

# Metabolic profiling reveals local and systemic responses of kiwifruit to *Pseudomonas syringae* pv. *actinidiae*

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## Abstract

*Pseudomonas syringae* pv. *actinidiae* (Psa), a bacterial pathogen, causes bacterial canker disease in kiwifruit. To elucidate the local and systemic influences of Psa infection on kiwifruit, comprehensive analyses were conducted by combining metabolomic and physiological approach under Psa-infected treatment and control in leaves, stems and bleeding saps. Our results show that Psa infection stimulated kiwifruit metabolic reprogramming. Levels of sugars, fumarate, and malic acid were decreased in Psa-infected leaves and stems, accompanied by the increased level of amino acids (AAs), which is associated with energy metabolism and defense metabolism. Arg, Gln, and pyroglutamic acid systematically were accumulated in long-distance leaves, which probably confers to systemic acquired resistance (SAR). In situ zymography analysis showed that Psa-infection increased N-acetyl-glucosaminidases (chitinases) activity and was highest at the root tips, indicating Psa-inoculation accelerated the nitrogen (N) cycling in kiwifruit. Moreover, phenolic compounds were negatively and lignin was positively related to kiwifruit Psa-resistance, respectively. Our results first reveal that Psa enhances infection by manipulating carbon (C)/N metabolism and sweet immunity, and that host lignin synthesis is a major physical barrier for restricting bacterial infection. This study provides an insight into the complex remodeling of plant metabolic response to Psa stress.

## Introduction

Kiwifruit, the genus of *Actinidia*, is an economically important fruit crop with primary production in China, Italy, New Zealand, and Chile. As an economically damaging trunk bacterial disease, bacterial canker of kiwifruit caused by the bacterial pathogen *Pseudomonas syringae* pv. *actinidiae* (Psa) is a major constraint to the production of kiwifruit. The cultivars of *A. chinensis* var. *chinensis* and *deliciosa* are generally sensitive to Psa, such as ‘Hort 16A’, ‘Jintao’, and ‘Hongyang’. As an epiphytic bacterium, the pathogen of Psa can be colonized on the surface of the plant tissues, including flowers/pollen, leaves, and stems, but it can not induce any visible symptoms until an “optimal state” is achieved (Donati et al., 2018; Straub et al., 2018). After it enters the kiwifruit by natural orifice (stomata) or wounds, Psa, as an endophytic phyto bacterium, can infect the flowers (pollen), leaves, and stems, then successively colonizes and occludes the vascular system (xylem ducts and phloem sieve tubes) for systemic infection (Vanneste 2017; Xin, Kvitko, & He, 2018), causing symptoms including dark brown spots surrounded by yellow halos on leaves, shoot dieback, wilting of buds, and oozing of opalescent or red rusty exudates on canes and trunks (<http://www.kvh.org.nz/>). The infected male flowers produced contaminated pollen, which could transmit Psa to healthy plants. Meanwhile, pollinators (*Apis mellifera* and *Bombus terrestris*) were reported to be contaminated with Psa (Donati et al., 2018). Previous studies reported that when average temperatures fluctuated between 10°C and 18°C for more than 10 days, Psa infections progressed rapidly, that Psa had an optimal growing temperature of 15°C ( $\pm 3^\circ\text{C}$ ), that infection and production of exudate decreased progressively as temperature rose from 18°C to

23°C, and that above 25°C, infection did not occur (Vanneste 2017). Therefore, spring and autumn are the most favorable seasons for *Psa* infection (Gao et al., 2016).

The growth of plant, as sessile organisms, is often influenced by abiotic and biotic stresses. Plants have evolved a set of intricate constitutive barriers including biochemical and physiological change, and production of antimicrobial compounds to prevent and defend pathogen attack. When these barriers fail to prevent entry of pathogen, plants are able to develop an induced resistance, such as systemic acquired resistance (SAR) (Sticher, Mauch-Mani, & Métraux, et al., 1997). These defenses included pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI), and specific bacterial effector-triggered immunity (ETI) (Návarová, Bernsdorff, Döring, & Zeier, 2012). ETI is usually associated with the hypersensitivity reaction (HR) that follows a massive burst of reactive oxygen species (ROS) at pathogen inoculation sites. Many natural metabolites such as the phenolic salicylic acid (SA) act as regulatory components of plant innate immunity processes and SAR. SA is a key regulator of plant basal immunity to biotrophic and hemibiotrophic pathogens. Upon pathogen attack, chorismate-derived SA accumulates in free and glycosidic forms systemically in the foliage and induces the expression of a set of SAR-related genes (Hartmann et al., 2017; 2018; Wang et al., 2018). Recently, there is increasing interests in the roles of carbohydrates and amino acids (AAs) in plant immunity. Plant AAs play a central role in plant-bacterial interactions as major growth-limiting nutrients and as precursors for the production of many plant defense compounds (Zeier, 2013). Carbohydrates produced by photosynthesis are well known for their essential role as vital sources of energy required for defenses and carbon skeletons of organic compounds and storage components, and carbohydrates serve as signals for the regulation of defense genes (Bolouri-Moghaddam and Van den Ende 2012; Yamada, Saijo, Nakagami, & Takano, 2016). In addition, sugars, especially disaccharides sucrose, trehalose, raffinose family oligosaccharides, and fructans play a role in ROS production (Trouvelot et al., 2014).

As the gram-negative hemibiotrophic bacterial pathogen, *P. syringae* has been studied as a model for understanding host-microorganism interactions since the early 1980s (Xin and He 2013). Metabolomics has become a powerful tool for better understanding the biochemical and molecular mechanisms of resistance, providing a comprehensive quantitative and qualitative metabolic picture of a living organism under biotic stress. Nuclear Magnetic Resonance (NMR), Gas Chromatography-Mass Spectrometer (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS) are the most common methods for metabolic profiling analyses (Luo 2015; Moros, Chatziioannou, Gika, Raikos, & Theodoridis, 2017). Extensive studies have been carried out on the change of metabolites in plant-*P. syringae* interaction (López-Gresa et al., 2011; Qian et al., 2015). *P. syringae* infection induced the accumulation of hydroxycinnamic acid amides, chlorogenic acid, and rutin in tomato leaves (López-Gresa et al., 2011). NMR-based metabolomics showed that the presence of the bacterium *Candidatus Liberibacter asiaticus* has a substantial effect on the metabolite composition of the citrus fruit (Chin, Mishchuk, Breksa, & Slupsky, 2014). LC-MS and GC-MS analysis showed that biosynthetic pathways of acetophenone, xanthophylls, fatty acids, alkaloids, glutathione, carbohydrate, and lipid were affected by *Xanthomonas oryzae* pv. *oryzae* infection in rice (Sana et al., 2010). Differentially accumulated metabolites, including sugars (monosaccharides and oligosaccharides), organic acids (oxalic acid and cuminic acid), AA derivatives, and other secondary metabolites (mannitol, octanal, hypoxanthine, and daidzein etc.) may participate in the metabolic-level defense response of soybean to *Phytophthora sojae* infection (Zhu et al., 2018).

In this study, combining <sup>1</sup>H-NMR spectroscopy with GC-MS, we carried out a comprehensive analysis of leaves, stems, and bleeding saps in both infected group and mock-inoculation control group of *A. chinensis* var. *chinensis* cultivar ‘Hongyang’. The roles of phenolic compounds and lignin in *Psa* -kiwifruit interaction were further analyzed.

## Materials and methods

### Biological material

Experiments were performed on *A. chinensis* var. *chinensis* cultivar ‘Hongyang’ (*Psa* susceptible) and *A. chinensis* var. *deliciosa* cultivar ‘Jinkui’ (*Psa* resistant) using three-year-old potted plants (75 cm diameter),

which were grown in greenhouse of Anhui Agricultural University (31°51'36'', 117°15'26'') at 15 ± 3°C and relative humidity of 85 ± 10%, with long-day photoperiod (16-h light/8-h dark) for growth, and short-day photoperiod (8-h light/16-h dark) for *Psa* infection.

*P. syringae* pv. *actinidiae* (*Psa*) strains JF8 (CCTCC AB2018305, biovar 3) was used for artificial inoculation, which isolated from cultivar 'Jinfeng' in Yuexi, Anhui, P.R. China. The inocula were cultured in nutrient-sucrose agar (NSA) at 25 °C for 48 h, and then were resuspended ( $1-2 \times 10^7$  cfu/mL) in 10 mM MgCl<sub>2</sub>. The strains were inoculated via the stomata into abaxial side of leaflet with a 1 mL sterilized plastic syringe without needle. The 10 mM MgCl<sub>2</sub> was used as a mock treatment.

Twenty days post-inoculation (dpi), leaves and stems were cut from plants at the base of the lamina, and bleeding saps were collected from cutting branches as described previously (Ferguson, 1980). Those samples were labeled as **A**: JY 1-6 (mock leaves), **B**: JZ 1-6 (mock stems), **C**: BY 1-6 (local leaves), **D**: BZ 1-6 (local stems), **E**: JY 1-6 (long-distances of non-infected leaves); **F**: JSL 1-3 (mock shoot bleeding saps) and **G**: BSL 1-3 (local shoot bleeding saps). Experiments were repeated for six times with biologically independent samples except three for F and G.

### NMR analysis

The samples were ground with liquid nitrogen, and then freeze dried, extracted by 50% methanol, and sonicated (4 s on/off cycling, for 8 cycles). Samples were centrifuged at 13,000 rpm for 15 min. The supernatant was lyophilized, then re-dissolved in 450 µL water. Subsequently, 50 µL DSS Standard Solution (DSS) (Anachro, Canada) was added into 450 µL aqueous layer, and the mixture was homogenized for 10 s. Samples were transferred into 5 mm NMR tube. The metabolites were analysed by Bruker AV III 600 MHz spectrometer with 600.13 MHz.

The collected Free Induction Decay (FID) signal was automatically zero filled and fourier transformed in processing module in Chenomx NMR Suite 8.3 (Chenomx Inc., Edmonton, Canada). And then, the data were phased, baseline corrected and analysed with DSS as the internal standard in Chenomx Processor against Chenomx Compound Library. NMR analysis was carried out with reference compounds as internal standard. Public databases were used to identify metabolites with chemical shifts that were not available in our database (BMRB: [www.bmrwisc.edu](http://www.bmrwisc.edu) and MDL: <http://www.liu.se/hu/mdl/main/>).

### GC-MS analysis

The metabolic profiling was analyzed according to the method described in previous study with minor modification using Agilent 5975C MSD mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) coupled with Agilent 7890A GC system containing a fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 µm DB-5 MS stationary phase). Samples were extracted and analyzed as described by Moros et al. (2017).

### Phenolic compound analysis

One gram fresh weight (FW) of leaf tissue was ground to a fine powder in liquid nitrogen and extracted as described by Gómez-Romero, Segura-Carretero, and Fernández-Gutiérrez (2010). Samples were analyzed on an Agilent 1260 (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector (DAD). A Nucleosil Agilent Proshell 120 EC-C<sub>18</sub> (4.6 × 100 mm, 2.7 µm) was used with the following gradient elution program (solution A, 0.1% formic acid, and solution B, acetonitrile with 0.1% formic acid): 0 min, 5 % B; 30 min, 15 % B; 60 min, 22 % B; and 70 min, 5 % B. The flow rate was 0.8 mL/min, and the injection volume was 10 µL. The phenolic compounds were detected at 280 nm. Identification was carried out by comparing reference phenolic compounds, i.e. arbutin, gallic acid, chlorogenic acid, caffeic acid, epicatechin, vanillin, *p*-coumaric acid, ferulic acid, 1,2-dihydroxybenzene, phloridzindihydrate, naringin, trans-cinnamic acid, and quercetin (Sigma-Aldrich). Results were presented as mg/g.FW.

### Antimicrobial activity of phenolic compounds

The 20  $\mu$ L *Psa* strain JF8 suspension ( $10^8$  CFU/mL) and different concentrations (40, 80, 160, 320 and 640  $\mu$ g/mL) of *p*-coumaric acid, ferulic acid, caffeic acid or epicatechin were mixed in 4.98 mL KB liquid medium in tube. Bacterial strain was grown for 24 h at 25 °C with 180 rpm. Growth concentration of *Psa* was measured spectrophotometrically at OD<sub>600</sub> nm.

### Analysis of PAL and POD activity and lignin content

PAL and POD activities in leaves were quantified at 1, 2, 4 and 8 day post infection (dpi). Samples were analyzed by using Phenylalanine ammoniolyase and Polyphenol Oxidase Kit (Nanjing Jiancheng Bioengineering Institute, China). The lignin content was quantified by the method described by Syros, Yupsanis, Zafiriadis, and Economou (2004).

### *In situ* zymography, image processing and analysis

Seeds of *A. chinensis* var. *chinensis* were sterilization in NaClO 2% for 10 min and rinsed four times with sterilized water. The seeds were germinated on agar medium for 20 days, and then seedlings was transplanted in new MSR medium ( $10 \times$ ). *P. syringae* pv. *actinidiae* inoculation method as described above. MUF-cellulose and MUF-N-acetyl-glucosaminide were used to measure the distribution and activity of cellulases (C) and chitinases (N). The methods of *in situ* zymography was followed the protocol improved by Razavi, Zarebanadkouki, Blagodatskaya, and Kuzyakov (2016), and Liu et al. (2017). The experiment was repeated three times.

### Statistical analysis

Supervised models of Partial least square-discriminant analysis (PLS-DA) were constructed. The PLS-DA loadings plots along with the Variable Importance in Projection (VIP) were used to ascertain the statistical weight that spectral variables/bins accounted for.

The statistically significant difference in spectral intensities of the metabolite were assayed through a Student's t-test. Significant differences in the metabolite levels were reported.

## Results

### NMR spectroscopy of kiwifruit extracts response to *Psainoculation*

To gain the insight into the effect of bacterial pathogen on the metabolic homeostasis, comparative metabolomic analysis using NMR was performed to quantify the primary metabolites from independent sets of *Psa*-infected and mock-inoculated plants. From the 42 spectra, totally 41 metabolites were detected and quantified (Supplemental Figure S1), including amino acids (AAs), amines and ammoniums compounds, organic acids, sugars, and other metabolic intermediates (epicatechin, myo-inositol, 1,3-dimethylurate, methanol, UDP-galactose and UDP-glucose). Quantification of the metabolites was performed by comparing the signal integral with the reference integral, and quantities were expressed as mg/g. FW for leaves and stems samples, and mM for bleeding saps samples. The concentration information of all metabolites was listed in Supplemental Table S1.

To analyze the metabolic data of *Psa-infected group* and mock-inoculated control group under biotic stress, partial least square-discriminant analysis (PLS-DA) were conducted with the NMR spectral sets. Significant differences were observed between A and C/E ( $R^2$  of 0.948 and a  $Q^2$  of 0.6996), B and D ( $R^2$  of 0.7475 and a  $Q^2$  of 0.4771), which suggested that *Psa* infection resulted in clear metabolic shift on leaves and stems. However, partial overlappings between C and E, F and G, were observed, indicating that there were similar metabolic processes among those groups (Figure. 1). Those results explained why there were less significant differences between local and systemic leaves (SAR), infected and non-infected stem exudates, which was consistent with the results of *Arabidopsis* -*P. syringae* interaction (Návarová et al., 2012).

A 10-fold cross-validation to the PLS-DA model indicated the credibility of the PLS-DA model. The loading plot generated from the PLS-DA reflected the contributions of the different metabolites from the treatments to the differences (Figure. 2). The points farther away from the center contributed more than the nearby

plots. The Variable Importance in Projection (VIP) values were calculated through PLS-DA. The larger the VIP values were, the greater their contributions were. Generally, VIP > 1 represented significant differences. On the basis of the loading plot, when the VIP values >1.0, and the False Discovery Rate (FDR)  $\leq$  0.05, the points representing myo-inositol, sucrose, glucose, arginine (Arg), asparagine (Asn), aspartate (Asp), glutamine (Gln), and glutamate (Glu) were identified in A (mock leaves) vs C (local leaves). Among them, *Psa* -infection significantly increased the levels of Gln, Glu, Arg, Asp, and alanine (Ala), but reduced myo-inositol, sucrose, and glucose in leaves. The points representing sucrose, myo-inositol, fumarate, Glu, Asp, and isoleucine (Ile) were identified by comparing B (mock stems) and D (local stems). Consistent with the leaf comparison result between A and C, *Psa* -infection significantly reduced the levels of myo-inositol, sucrose, fumarate, and Glu, and increased the levels of Ile and Asp. By comparing F (mock shoot bleeding saps) and G (local shoot bleeding saps), the points representing glucose, fumarate, Glu, Pro, and pyruvate were identified in bleeding saps (Figure. 2)

### GC-MS analysis of kiwifruit extracts response to *Psa* inoculation

GC-MS provided a complementary approach to identify small polar metabolites associated with plant responses to *Psa* -infection. By GC-MS, a total of 425 metabolites were detected, and on the basis of retention index and mass spectrum, 68 metabolites were identified by the Binbase identifier (Supplemental Table S2 and Figure S2). Consistent with the NMR spectral sets, the score plot of PLS-DA indicated that *Psa* -infection obviously induced the alteration in production of metabolites in leaves, stems, and bleeding saps, which was different from the tendency exhibited by the control group (A vs C vs E, B vs D, F vs G). Those results indicated that some metabolite content levels changed when kiwifruit was infected by *Psa* in leaves, stems and bleeding saps (Figure. 3).

Sorbose (254), galactose (272), psicose (260), idose (273), mannose (278), glucose (281), glycerolaldopyranosid (330), and helicin (salicylaldehyde-beta-D-glucopyranoside) (359) were identified in leaves by comparing A with C/E with the VIP values >1.0 and the FDR  $\leq$  0.05 (Fig S1). Only helicin, the precursor of salicylates, displayed higher content in *Psa* -infected leaves than in that in control group leaves, while the content levels of sorbose, galactose, psicose, idose, mannose, glucose, glycerolaldopyranosid decreased in *Psa* -infected leaves. Furthermore, *Psa* -infection significantly increased the levels of pyroglutamic acid (160) and isocitric acid (229) in systemic leaves of kiwifruit.

The content levels of galactose (272), sorbose (254), idose (273), psicose (260), mannose (278), quinic acid (248), fructose (264), glucose (281), epicatechin (410), and N-(3-aminopropyl)morpholine (173) were lower in infected stems than in mock-inoculated stems (VIP >1.0; FDR  $\leq$  0.05), while the levels of pyroglutamic acid (160) were higher in infected stems than in mock-inoculated stems.

The metabolites of sorbose (254), idose (273), psicose (260), galactose (272), psicose (263), 2-oxo-gulonic acid (265), and glucose (281) were increased whereas fructose (264), pyroglutamic acid (163), and succinic acid (97) were decreased after *Psa* -infection (VIP >1.0; FDR  $\leq$  0.05) in kiwifruit bleeding saps, compared with those in mock-inoculated group.

### Comprehensive analysis of GC-MS and NMR data

By combining the data of NMR and GC-MS, totally 18, 16, and 13 metabolites were enriched in leaves, stems and bleeding saps, respectively. Among them, glucose, galactose, sorbose, idose, psicose, fumarate, and Glu altered in all tested tissues. In addition, sucrose, mannose, myo-inositol, Asp, and Ile were observed to in both leaves and stems. Metabo Analyst 4.0 (Chong et al., 2018) analysis indicated that a total of 28, 26, and 21 pathways were identified (Table S3) respectively in leaves, stems, and bleeding saps after *Psa* -infection, including AA metabolism, nitrogen (N) metabolism, starch and sucrose metabolism, tricarboxylic acid (TCA) cycle, carbon fixation in photosynthetic organisms, glutathione metabolism, and porphyrin and chlorophyll metabolism, (Supplemental Table S3). The metabolites ethanolamine, Arg, Asn, Gln, glycerolaldopyranosid, and helicin were induced merely in leaves, whereas quinic acid, epicatechin, and N-(3-aminopropyl) morpholine were induced solely in stems (Figure.4).

### ***Psa* infection induces systemic changes in metabolite composition**

*Psa* -infection significantly induced metabolic difference in systemic leaves of kiwifruit by comparing A with C/E, and sucrose, glucose, myo-inositol, Gln, Glu, Arg, Asn, Asp, Ala, choline and ethanolamine were identified (VIP >1.0; FDR  $\leq$  0.05). Among them, *Psa* -infection significantly increased the contents of Gln, Arg, and pyroglutamic acid in the distant leaves, but reduced the contents of myo-inositol, sucrose, glucose, galactose, sorbose, idose, psicose, and mannose (Figure. 4). The results indicated that *Psa* -infection directly/indirectly inhibited carbon fixation in photosynthetic organisms, starch and sucrose metabolism, and fructose and mannose metabolism. Particularly, the low concentration of the myo-inositol was observed in local and distant leaves of *Psa* -infection plant, which was consistent with the previous study results of its role in cell wall biosynthesis and stress response (Valluru and Van den Ende, 2011).

### ***Psa* infection induces metabolic changes in xylem**

The xylem in plants is the main conduit for water and minerals from roots to shoots. It is composed of cell wall materials facilitating the movement of water through the plant. The bacterium of *Psa* can systemically colonize in root and stem xylem. Different from those in leaves and stems, glucose, galactose, sucrose, sorbose, idose, psicose, Pro, 2-oxo-gulonic acid, pyruvate, 3-hydroxytetradecanoic acid accumulated in stem exudates from infected branches. Particularly, Pro, 2-oxo-gulonic acid, glutamic acid, pyruvate, 3-hydroxytetradecanoic acid, succinic acid and fumarate were solely induced in bleeding saps. But fructose, Glu, Val, fumarate, succinic acid, and glutamic acid were reduced. The results showed the tissue-specific metabolite production after *Psa* -infection. The pathogen induced not only the disorder of sugar and protein, but also leads to metabolic disturbance of energy, immune system, AA, and various metabolic systems. It should be noted that high concentration of sugar is conducive to *Psa* proliferation and spread from pathogen-inoculation sites.

### ***Psa* infection induces N cycling enzyme activities around the roots**

*In situ* zymography is a novel method for analysis extracellular cellulase and N-acetyl-glucosaminidases (chitinases) activity in the presence of living kiwifruit root. The spatial and temporal distribution of the activity two exoenzymes of cellulases and chitinases are involved in plant C and N cycling in presence of living roots (Spohn and Kuzyakov 2014). After inoculation *Psa* -infection with 10 days, chitinase activity increased and highest at the root tips. Meanwhile, there is not obvious for cellulase (Figure. 5A, B and C), which indicated that *Psa* - inoculation accelerated the N cycling in kiwifruit. 0.5mM Urea ((NH<sub>2</sub>)<sub>2</sub>CO) was added to MSR medium to study the effects of exogenous N-supply on the resistance of kiwifruit to *Psa* (Figure. 5D). This is in accordance with observations in the field indicate that exogenous N-supply enhanced plant sensitivity to *Psa*-infection.

### ***Psa* infection induces phenolic compounds and lignin changes in kiwifruit**

Plant phenolics, such as flavonoids and lignin precursors, are usually accumulated in response to various biotic and abiotic stresses. Based on the research above, quinic acid and epicatechin have been observed to respond to *Psa* inoculation in leaves and stems (Figure. 4). In order to further reveal their contributions to the disease resistance, the concentrations of individual phenolic compounds were compared between disease-resistant variety 'Jinkui' and susceptible variety 'Hongyang' (Figure. 6A). A total of 13 phenolic compounds were quantified by HPLC-DAD. The main phenolic compounds of disease-resistant variety 'Jinkui' included 1,2-dihydroxybenzene, epicatechin, and trans-cinnamic, whereas epicatechin, trans-cinnamic acid, *p* -coumaric acid, and gallic acid represented the major phenolic compounds of susceptible variety 'Hongyang'. The genotypes of 'Hongyang' presented higher levels of quantified phenolic compounds, compared to those of 'Jinkui'. Under *Psa* -inoculation, the most of phenolic compounds were reduced in 'Jinkui'. On the contrary, phenolics, gallic acid and chlorogenic acid, were enhanced in 'Hongyang' after *Psa* -inoculation. The genotypes resistance to *Psa* was negatively correlated with the levels of phenolic compounds (Figure. 6B).

*In vitro* , the direct activities against *Psa* bacterium of epicatechin, ferulic acid, *p* -coumaric acid, and caffeic acid were tested. Only the antimicrobial activities of epicatechin at all test concentrations were

found. Ferulic acid promoted the bacterium growth when its concentration was lower than  $80 \mu\text{g/mL}$ , while it inhibited the bacterium growth when its concentration was over  $160 \mu\text{g/mL}$ . Similar pattern was observed for caffeic acid and *p*-coumaric acid (Supplemental Figure S3). Consistent with above results, phenolics extracted from kiwifruit leaves promoted bacterium *Psa* proliferation with an equal concentration in leaf, which could explain why phenolics might be used directly by *Psa* or they could serve as quorum-sensing (QS) substances. Phenylalanine ammonia-lyase (PAL) activity was found to be increased rapidly in ‘Hongyang’, and was decreased in ‘Jinkui’ at 2 days post inoculation (dpi). Similar result was observed for POD when ‘Hongyang’ was infected by *Psa*, whereas the activity of POD was obviously lower in ‘Jinkui’ than in ‘Hongyang’ (Supplemental Figure S4).

As a wood-infecting pathogen, *Psa* has evolved a system enabling the breakdown of woody tissue of tree. Cell wall is a recalcitrant network of polysaccharides including cellulose, hemicellulose (such as xyloglucan and arabinoxylan), lignin, and pectin. Upon pathogen attack, plant often deposited callose-rich cell wall appositions (i.e. papillae) at the sites of pathogen penetration, accumulated phenolic compounds and various toxins in the cell wall and synthesized lignin like polymers to reinforce the cell wall. In kiwifruit, we found that the content of lignin was obviously induced after *Psa* inoculation in ‘Jinkui’, but not in ‘Hongyang’ (Figure. 6C). The content of lignin in the genotypes of kiwifruit was positively correlated with *Psa* resistance.

## Discussion

At present, bacterial canker of kiwifruit caused by *Psa* has been reported at least in 15 counties, resulting in serious damage. The *Psa* strains are currently classified into five biovars (biovar 1, 2, 3, 5 and 6), based on the differences in virulence and composition of pathogenicity-related genes (Vanneste 2017). Of them, biovar 3 is of strong virulence and has posed a major threat to kiwifruit production around the world. In this study, we performed a comprehensive analysis of local and systemic tissues of kiwifruit to capture a series of metabolite changes associated with disease development following infection with *Psa* by NMR and GC-MS approaches. As expected, a total of 18, 16 and 13 metabolites were identified from leaves, stems and bleeding saps, respectively, by comparing *Psa*-infection with mock-inoculated control based on NMR and GC-MS data. The PLS-DA result showed that *Psa* triggered significant metabolic changes in local kiwifruit leaves, stems, and bleeding saps respectively, particularly the change in metabolites such as glucose, galactose, sorbose, idose, psicose (also known as D-allulose, non-metabolizable fructose analogue), fumarate, and Glu.

In kiwifruit, *Psa* mainly lived in leaves (apoplasm), shoot, and trunks (vascular system). In leaves, *Psa*-infection significantly increased the content of Gln, Glu, Arg, Asp, Ala, pyroglutamic acid and helicin in the leaves, but reduced the content of myo-inositol, sucrose, glucose, galactose, sorbose, idose, psicose, mannose, and glycerolaldopyranosid. Similar results were observed in stems. Namely, *Psa*-infection inhibited the accumulation of sugars, fumarate, and malic acid, but increased content of AAs in stems. After *Psa*-infection, the above-mentioned substances in bleeding saps exhibited the change opposite to that in leaves. On one hand, the results denoted host modulation of primary and intermediary metabolism in response to bacterial infection. On the other hand, plant pathogen that perturbed host central metabolism derived nutrients from their hosts to promote its reproduction and virulence via secreting type III secretion effectors (T3SE) or toxins (Rico, McCraw, & Preston, 2011). The results were consistent with the symptoms of bacterial canker of kiwifruit, including suppression of photosynthesis, and dark brown spots surrounded by yellow halos. Such symptoms led to the reduction in carbohydrates synthesis in the chloroplasts of *Psa*-infected tissues. In these tissues, carbohydrates supplied the energy and carbon for kiwifruit defenses, since plant defense was highly energy-demanding processes (Trouvelot et al., 2014), and carbohydrates served as signals for the regulation of defense genes. Plant defense heavily drained TCA cycle-generated energy and intermediates (Bolton, 2009) to support the costly defense-related metabolic pathways. For example, phenylpropanoid metabolism consumed up to 20% of the total photosynthetic carbon in plant. This huge demand highlighted the significance of anaplerotic reactions (i.e., filling-up reactions) (Chu et al., 2019), such as the Glu and Asp metabolism. The anaplerotic reactions replenished the cycle energy and ensured the constant functionality of the TCA cycle in this circumstances (Figure. 7). The present study showed that *Psa* infection inhibited photosynthesis. Interestingly, *Psa* infection inhibited the accumulation of most investigated sugars in leaves

and stems, but not inhibited in bleeding saps (Figure. 7). The inconsistent results might be attributed to the induced phloem transport of carbon out of leaves, which benefited the movement of bacterium from leaves to stems. As precursor of salicylates, helicin (salicylaldehyde  $\beta$ -D-glucoside) displayed high content in *Psa* -infected leaves. Previous study showed that the hydrolysis of helicon resulted in the release of salicylaldehyde which was toxic to bacteria and served as a defense compound (Caboni et al., 2013).

Bacterial pathogens strategically acquired metabolites from their hosts during infection. The *P. syringae* pv. *tomato* (*Pst*) was reported to promote sugar efflux to the apoplast by manipulating host plant sugar transporter. Meanwhile, the intervention of host could prevent such metabolite loss via sugars transporters and glycoside hydrolases manipulated by pathogens (Yamada et al., 2016). The key roles of some sugars and the sugar metabolites including sucrose, D-psicose, D-allose and the sugar-like 2,5-dideoxy-2,5-imino-D-mannitol (DMDP), a fructose analogue, have been confirmed in stimulating the plant immunity system, such as sweet immunity and sugar-enhanced defense, and in regulating defence-related genes (Bolouri-Moghaddam and Van den Ende, 2012). Sucrose and myo-inositol were often observed under (a)biotic stresses (Valluru and Van den Ende, 2011). As a major form of carbohydrate and transporter from source to sink tissues by the phloem, sucrose emerged as a candidate signaling molecule in plant innate immunity, and sucrose induced isoflavonoids synthesis and enhanced resistance against *Fusarium oxysporum* (Morkunas, Marczak, Stachowiak, & Stobiecki, 2005). Sucrose and glucose induced the PR-gene transcripts, PR-2 and PR-5, in *Arabidopsis* via SA-dependent pathway (Thibaud, Gineste, Nussaume, & Robaglia, 2004), and those sugars could be efficiently utilized by *X. campestris* pv. *campestris* via SWEET systems (Zhang et al., 2019). D-psicose conferred a significant resistance to rice bacterial blight (Kano et al., 2011). D-idose was reported to have an inhibitory effect on *Caenorhabditis elegans* growth (Sakoguchi, Yoshihara, Izumori, & Sato, 2016). In addition, the response of carbohydrates accumulation to *Psa* infection included physical disturbance of the cell wall.

Amino acids (AAs) were accurately controlled to be in steady state in normal plant tissues. Emerging evidence indicated that AAs play crucial roles in plant-bacterium interaction, in growth-limiting nutrient (N/C metabolism) and phytohormone precursor synthesis (SA and pipecolic acid), and in defense-related compound synthesis (glucosinolates, N<sup>6</sup>-acetyl-ornithine, and camalexin) (Zeier, 2013). The increase in AAs (Gly, Arg, Asn, Asp, Glu, Gln, and Ile) and carbohydrate starvation in leaves and stems reflected the inhibition of N assimilation. Among these AAs, Asn, Gln, and Arg were common compounds for N storage and transport. Our results were consistent with previous study results that *P. syringae* inoculation increased the accumulation of branched-chain AAs (Val, Leu, Ile), aromatic AAs (Phe, Tyr, Trp), and Lysin SAR, whereas decreased Asp in leaves of *Arabidopsis*, that *P. syringae* pv. *tomato* was well-adapted to metabolizing six most abundant AAs into N and C sources, but not those the low-abundant AAs in tomato (Rico and Preston 2008). Glu played a pivotal role in plant defense against pathogens (Seifi, Van, Angenon, & Höfte, 2013), and the knockout of LYSINE HISTIDINE TRANSPORTER1 (LHT1) increased the resistance to *P. syringae* (Liu et al., 2010). The downregulation of arginine biosynthesis is associated with the inhibitory effect of phaseolotoxin during the *P. syringae* pv. *phaseolicola* - in vetch interaction. Moreover, our study showed that *Psa* infection increased content of pyroglutamic acid in leaves and stems. Pyroglutamic acid, a cyclization product of Glu, was a precursor of gamma-amino butyric acid (GABA). It has been reported that pyroglutamic acid played a role in non-enzymatic browning reactions by promoting the color formation of carbohydrate, which explained the red rusty exudates on kiwifruit trunks. Our study implied that reducing N concentrations in kiwifruit could decrease susceptibility against *Psa*, and that N redundancy inhibited triacylglycerol (TAG) synthesis and TCA cycle.

The 2-oxo-gulonic acid and Pro accumulations were obviously observed in kiwifruit bleeding saps after the inoculation with *Psa*, but not in leaves and stems. One previous study reported that 2-oxo-gulonic acid acting as nitrogen starvation signal triggered a cascade response in cyanobacteria *Anabaena* sp. PCC7120 (Liu et al., 2013). Pro synthesized in the cytosol from Glu was usually induced by abiotic stress in *Arabidopsis* (Seifi et al., 2013). Since the elevations of leaf Pro levels partly depended on an intact SA signaling pathway and ROS, the stress-induced and exogenous Pro treatment induced ROS formation, SA accumulation, PR gene expression, and hypersensitive cell death.



Plant phenolics, such as flavonoids and lignin precursors, could accumulate in response to various biotic and abiotic stresses. Flavonoids, as precursors, also provided defense against phytopathogens to promote the synthesis of tissue lignification around infection sites. *p*-Coumaric acid inhibited bacterial growth by repressing the expression of type III secretion system genes (Li et al., 2009; Vargas et al., 2013). Meanwhile, pathogens have evolved mechanisms to degrade, detoxify, and tolerate phenolic compounds in the process of co-evolution, or pathogens responded to phenolic compounds as signals. Degradation of flavonols (such as quercetin) by bacteria has been well-documented (Braune, Gutschow, Engst, & Blaut, 2001). Arbutin might be involved in fire blight *Erwinia amylovora* resistance in apple (Petkou, Diamantidis, & Vasilakakis, 2002). Our study showed that *Psa*-infection specifically affected the contents of phenolic compounds and lignin, that phenolic compounds (quinic acid and epicatechin) were negatively related to kiwifruit *Psa*-resistance, and that lignin was positively related to kiwifruit *Psa*-resistance. In moderate concentration, ferulic acid, caffeic acid, *p*-coumaric acid, or crude extract of phenolics from kiwifruit induced bacterium proliferation. Consistent results were observed by enzyme activity analysis.

Systemic acquired resistance (SAR) is an inducible immune response that confers a broad spectrum of disease resistance against biotrophic and hemibiotrophic pathogens at the whole plant levels (Sticher et al., 1997). SAR is activated by a local primary leaf inoculation with virulent or virulent pathogens, and SAR renders non-inoculated, distal leaves more resistant to subsequent infection (Mishina and Zeier 2007). Evidence from several lines indicated that long-distance communication of SAR was brought about by metabolic signals that were transmitted from inoculated leaves to distant tissue via the shoot vasculature. Several compounds have been reported to participate in SAR long-distance communication. These compounds included the putative lipid transfer protein DEFECTIVE IN INDUCED RESISTANCE1 (DIR1), the methyl ester of SA (MeSA), glycerol-3-phosphate (G3P), the diterpenoid dehydroabietinal, pipecolic acid, and the dicarboxylic acid azelaic acid (Wang et al., 2018; Wenig et al., 2019). Systemic effects of *Psa* infection were demonstrated by correlation-based network analysis as well as independent component analysis. *Psa*-infection significantly increased the contents of Gln, Arg, pyroglutamic acid, and isocitric acid, but reduced the contents of myo-inositol, sucrose, glucose, galactose, sorbose, idose, psicose, and mannose in the distant leaves.

## Conclusion

This study indicated that *Psa*-infection resulted in AAs anaplerotic reactions, in turn leading to an extensive C and N reprogramming in various metabolic pathways in leaves, stems and bleeding saps by inhibiting photosynthesis, C fixation, and TCA cycle (Figure. 7). *In situ* zymography analysis showed that *Psa*-infection increased N-acetyl-glucosaminidases (chitinases) activity and was highest at the root tips, indicating *Psa*-inoculation accelerated the nitrogen (N) cycling in kiwifruit. The concerted metabolic changes between different tissues may be due to a compromise between tissues to response to *Psa*-infection, which was reflected by pathogen manipulating C/N metabolism and sweet immunity to benefit infection. Host lignin synthesis is a major physical barrier for restricting bacterial infection. To our knowledge, this work is the first integrative analyses of metabolic and biochemical mechanism underlying *Psa* stress in kiwifruit. Psicose, myo-inositol, and helicon are promising for diagnostic and detailed metabolic analyses.

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## Conflict of Interest Statement

We declare that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome. The manuscript has been read and approved by all named authors.

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## Figure Legends

**Figure. 1 PLS-DA score plots of metabolomic changes in kiwifruit by NMR.** A, mock inoculation leaves; B, mock inoculation stems; C, infected leaves; D, infected stems; E, normal branch leaves from infected trees; F, mock inoculation bleeding saps; G, infected shoot bleeding saps.

**Figure. 2 ANOVA score plots of metabolomic changes in kiwifruit by NMR.**

**Figure. 3 PLS-DA score plots and ANOVA of metabolomic changes in kiwifruit by GC-MS.** A, mock inoculation leaves; B, mock inoculation stems; C, infected leaves; D, infected stems; E, normal branch leaves from infected trees; F, mock inoculation bleeding saps; G, infected shoot bleeding saps.

**Figure. 4 VIP (Variable Importance in Projection) score plots of metabolomic changes in kiwifruit by NMR and GC-MS.** A, mock inoculation leaves; B, mock inoculation stems; C, infected leaves; D, infected stems; E, normal branch leaves from infected trees; F, mock inoculation bleeding saps; G, infected shoot bleeding saps.

**Figure. 5. *Pseudomonas syringae* pv. *actinidiae* enhance N cycling.** A, B and C. Cellulase and N-acetylglucosaminidase (chitinase) activity in living roots 10 days after inoculation of *Pseudomonas syringae* pv. *actinidiae* in leaf. D. The calibration line for the cellulase activity is presented at the bottom. Exogenous N-supply enhanced plant sensitivity to Psa-infection.

**Figure. 6. Phenolic compounds and lignin content analysis in ‘Hongyang’ and ‘Jinkui’.** A. The symptoms of Psa-infected ‘Hongyang’ and ‘Jinkui’. B. Phenolic compounds analysis. C. Lignin content analysis.

**Figure. 7. Possible scheme for the metabolism following pathogen attack.** The red and blue letters refer to increase and decrease of variables under Psa-infection, respectively; black and gray letters refer to unchanged and unidentified metabolites, respectively. 2OB, 2-oxobutyrate; 2OG, 2-oxoglutarate; 3MP, 3-mercaptopyruvate; ETF, electron-transfer flavoprotein; ETFQO, electron-transfer flavoprotein: ubiquinone oxidoreductase; GABA,  $\gamma$ -aminobutyric acid; OA, oxaloacetate; P5C, 1-pyrroline-5-carboxylate; ProDH, proline dehydrogenase; TCA, tricarboxylic acid cycle. PEP: phosphoenolpyruvate.

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** A representative  $^1\text{H}$  NMR spectrum of kiwifruit instems.

**Supplemental Figure S2.** GC-MS total ion chromatogram (A) and Agilent ChromStation retention time alignment (B) of a kiwifruit leaves extract.

**Supplemental Figure S3.** Effects of the phenolic compounds of p-coumaric acid, ferulic acid, caffeic acid and epicatechin on growth of Psa bacterium.

**Supplemental Figure S4.** Enzyme activity of PAL and POD response to Psa bacterium in ‘Hongyang (HY)’ and ‘Jinkui (JK)’.

**Supplemental Table S1.** Fold change in the levels of the different metabolites detected (NMR).

**Supplemental Table S2.** Fold change in the levels of the different metabolites detected (GC-MS).

**Supplemental Table S3.** Metabo Analyst 4.0 analysis of the different metabolites detected.





