.81	↓	1.21	1.52E-04	1.67	↓
(-)-Jasmonoyl-L-Isoleucine	1.22	6.05E-06	1.89	↓	1.27	6.87E-05	1.79	↓	1.22	1.14E-03	1.99	↓	1.23	1.65E-06	2.53	↓

Appendix Table 1 Common up- and down-regulated metabolites (lipids) exhibited in four comparisons (Continued).

Compounds	LeafCK vs. LeafS1				LeafCK vs. LeafS2				LeafCK vs. LeafS3				LeafCK vs. LeafS4			
	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T
Punicic acid	1.22	5.01E-04	2.23	↓	1.28	9.20E-04	2.27	↓	1.23	7.78E-04	2.58	↓	1.24	2.24E-04	3.45	↓
(9Z,11E)-Octadecadienoic acid	1.22	7.57E-03	10.8	↓	1.28	7.57E-03	10.8	↓	1.23	5.63E-04	11.25	↓	1.24	1.32E-02	11.12	↓
Linoleic acid	1.22	1.13E-02	3.34	↓	1.27	5.99E-03	3.67	↓	1.23	2.37E-03	3.76	↓	1.23	7.27E-03	3.66	↓
Petroselinic acid	1.22	6.91E-04	2.80	↓	1.27	5.13E-03	3.12	↓	1.23	2.56E-03	3.43	↓	1.23	1.46E-02	3.33	↓
11-Octadecanoic acid	1.22	1.80E-03	2.77	↓	1.28	8.61E-04	3.11	↓	1.23	7.77E-04	3.42	↓	1.23	9.61E-03	3.25	↓
Elaidic Acid	1.22	8.81E-03	2.77	↓	1.27	1.74E-02	3.07	↓	1.23	1.83E-03	3.42	↓	1.23	4.19E-03	3.26	↓
Methyl linolenate	1.22	5.10E-04	2.06	↓	1.27	1.52E-03	1.92	↓	1.22	2.35E-03	2.58	↓	1.23	2.65E-04	3.53	↓
17-Hydroxylinolenic acid	1.22	5.39E-04	2.41	↓	1.28	9.95E-05	2.54	↓	1.23	1.15E-03	3.11	↓	1.24	2.28E-06	3.52	↓
2R-hydroxy-9Z,12Z,15Z-octadecatrienoic acid	1.22	2.40E-04	2.48	↓	1.28	1.88E-08	2.43	↓	1.23	2.03E-04	3.00	↓	1.24	8.30E-05	3.66	↓
Ricinoleic acid	1.22	6.27E-04	1.70	↓	1.28	1.16E-04	2.24	↓	1.23	4.57E-03	2.33	↓	1.23	3.16E-03	2.39	↓
1-Eicosanol	1.22	2.13E-05	1.80	↓	1.27	1.02E-04	2.30	↓	1.22	2.25E-03	2.41	↓	1.23	4.50E-03	2.42	↓
15(R)-Hydroxylinoleic Acid	1.20	2.93E-04	1.63	↓	1.23	4.14E-03	1.36	↓	1.21	2.55E-04	2.24	↓	1.22	2.25E-04	2.46	↓

Appendix Table 1 Common up- and down-regulated metabolites (lipids) exhibited in four comparisons (Continued).

Compounds	LeafCK vs. LeafS1				LeafCK vs. LeafS2				LeafCK vs. LeafS3				LeafCK vs. LeafS4			
	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T
Octadecenoic acid	1.22	3.99E-04	1.90	↓	1.27	1.65E-04	1.67	↓	1.23	6.06E-05	2.51	↓	1.23	9.21E-07	2.65	↓
9S-Hydroxy-10E,12Z-octadecadienoic acid	1.22	1.40E-03	2.59	↓	1.28	7.72E-05	2.75	↓	1.23	7.44E-04	3.13	↓	1.24	6.71E-04	3.79	↓
Epoxyoctadecenoic acid	1.22	2.52E-04	2.26	↓	1.27	2.68E-04	2.51	↓	1.23	3.98E-04	2.76	↓	1.23	6.79E-04	3.21	↓
12,13-Epoxy-9-Octadecenoic Acid	1.22	1.07E-02	10.04	↓	1.28	1.03E-03	10.39	↓	1.23	7.83E-04	10.78	↓	1.24	3.60E-03	11.09	↓
13(S)-HODE;13(S)-Hydroxyoctadeca-9Z,11E-dienoic acid	1.22	2.41E-04	2.56	↓	1.28	4.24E-06	2.73	↓	1.23	4.24E-05	3.13	↓	1.24	9.50E-04	3.77	↓
9-Hydroperoxy-9Z,11E-Octadecadienoic Acid	1.19	1.09E-02	2.94	↓	1.25	2.68E-05	3.26	↓	1.20	3.59E-04	3.20	↓	1.22	4.20E-04	4.12	↓
2-α-Linolenoyl-glycerol*	1.22	5.63E-04	1.25	↓	1.27	1.18E-04	1.77	↓	1.22	6.23E-04	1.69	↓	1.23	3.36E-04	1.70	↓
Eicosadienoic acid	1.22	5.55E-05	13.58	↓	1.28	2.27E-03	14.09	↓	1.23	2.37E-03	14.15	↓	1.24	7.88E-03	14.43	↓
octadecadienoic acid	1.21	4.17E-05	2.43	↓	1.26	1.79E-04	2.22	↓	1.22	5.46E-06	3.08	↓	1.23	4.66E-04	3.33	↓
Dibutyl sebacate	1.14	2.29E-03	1.65	↓	1.25	7.22E-05	2.86	↓	1.19	1.88E-02	2.99	↓	1.21	9.73E-04	3.00	↓
9-Hydroxy-13-oxo-10-octadecenoic Acid	1.21	4.67E-05	1.87	↓	1.26	6.66E-05	1.79	↓	1.22	9.46E-04	2.30	↓	1.23	2.21E-04	2.66	↓

Appendix Table 1 Common up- and down-regulated metabolites (lipids and others) exhibited in four comparisons (Continued).

Compounds	LeafCK vs. LeafS1				LeafCK vs. LeafS2				LeafCK vs. LeafS3				LeafCK vs. LeafS4			
	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T	VIP	p-value	Log_2FC	T
13S-Hydroperoxy-9Z,11E-octadecadienoic acid	1.22	2.11E-05	2.20	↓	1.27	2.35E-04	1.74	↓	1.23	7.04E-04	2.48	↓	1.23	1.15E-03	3.35	↓
9,10,13-Trihydroxy-11-Octadecenoic Acid	1.22	1.91E-06	1.39	↓	1.27	9.45E-05	1.06	↓	1.22	4.30E-03	1.69	↓	1.24	7.91E-04	2.48	↓
2-Linoleoylglycerol*	1.22	4.76E-04	2.42	↓	1.28	1.83E-03	2.87	↓	1.23	6.40E-04	2.52	↓	1.24	3.47E-04	2.09	↓
1-Linoleoylglycerol*	1.22	6.80E-04	2.45	↓	1.27	1.03E-03	2.98	↓	1.22	4.51E-03	2.56	↓	1.23	2.46E-06	2.08	↓
LysoPC 15:0	1.22	1.85E-05	1.33	↓	1.26	4.29E-04	1.13	↓	1.21	6.57E-04	1.08	↓	1.21	1.35E-02	1.24	↓
LysoPG 16:1	1.21	2.31E-04	1.30	↓	1.27	1.13E-03	1.91	↓	1.22	1.74E-03	1.78	↓	1.23	4.70E-03	2.01	↓
LysoPC 16:1(2n isomer)*	1.22	2.63E-04	2.05	↓	1.27	2.52E-03	1.82	↓	1.22	1.59E-03	2.11	↓	1.23	1.33E-03	2.34	↓
LysoPC 16:0(2n isomer)*	1.21	7.19E-03	1.52	↓	1.26	7.93E-03	1.08	↓	1.22	1.35E-03	1.40	↓	1.21	2.74E-02	1.42	↓
Meso-Erythritol	1.22	7.24E-03	12.37	↓	1.28	1.55E-03	12.61	↓	1.23	1.79E-02	12.54	↓	1.24	7.79E-03	11.91	↓
D-Mannitol*	1.22	4.57E-03	5.22	↓	1.27	3.44E-03	5.47	↓	1.22	4.57E-03	5.22	↓	1.23	2.22E-03	5.19	↓
D-Sorbitol*	1.22	6.55E-03	4.47	↓	1.28	1.99E-03	4.77	↓	1.23	1.30E-03	4.84	↓	1.24	2.88E-05	4.61	↓
Dulcitol*	1.22	3.56E-03	4.78	↓	1.27	1.15E-03	4.91	↓	1.23	1.96E-03	5.12	↓	1.23	3.97E-03	4.74	↓
N-(beta-D-Glucosyl) nicotinate	1.22	5.48E-05	2.97	↓	1.27	7.06E-04	3.57	↓	1.22	4.75E-04	3.88	↓	1.23	2.58E-03	3.69	↓
Roseoside	1.28	9.51E-04	-2.44	↑	1.26	1.17E-03	-1.59	↑	1.27	1.84E-04	-1.13	↑	1.29	1.94E-04	-1.28	↑

Appendix Table 2 KEGG enrichment of all tea leaves.

Pathways	ID	Pathways	ID	Pathways	ID
Linoleic acid metabolism	ko00591	Polyketide sugar unit biosynthesis	ko00523	C5-Branched dibasic acid metabolism	ko00630
Fructose and mannose metabolism	ko00051	Galactose metabolism	ko00052	Plant hormone signal transduction	ko04075
2-Oxocarboxylic acid metabolism	ko01210	Starch and sucrose metabolism	ko00500	Biotin metabolism	ko00780
Caffeine metabolism	ko00232	Biosynthesis of secondary metabolites	ko01110	Sphingolipid metabolism	ko00600
Betalain biosynthesis	ko00965	Phenylpropanoid biosynthesis	ko00940	Thiamine metabolism	ko00730
alpha-Linolenic acid metabolism	ko00592	Biosynthesis of amino acids	ko01230	Indole alkaloid biosynthesis	ko00901
Glucosinolate biosynthesis	ko00966	Nitrogen metabolism	ko00910	Phenylalanine metabolism	ko00360
Glycolysis/Gluconeogenesis	ko00010	Biosynthesis of unsaturated fatty acids	ko01040	Aminoacyl-tRNA biosynthesis	ko00970
Stilbenoid, diarylheptanoid and gingerol biosynthesis	ko00945	Neomycin, kanamycin and gentamicin biosynthesis	ko00524	Glyoxylate and dicarboxylate metabolism	ko00660
Carbapenem biosynthesis	ko00332	Porphyrin and chlorophyll metabolism	ko00860	Flavonoid biosynthesis	ko00941
Valine, leucine and isoleucine biosynthesis	ko00290	Biosynthesis of various secondary metabolites - part 2	ko00998	Phosphonate and phosphinate metabolism	ko00440
Alanine, aspartate and glutamate metabolism	ko00250	Phenylalanine, tyrosine and tryptophan biosynthesis	ko00400	Taurine and hypotaurine metabolism	ko00430
Butanoate metabolism	ko00650	Glycerophospholipid metabolism	ko00564	Propanoate metabolism	ko00640
Isoquinoline alkaloid biosynthesis	ko00950	Carbon metabolism	ko01200	Citrate cycle (TCA cycle)	ko00020

Appendix Table 2 KEGG enrichment of all tea leaves (Continued).

Pathways	ID	Pathways	ID	Pathways	ID
Vitamin B6 metabolism	ko00750	Lysine degradation	ko00310	D-Arginine and D-ornithine metabolism	ko00472
Ubiquinone and other terpenoid-quinone biosynthesis	ko00130	Amino sugar and nucleotide sugar metabolism	ko00520	Carbon fixation in photosynthetic organisms	ko00710
Valine, leucine and isoleucine degradation	ko00280	Glycine, serine and threonine metabolism	ko00260	Tropane, piperidine and pyridine alkaloid biosynthesis	ko00960
Tryptophan metabolism	ko00380	Oxidative phosphorylation	ko00190	Cyanoamino acid metabolism	ko00460
Tyrosine metabolism	ko00350	Fatty acid biosynthesis	ko00061	Nicotinate and nicotinamide metabolism	ko00760
Glutathione metabolism	ko00480	Fatty acid degradation	ko00071	Pentose and glucuronate interconversions	ko00040
Monobactam biosynthesis	ko00261	Fatty acid elongation	ko00062	Biosynthesis of cofactors	ko01240
Flavone and flavonol biosynthesis	ko00944	ABC transporters	ko02010	GPI-anchor biosynthesis	ko00563
Pyruvate metabolism	ko00620	Isoflavonoid biosynthesis	ko00943	Inositol phosphate metabolism	ko00562
Arginine and proline metabolism	ko00330	Fatty acid metabolism	ko01212	Cutin, suberine and wax biosynthesis	ko00073
Metabolic pathways	ko01100	Purine metabolism	ko00230	Histidine metabolism	ko00340
Glycerolipid metabolism	ko00561	Riboflavin metabolism	ko00740	Pentose phosphate pathway	ko00030
Lysine biosynthesis	ko00300	Zeatin biosynthesis	ko00908	Pantothenate and CoA biosynthesis	ko00770
Pyrimidine metabolism	ko00240	Arginine biosynthesis	ko00220	beta-Alanine metabolism	ko00410
Ascorbate and aldarate metabolism	ko00053				

Appendix Table 3 Primers for qRT-PCR analysis.

Gene ID	Gene Name	Primer type	Sequence (5'-3')	Product size
evm.TU.Cha13g007060	AP2	F	AGTCCTACAGAGGGGTTCGG	109bp
		R	TCCGCACTATCAAACGTCCC	
evm.TU.Cha02g016090	bZIP	F	GCTCGGAAACAGGCCTATAC	183bp
		R	GGGACCTGTCAACGTTCTCC	
evm.TU.Cha07g014430	WRKY6	F	CCAGTGCGCAAACAAGTTCAA	164bp
		R	TCTGCACTTGACATCGAGCC	
Camellia_sinensis_newGene_41861	MYB	F	GCACAACATAGCAAGGGCAG	165bp
		R	AACCTCAGAAGCCTCCGTTC	
evm.TU.Cha04g000480	RPM1	F	AAATCCCGAGCAAGTCCTGG	190bp
		R	CGCGGACGACGAACAATTAC	
evm.TU.ChaUn15033.3	RPS2	F	ATGCATGATGTAGTGCGCGAT	100bp
		R	TCTTTGGCCACTCTCTCGAT	
Actin	-	F	CCTGTGCTGCTAACTGAAGC	138bp
		R	GCGACCACTGGCATAAAGAG	

Note: "F" and "R" represent forward primer and reverse primer, respectively.

Appendix Table 4 Conditions for qRT-PCR analysis.

component	Volume/reaction	Final concentration
Reaction mix 2x SYBR Green PCR Master Mix	5 µl	1 x
QN ROX Reference Dye （Applied Biosystems instruments only）	0.05 µl	1 x
Primer A	0.7 µl	0.7 µM
Primer B	0.7 µl	0.7 µM
RNase-free water	2.55 µl	-
Template gDNA or cDNA	1 µl	≤100ng/reaction
Total reaction volume	10 µl	-

Appendix Table 5 Procedures for qRT-PCR analysis.

Step	Time	Temperature	Number of cycle
PCR initial heat activation	2 min	95°C	1
Denaturation	5 s	95°C	40
Combined annealing/extension	30 s	60°C	
Melting curve analysis	-		-

Appendix Table 6 Statistics of transcriptome sequencing.

Sample	Raw reads	Clean reads	Clean bases (G)	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
LeafCK1	21841294	20,801,232	6.19	0.02	97.12	94.29	44.42
LeafCK2	22715275	21,633,595	6.46	0.02	98.26	95.40	44.49
LeafCK3	38296088	36,472,465	10.86	0.02	97.67	94.83	44.26
LeafS11	34045726	32,424,501	9.66	0.03	97.47	94.63	44.57
LeafS12	38007951	36,198,049	10.76	0.02	97.58	94.74	44.64
LeafS13	23103111	22,002,963	6.54	0.01	97.89	95.04	44.74
LeafS21	41404140	39,432,514	11.73	0.02	97.86	95.01	44.64
LeafS22	36708756	34,960,720	10.42	0.03	97.09	94.26	44.59
LeafS23	40690023	38,752,403	11.54	0.02	97.54	94.70	44.72
LeafS31	41807558	39,816,722	11.83	0.01	97.95	95.10	44.70
LeafS32	36491854	34,754,147	10.38	0.02	98.18	95.32	44.44
LeafS33	21616326	20,586,977	6.13	0.02	98.08	95.22	44.59
LeafS41	39865282	37,966,935	11.31	0.01	98.41	95.54	44.29
LeafS42	23246116	22,139,158	6.59	0.02	95.93	93.14	44.41
LeafS43	21933491	20,889,039	6.22	0.03	97.72	94.87	44.53

Appendix Table 7 Sequence alignment of reference genomes.

Sample	Total Reads	Mapped Reads	Unique Mapped	Multiple Map Reads	Reads Map to '+'	Reads Map to '-'
LeafCK1	41,602,464	35,999,686 (86.53%)	34,553,302 (83.06%)	1,446,384 (3.48%)	18,932,445 (45.51%)	18,976,010 (45.61%)
LeafCK2	43,267,190	37,554,060 (86.80%)	36,020,787 (83.25%)	1,533,273 (3.54%)	19,779,741 (45.72%)	19,816,454 (45.80%)
LeafCK3	72,944,930	63,400,755 (86.92%)	60,771,743 (83.31%)	2,629,012 (3.60%)	33,422,189 (45.82%)	33,479,075 (45.90%)
LeafS11	64,849,002	50,010,740 (77.12%)	48,170,221 (74.28%)	1,840,519 (2.84%)	26,210,130 (40.42%)	26,267,300 (40.51%)
LeafS12	72,396,098	55,759,793 (77.02%)	53,669,196 (74.13%)	2,090,597 (2.89%)	29,247,363 (40.40%)	29,318,102 (40.50%)
LeafS13	44,005,926	33,412,022 (75.93%)	32,201,361 (73.18%)	1,210,661 (2.75%)	17,500,066 (39.77%)	17,546,845 (39.87%)
LeafS21	78,865,028	58,851,425 (74.62%)	56,507,412 (71.65%)	2,344,013 (2.97%)	30,955,840 (39.25%)	31,008,699 (39.32%)
LeafS22	69,921,440	52,136,863 (74.56%)	50,094,607 (71.64%)	2,042,256 (2.92%)	27,384,230 (39.16%)	27,439,225 (39.24%)
LeafS23	77,504,806	58,012,921 (74.85%)	55,695,925 (71.86%)	2,316,996 (2.99%)	30,518,128 (39.38%)	30,573,484 (39.45%)
LeafS31	79,633,444	50,414,522 (63.31%)	48,411,944 (60.79%)	2,002,578 (2.51%)	26,500,890 (33.28%)	26,569,987 (33.37%)
LeafS32	69,508,294	43,676,090 (62.84%)	41,979,951 (60.40%)	1,696,139 (2.44%)	22,927,716 (32.99%)	22,981,272 (33.06%)
LeafS33	41,173,954	26,018,045 (63.19%)	25,036,476 (60.81%)	981,569 (2.38%)	13,636,408 (33.12%)	13,675,482 (33.21%)
LeafS41	75,933,870	65,538,640 (86.31%)	62,940,006 (82.89%)	2,598,634 (3.42%)	34,481,249 (45.41%)	34,539,444 (45.49%)
LeafS42	44,278,316	38,008,467 (85.84%)	36,585,423 (82.63%)	1,423,044 (3.21%)	19,902,487 (44.95%)	19,990,432 (45.15%)
LeafS43	41,778,078	36,158,651 (86.55%)	34,804,268 (83.31%)	1,354,383 (3.24%)	18,954,333 (45.37%)	19,001,758 (45.48%)

Appendix Table 8 The annotation information of unigenes.

Annotated databases	New Gene Number	Annotated Percent (%)
COG	11519	20.8
GO	35010	63.1
KEGG	28212	50.8
KOG	21699	39.1
Pfam	30383	54.7
Swiss-Prot	28022	50.5
eggNOG	34158	61.5
NR	45008	81.1
All	45133	81.3

Appendix Table 9 Top 20 GO terms among four pairwise comparisons.

Category	Term	Name	*p*-value
Biological process	GO:0009765	photosynthesis, light harvesting	2.65E-09
	GO:0015979	photosynthesis	1.62E-08
	GO:0010411	xyloglucan metabolic process	1.17E-05
	GO:0016998	cell wall macromolecule catabolic process	4.87E-04
Cellular component	GO:0009535	chloroplast thylakoid membrane	8.31E-13
	GO:0009523	photosystem II	9.24E-09
	GO:0009507	chloroplast	9.23E-08
	GO:0016021	integral component of membrane	3.89E-06
	GO:0009536	plastid	5.15E-06
	GO:0009522	photosystem I	6.58E-06
	GO:0044434	chloroplast part	1.09E-05
	GO:0009534	chloroplast thylakoid	3.04E-05
	GO:0005618	cell wall	3.04E-05
	GO:0031976	plastid thylakoid	4.05E-05
Molecular function	GO:0016168	chlorophyll binding	1.64E-06
	GO:0020037	heme binding	3.59E-05
	GO:0003700	transcription factor activity, sequence-specific DNA binding	4.04E-05
	GO:0016762	xyloglucan:xyloglucosyl transferase activity	5.80E-05
	GO:0004497	monooxygenase activity	8.93E-05
	GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	1.67E-04

Note: Top 20 GO terms in LeafCK vs. LeafS1.

Appendix Table 9 Top 20 GO terms among four pairwise comparisons (Continued).

Category	Term	Name	*p*-value
Biological process	GO:0010411	xyloglucan metabolic process	1.47E-04
	GO:0006952	defense response	3.79E-04
	GO:0009628	response to abiotic stimulus	8.13E-04
	GO:0008299	isoprenoid biosynthetic process	2.05E-03
	GO:0042493	response to drug	2.05E-03
Cellular component	GO:0016021	integral component of membrane	5.17E-10
	GO:0005618	cell wall	4.00E-04
	GO:0009522	photosystem I	8.32E-04
	GO:0009523	photosystem II	8.99E-03
	GO:0005968	Rab-protein geranylgeranyltransferase complex	9.99E-03
Molecular function	GO:0008194	UDP-glycosyltransferase activity	2.88E-08
	GO:0020037	heme binding	2.88E-08
	GO:0004497	monooxygenase activity	1.35E-07
	GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	1.35E-07
	GO:0005506	iron ion binding	4.67E-07
	GO:0016747	transferase activity, transferring acyl groups other than amino-acyl groups	1.68E-05
	GO:0003700	transcription factor activity, sequence-specific DNA binding	2.28E-05
	GO:0016168	chlorophyll binding	1.16E-04
	GO:0016762	xyloglucan:xyloglucosyl transferase activity	5.37E-04
	GO:0010333	terpene synthase activity	7.84E-03

Note: Top 20 GO terms in LeafCK vs. LeafS2.

Appendix Table 9 Top 20 GO terms among four pairwise comparisons (Continued).

Category	Term	Name	*p*-value
Biological process	GO:0009765	photosynthesis, light harvesting	3.81E-07
	GO:0048544	recognition of pollen	3.81E-07
	GO:0015979	photosynthesis	4.70E-05
	GO:0009404	toxin metabolic process	5.14E-05
	GO:0006749	glutathione metabolic process	5.14E-05
	GO:0009636	response to toxic substance	7.13E-05
	GO:0006575	cellular modified amino acid metabolic process	7.13E-05
Cellular component	GO:0016021	integral component of membrane	1.39E-16
	GO:0005618	cell wall	1.42E-09
	GO:0009523	photosystem II	2.10E-06
	GO:0048046	apoplast	2.10E-06
	GO:0005576	extracellular region	3.72E-05
Molecular function	GO:0020037	heme binding	7.45E-13
	GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	2.17E-11
	GO:0005506	iron ion binding	2.49E-11
	GO:0004497	monooxygenase activity	4.56E-11
	GO:0008194	UDP-glycosyltransferase activity	1.60E-07
	GO:0004674	protein serine/threonine kinase activity	1.60E-07
	GO:0004672	protein kinase activity	4.57E-05
	GO:0016168	chlorophyll binding	4.57E-05

Note: Top 20 GO terms in LeafCK vs. LeafS3.

Appendix Table 9 Top 20 GO terms among four pairwise comparisons (Continued).

Category	Term	Name	*p*-value
Biological process	GO:0009765	photosynthesis, light harvesting	1.74E-11
	GO:0015979	photosynthesis	1.61E-08
	GO:0048544	recognition of pollen	7.10E-08
	GO:0042493	response to drug	1.15E-04
	GO:0018298	protein-chromophore linkage	2.38E-04
	GO:0009636	response to toxic substance	3.07E-04
	GO:0006952	defense response	3.25E-04
Cellular component	GO:0016021	integral component of membrane	1.71E-14
	GO:0009523	photosystem II	2.89E-10
	GO:0009522	photosystem I	6.39E-09
	GO:0009535	chloroplast thylakoid membrane	1.00E-08
Molecular function	GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	4.30E-11
	GO:0004497	monooxygenase activity	4.30E-11
	GO:0020037	heme binding	4.30E-11
	GO:0005506	iron ion binding	1.08E-09
	GO:0016168	chlorophyll binding	1.31E-08
	GO:0004672	protein kinase activity	1.06E-07
	GO:0003700	transcription factor activity, sequence-specific DNA binding	1.77E-07
	GO:0004674	protein serine/threonine kinase activity	2.22E-07
	GO:0005524	ATP binding	2.50E-05

Note: Top 20 GO terms in LeafCK vs. LeafS4.

Appendix Table 10 Top 20 enriched KEGG pathways of differentially expressed genes (DEGs).

ID	Pathways	Gene ratio	p-value	Gene number	Type
ko00196	Photosynthesis - antenna proteins	0.86%	3.19E-11	19	↓
ko04016	MAPK signaling pathway - plant	7.61%	4.98E-10	169	↑ / ↓
ko04075	Plant hormone signal transduction	9.86%	2.84E-09	219	↑ / ↓
ko00195	Photosynthesis	1.40%	1.20E-07	31	↓
ko00940	Phenylpropanoid biosynthesis	5.13%	5.91E-06	114	↑ / ↓
ko00500	Starch and sucrose metabolism	5.22%	5.14E-05	116	↑ / ↓
ko00906	Carotenoid biosynthesis	1.31%	5.36E-05	29	↑ / ↓
ko00966	Glucosinolate biosynthesis	0.50%	0.000103	11	↑ / ↓
ko04626	Plant-pathogen interaction	12.47%	0.000143	277	↑ / ↓
ko00904	Diterpenoid biosynthesis	1.04%	0.00099	23	↑ / ↓
ko00130	Ubiquinone and other terpenoid-quinone biosynthesis	1.26%	0.015649	28	↑ / ↓
ko00710	Carbon fixation in photosynthetic organisms	1.98%	0.024137	44	↑ / ↓
ko00261	Monobactam biosynthesis	0.36%	0.025128	8	↑
ko00280	Valine, leucine and isoleucine degradation	1.40%	0.028721	31	↑ / ↓
ko01210	2-Oxocarboxylic acid metabolism	1.26%	0.045789	28	↑ / ↓
ko00910	Nitrogen metabolism	0.77%	0.050036	17	↑ / ↓
ko00250	Alanine, aspartate and glutamate metabolism	1.04%	0.050734	23	↑ / ↓
ko00592	alpha-Linolenic acid metabolism	1.08%	0.053585	24	↑ / ↓
ko00903	Limonene and pinene degradation	0.23%	0.054769	5	↑
ko00902	Monoterpenoid biosynthesis	0.63%	0.060515	14	↑ / ↓

Note: Top 20 enriched KEGG pathways of differentially expressed genes (DEGs) in LeafCK vs. LeafS1. ‘ ↑ / ↓ ’ indicates that the genes annotated to this metabolic pathway are up-regulated or down-regulated.

Appendix Table 10 Top 20 enriched KEGG pathways of DEGs (Continued).

ID	Pathways	Gene ratio	*p*-value	Gene number	Type
ko00940	Phenylpropanoid biosynthesis	5.69%	5.42E-09	124	↑ / ↓
ko04075	Plant hormone signal transduction	9.41%	4.14E-07	205	↑ / ↓
ko04016	MAPK signaling pathway - plant	6.98%	1.34E-06	152	↑ / ↓
ko00196	Photosynthesis - antenna proteins	0.64%	3.23E-06	14	↓
ko04626	Plant-pathogen interaction	12.94%	7.08E-06	282	↑ / ↓
ko00906	Carotenoid biosynthesis	1.38%	1.30E-05	30	↑ / ↓
ko00062	Fatty acid elongation	0.78%	0.000495	17	↑ / ↓
ko02010	ABC transporters	2.62%	0.000541	57	↑ / ↓
ko00941	Flavonoid biosynthesis	2.62%	0.000744	57	↑ / ↓
ko00905	Brassinosteroid biosynthesis	0.87%	0.001439	19	↑ / ↓
ko00910	Nitrogen metabolism	0.96%	0.00209	21	↑ / ↓
ko00966	Glucosinolate biosynthesis	0.41%	0.002364	9	↑ / ↓
ko00500	Starch and sucrose metabolism	4.77%	0.003234	104	↑ / ↓
ko00900	Terpenoid backbone biosynthesis	1.19%	0.00342	26	↑ / ↓
ko00944	Flavone and flavonol biosynthesis	0.60%	0.005222	13	↑ / ↓
ko00071	Fatty acid degradation	1.15%	0.011685	25	↑ / ↓
ko00902	Monoterpenoid biosynthesis	0.73%	0.011934	16	↑ / ↓
ko00943	Isoflavonoid biosynthesis	0.78%	0.012219	17	↑ / ↓
ko00072	Synthesis and degradation of ketone bodies	0.37%	0.012995	8	↑ / ↓
ko00030	Pentose phosphate pathway	1.61%	0.014397	35	↑ / ↓

Note: Top 20 enriched KEGG pathways of differentially expressed genes (DEGs) in LeafCK vs. LeafS2. ‘ ↑ / ↓ ’ indicates that the genes annotated to this metabolic pathway are up-regulated or down-regulated.

Appendix Table 10 Top 20 enriched KEGG pathways of DEGs (Continued).

ID	Pathways	Gene ratio	*p*-value	Gene number	Type
ko04626	Plant-pathogen interaction	14.53%	3.95E-13	343	↑ / ↓
ko04016	MAPK signaling pathway - plant	7.67%	4.62E-11	181	↑ / ↓
ko00940	Phenylpropanoid biosynthesis	5.59%	4.39E-09	132	↑ / ↓
ko00196	Photosynthesis - antenna proteins	0.72%	1.52E-08	17	↓
ko04075	Plant hormone signal transduction	9.15%	1.51E-06	216	↑ / ↓
ko00450	Selenocompound metabolism	1.14%	3.91E-05	27	↑ / ↓
ko00500	Starch and sucrose metabolism	5%	0.000276	118	↑ / ↓
ko00195	Photosynthesis	1.06%	0.000381	25	↑ / ↓
ko00592	alpha-Linolenic acid metabolism	1.36%	0.00076	32	↑ / ↓
ko00943	Isoflavonoid biosynthesis	0.89%	0.000894	21	↑ / ↓
ko00480	Glutathione metabolism	2.33%	0.000931	55	↑ / ↓
ko00942	Anthocyanin biosynthesis	0.30%	0.004046	7	↑ / ↓
ko00071	Fatty acid degradation	1.19%	0.00461	28	↑ / ↓
ko00460	Cyanoamino acid metabolism	1.48%	0.005709	35	↑ / ↓
ko00130	Ubiquinone and other terpenoid-quinone biosynthesis	1.31%	0.005918	31	↑ / ↓
ko00908	Zeatin biosynthesis	1.40%	0.007931	33	↑ / ↓
ko00906	Carotenoid biosynthesis	1.02%	0.009224	24	↑ / ↓
ko00590	Arachidonic acid metabolism	0.80%	0.009417	19	↑ / ↓
ko00904	Diterpenoid biosynthesis	0.89%	0.01075	21	↑ / ↓
ko00966	Glucosinolate biosynthesis	0.34%	0.015332	8	↑ / ↓

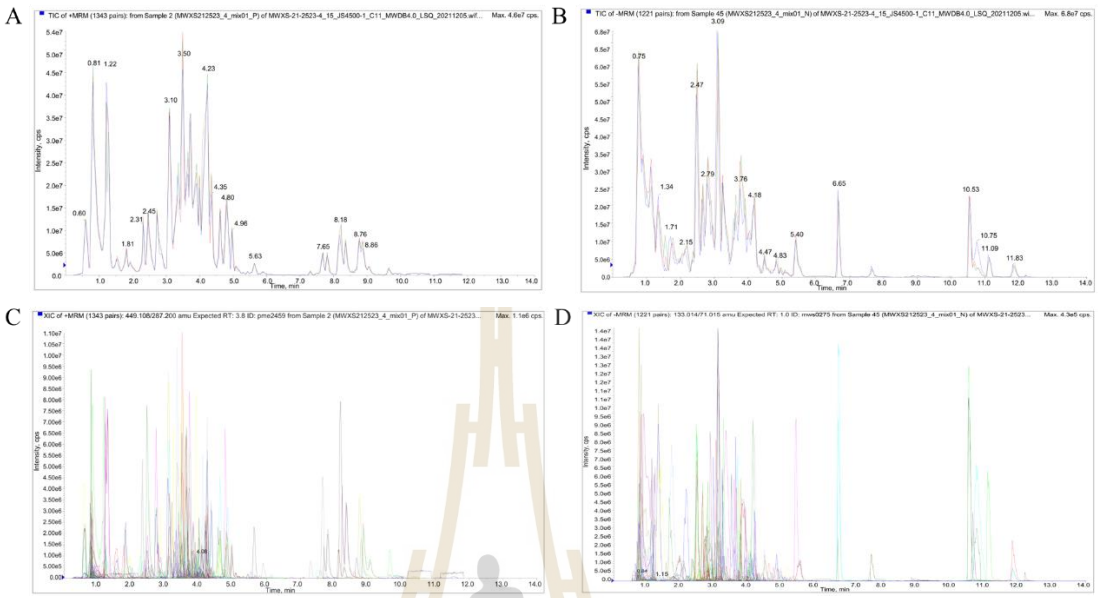
Note: Top 20 enriched KEGG pathways of differentially expressed genes (DEGs) in LeafCK vs. LeafS3. ‘ ↑ / ↓ ’ indicates that the genes annotated to this metabolic pathway are up-regulated or down-regulated.

Appendix Table 10 Top 20 enriched KEGG pathways of DEGs (Continued).

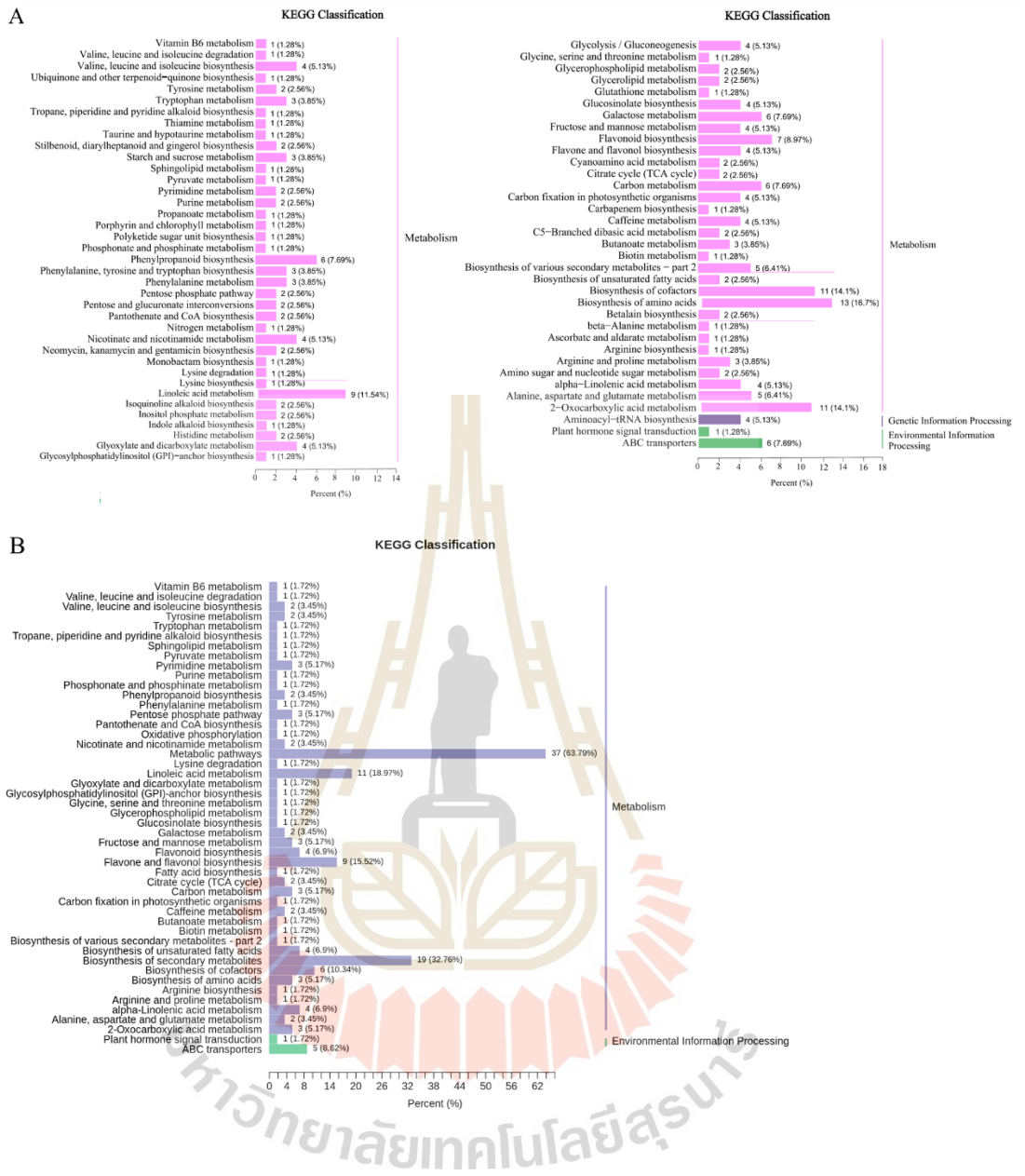
ID	Pathways	Gene ratio	*p*-value	Gene number	Type
ko04626	Plant-pathogen interaction	15.45%	3.81E-14	287	↑ / ↓
ko00196	Photosynthesis - antenna proteins	1.02%	1.24E-12	19	↓
ko04016	MAPK signaling pathway - plant	8.02%	1.83E-10	149	↑ / ↓
ko04075	Plant hormone signal transduction	9.85%	8.95E-08	183	↑ / ↓
ko00195	Photosynthesis	1.40%	1.94E-06	26	↓
ko00900	Terpenoid backbone biosynthesis	1.56%	1.66E-05	29	↑ / ↓
ko00966	Glucosinolate biosynthesis	0.59%	1.88E-05	11	↑ / ↓
ko00940	Phenylpropanoid biosynthesis	5.01%	0.000134	93	↑ / ↓
ko00943	Isoflavonoid biosynthesis	1.02%	0.000304	19	↑ / ↓
ko00592	alpha-Linolenic acid metabolism	1.51%	0.000336	28	↑ / ↓
ko00906	Carotenoid biosynthesis	1.24%	0.000878	23	↑ / ↓
ko00500	Starch and sucrose metabolism	5.06%	0.000897	94	↑ / ↓
ko00909	Sesquiterpenoid and triterpenoid biosynthesis	1.13%	0.000977	21	↑ / ↓
ko00280	Valine, leucine and isoleucine degradation	1.67%	0.00232	31	↑ / ↓
ko00071	Fatty acid degradation	1.29%	0.003136	24	↑ / ↓
ko00942	Anthocyanin biosynthesis	0.32%	0.005866	6	↑ / ↓
ko00902	Monoterpenoid biosynthesis	0.81%	0.006506	15	↑ / ↓
ko00073	Cutin, suberine and wax biosynthesis	1.02%	0.007452	19	↑ / ↓
ko00440	Phosphonate and phosphinate metabolism	0.32%	0.008707	6	↑
ko00261	Monobactam biosynthesis	0.43%	0.008965	8	↑

Note: Top 20 enriched KEGG pathways of differentially expressed genes (DEGs) in LeafCK vs. LeafS4. ‘ ↑ / ↓ ’ indicates that the genes annotated to this metabolic pathway are up-regulated or down-regulated.

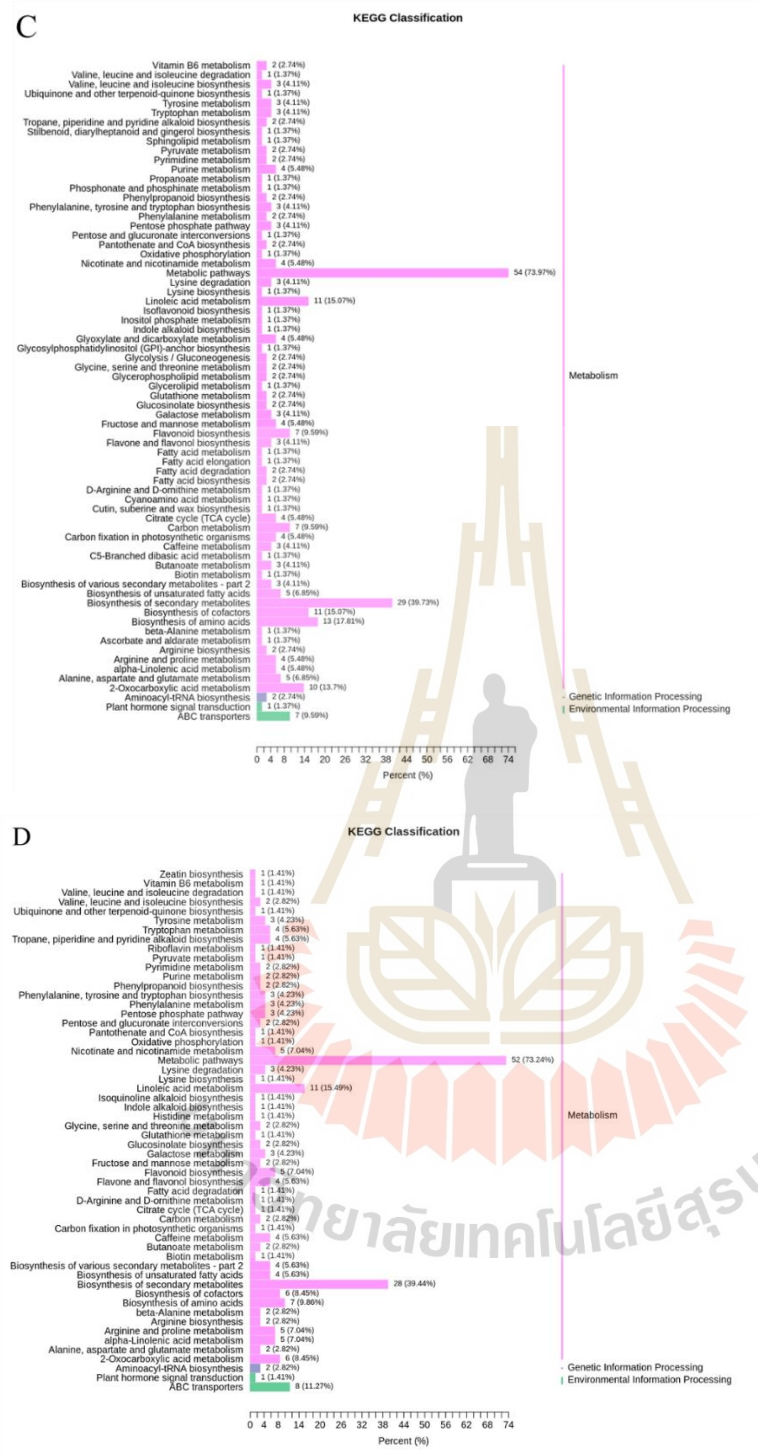
APPENDIX B



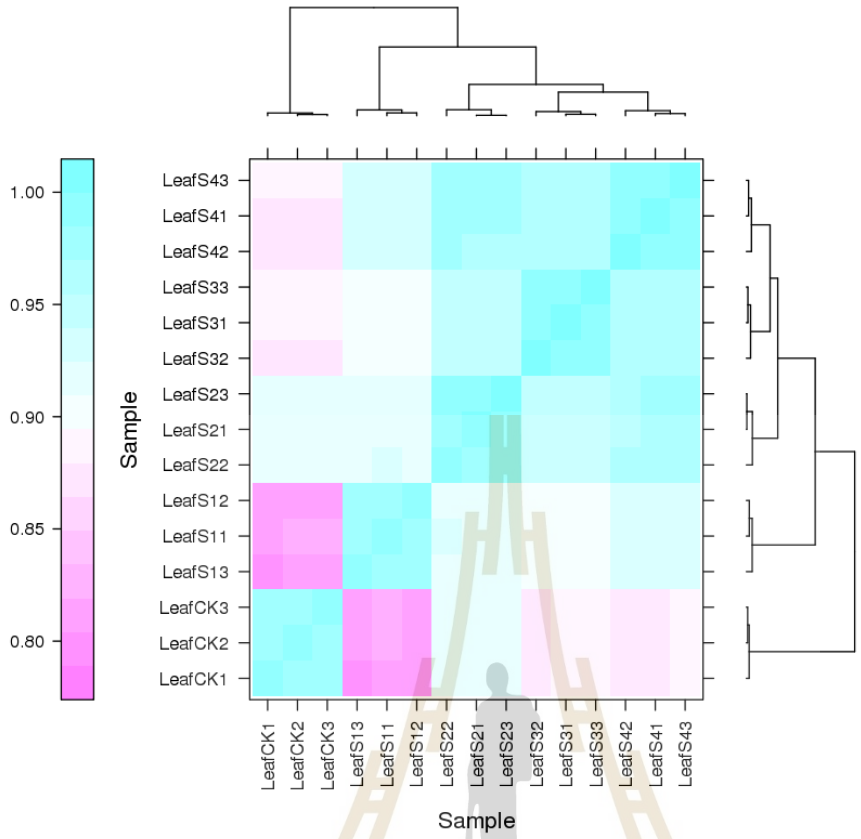
Appendix Figure 1 Total ion current of one quality control sample, as revealed by mass spectrometry detection in positive and negative modes (**A-B**), and a multi-peak detection plot of the metabolites in the multiple reaction monitoring (MRM) in positive and negative modes (**C-D**).



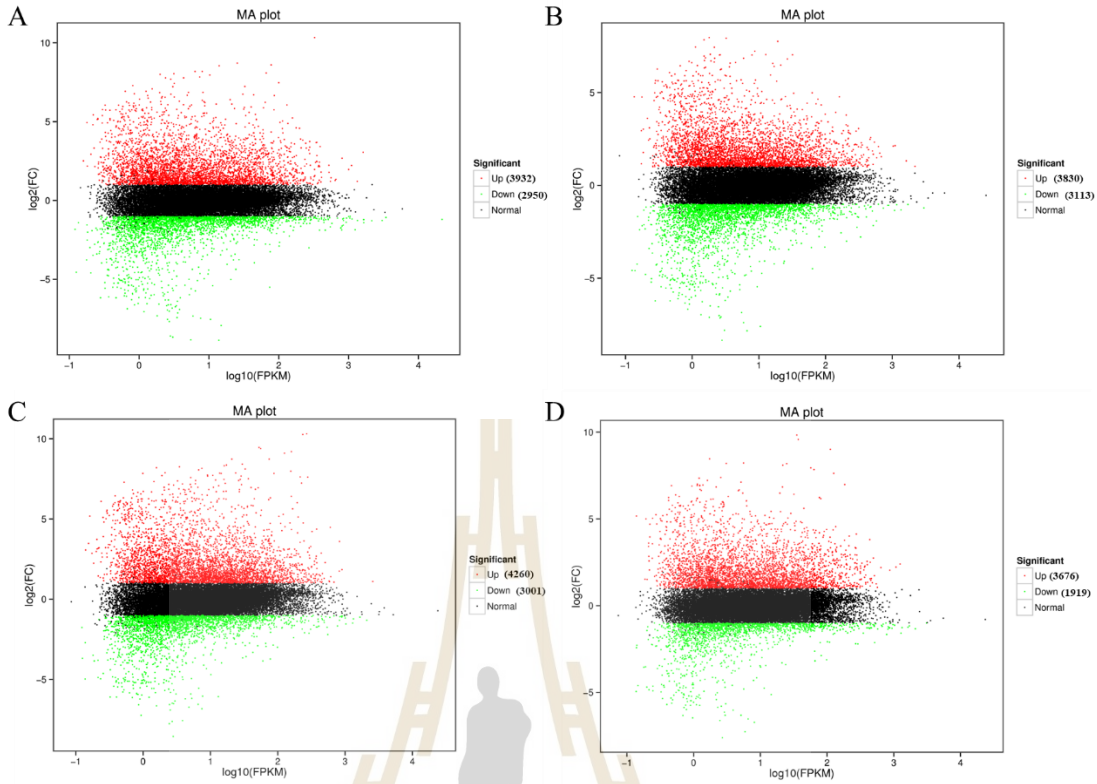
Appendix Figure 2 Substance annotated into KEGG pathways of (**A**) LeafCK vs. LeafS1, (**B**) LeafCK vs. LeafS2, (**C**) LeafCK vs. LeafS3, and (**D**) LeafCK vs. LeafS4.



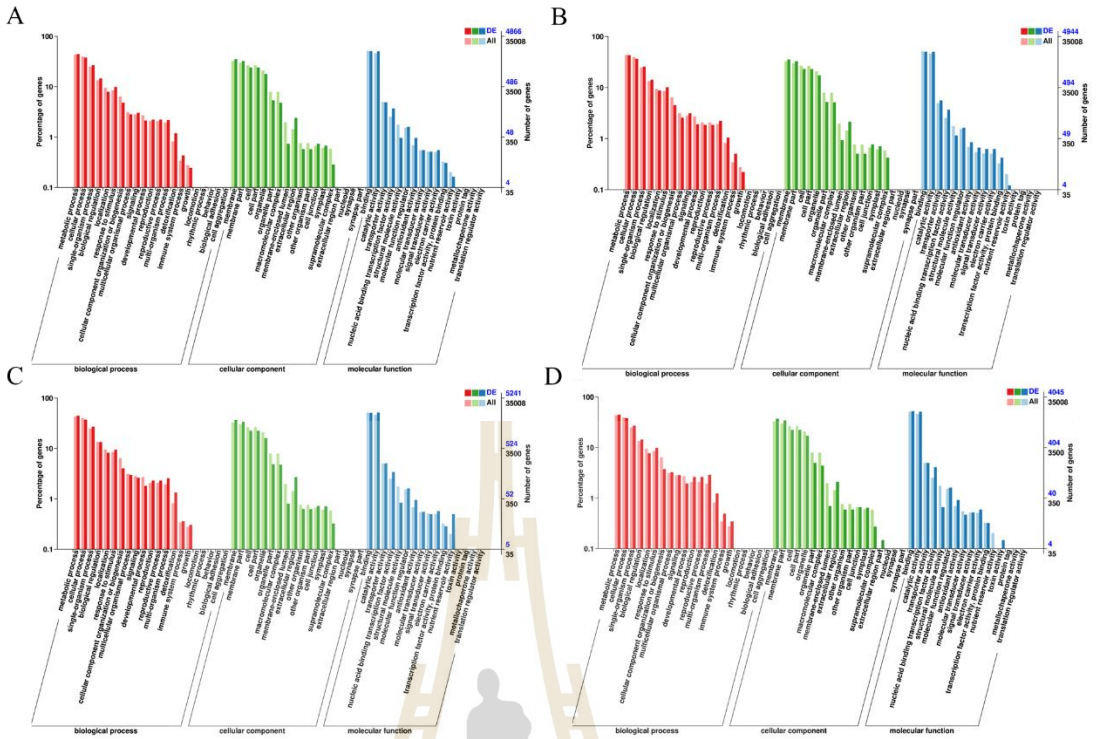
Appendix Figure 2 Substance annotated into KEGG pathways of (**A**) LeafCK vs. LeafS1, (**B**) LeafCK vs. LeafS2, (**C**) LeafCK vs. LeafS3, and (**D**) LeafCK vs. LeafS4 (Continued).



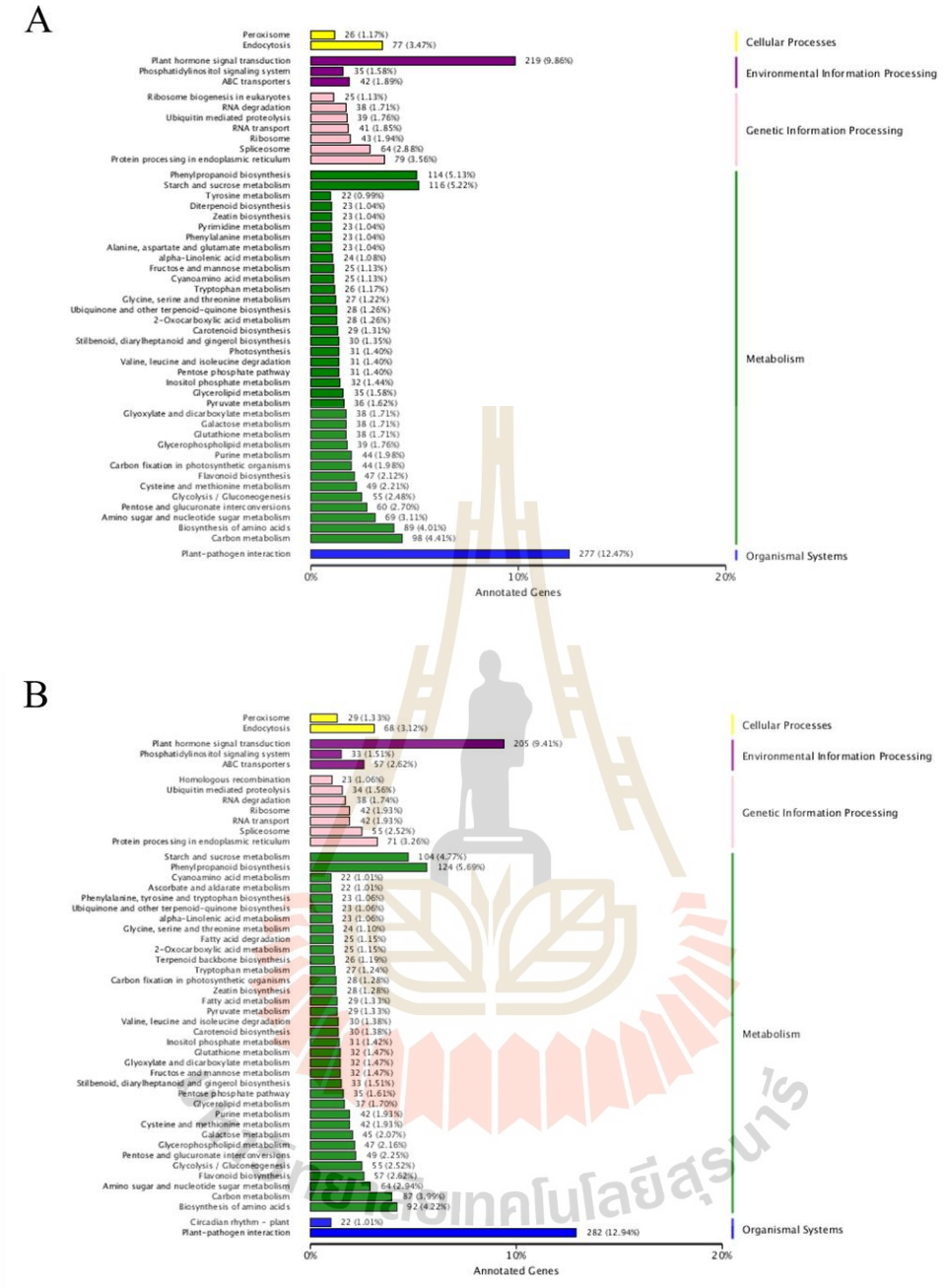
Appendix Figure 3 Correlation evaluation of biological replicates for transcriptome sequencing of tea plants with each treatment. The closer r2 is to 1, the stronger the correlation between two replicates.



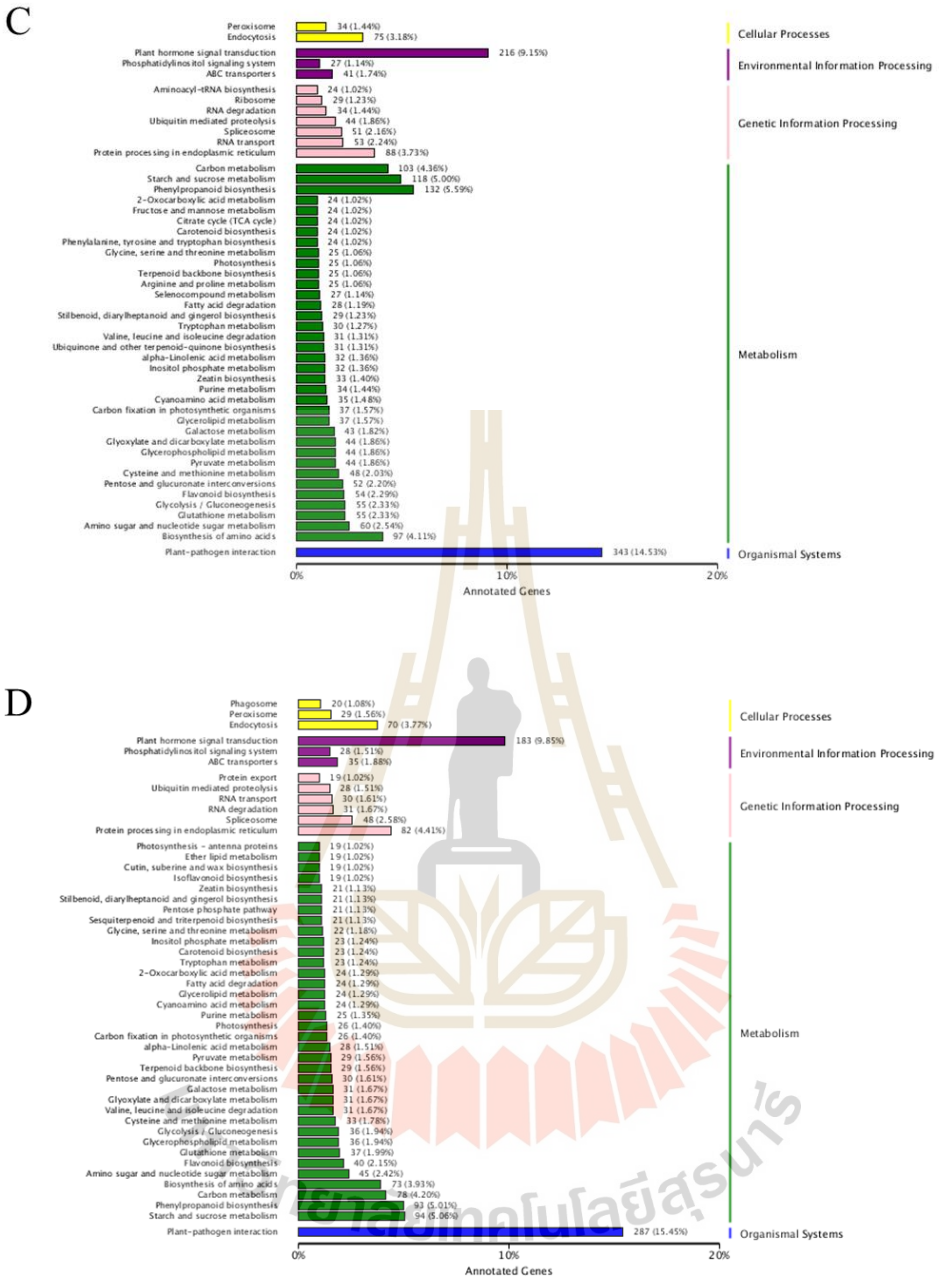
Appendix Figure 4 MA plots of differentially expressed genes between four comparisons of tea plants against *E. vexans*. (**A**) LeafCK vs. LeafS1, (**B**) LeafCK vs. LeafS2, (**C**) LeafCK vs. LeafS3, (**D**) LeafCK vs. LeafS4. Each point represents a detected gene.



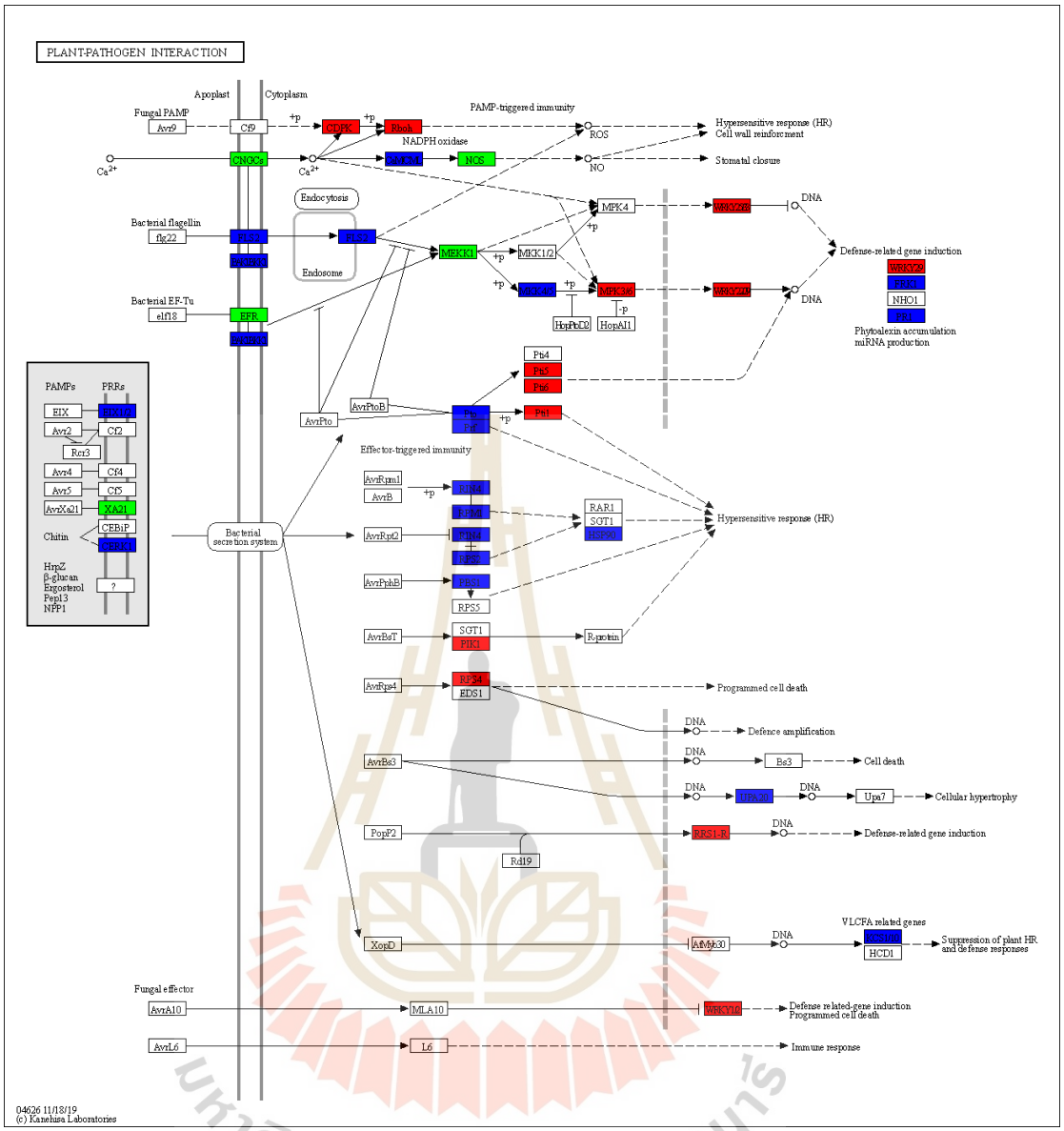
Appendix Figure 5 GO terms classification in (**A**) LeafCK vs. LeafS1, (**B**) LeafCK vs. LeafS2, (**C**) LeafCK vs. LeafS3, (**D**) LeafCK vs. LeafS4. The abscissa represents the functional, and the ordinate represents classification the number of genes annotated with each GO term. The red, green, and blue columns represent three GO-enriched categories of biological processes, molecular functions, and cellular components, respectively.



Appendix Figure 6 KEGG classification of differentially expressed genes in (**A**) LeafCK vs. LeafS1, (**B**) LeafCK vs. LeafS2, (**C**) LeafCK vs. LeafS3, and (**D**) LeafCK vs. LeafS4. The ordinate represents the KEGG metabolic pathway, and the abscissa is the number of genes annotated to the pathway and its proportion to the total number of genes annotated.



Appendix Figure 6 KEGG classification of differentially expressed genes in (**A**) LeafCK vs. LeafS1, (**B**) LeafCK vs. LeafS2, (**C**) LeafCK vs. LeafS3, and (**D**) LeafCK vs. LeafS4. The ordinate represents the KEGG metabolic pathway, and the abscissa is the number of genes annotated to the pathway and its proportion to the total number of genes annotated (Continued).



Appendix Figure 7 Plant pathogen interaction pathway map ko 04626. Red represents upregulation, green represents downregulation, and blue represents both upregulation and downregulation.

BIOGRAPHY

Miss Xiaolu Zhou was born on 17, Feb. 1991 in Jingzhou city, Hubei province, P. R. China. She received a M.S. degree from the College of Horticulture and Landscape Architecture of Hunan Agricultural University in June 2019. Miss Xiaolu Zhou is currently a Ph.D. candidate in Crop Science under the supervision of Asst. Prof. Dr. Kumrai Buensanteai in the School of Crop Production Technology, Institute of Agriculture Technology, Suranaree University of Technology, Thailand. In December 2021, she received SUT OROG Scholarship [D6300685, NO. 19/2564] to purpose a Doctoral Degree in Suranaree University of Technology, Nakhon Ratchasima, Thailand.