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RESEARCH PAPER

Amino acids implicated in plant <u>defense</u> are higher in *Candidatus* Liberibacter asiaticus-tolerant citrus varieties

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ABSTRACT

Citrus Huanglongbing (HLB), also known as citrus greening, has been threatening the citrus industry since the early 1900's and up to this date there are no effective cures for this disease. Field observations and greenhouse controlled studies demonstrated that some citrus genotypes are more tolerant to *Candidatus Liberibacter* asiaticus (*C*Las) pathogen than others. However, the mechanisms underpinning tolerance has not been determined yet. The phloem sap composition of *C*Las-tolerant and sensitive citrus varieties was studied to identify metabolites that could be responsible for their tolerance to *C*Las. The citrus phloem sap was collected by centrifugation and was analyzed with gas chromatography-mass spectrometry after methyl chloroformate derivatization. Thirty-three metabolites were detected in the phloem sap of the studied varieties: twenty 20 amino acids, eight 8 organic acids, and five 5 fatty acids. Interestingly, the levels of most amino acids, especially those implicated in plantdefense to pathogens such as phenylalanine, tyrosine, tryptophan, lysine, and asparagine were higher in tolerant varieties. Although the level of organic acids varied between cultivars, this variation was not correlated with citrus resistance to *C*Las and could be cultivar specific. The fatty acids were found in trace amounts and in most cases their levels were not significantly different among varieties. Better understanding of the mechanisms underpinning citrus tolerance to *C*Las will help in developing economically tolerant varieties.

Introduction

Citrus is one of the world's major fruit crops with an annual global production of more than 100 million metric tons.[1] Citrus fruits are a good source for carbohydrates and are rich in biological compounds that are essential for human health such as vitamins, dietary fiber, minerals, carotenoids, and flavonoids.[1] Unfortunately, nowadays many diseases threaten the survival of the citrus industry. Many of these diseases such as citrus stubborn and huanglongbing (HLB) are caused by phloem sap-restricted bacteria and are spread by phloem sap-piercing insects.[2] Currently, citrus greening disease is considered as the most destructive disease of citrus and it is threatening the citrus industry worldwide.

Candidatus Liberibacter which causes citrus greening is a phloem-limited, gram-negative bacterium.[3] Three species of *Candidatus* Liberibacter are associated with citrus greening: *Candidatus* Liberibacter asiaticus (*C*Las) (Asia, North America, and Brazil), *Candidatus* Liberibacter africanus (*C*Laf) (Africa), and *Candidatus* Liberibacter americanus (*C*Lam) (Brazil).[4] Although the citrus greening pathogen can be spread by grafting, psyllid vectors are the major transmission means in the field.[5] The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama transmits *C*Las and *C*Lam, while the African citrus psyllid *Trioza erytrea* (Del Guercio) transmits *C*Laf.[5]

ACP transmits the *C*Las while feeding on citrus phloem sap. Citrus and close citrus relatives are the main host plants for the ACP.[5] Although ACP can survive and multiply on many host plants, ACP prefers some hosts more than others. Field observations indicated that sweet orange and *Murraya paniculata* (L.) Jack (orange jasmine) were the most preferred host for ACP, while *Poncirus trifoliata* was an occasional host.[5] Although there are many observations about preferred host of ACP, comparative laboratory studies are limited. For instance, Tsai & Liu (2000) tested the preference of ACP to four 4 hosts: *Citrus jambhiri* Lushington (rough lemon), *Murraya paniculata*, *Citrus × paradisi* Macfad. (grapefruit), and *Citrus aurantium* L. (sour orange).[6] Grapefruit was the most preferable host, followed by the other hosts.[6] In a recent study, Richardson and Hall (2013) also showed that accessions of *Poncirus trifoliata* and x *Citroncirus* spp. were more tolerant to ACP than *Citrus macrophylla* Wester (Alemow); more eggs were laid on *C. macrophylla* than *P. trifoliata* accessions.[7] Richardson and Hall (2013) concluded that *P. trifoliata* may have antixenosis and antibiosis resistance to ACP.[7]

Field observations also indicated that some ACP hosts are more tolerant to *C*Las bacteria than others.[8-10] To test these observations, Folimonova et al. (2009) examined the responses of many citrus varieties to Florida *C*Las isolates, using graft inoculation, under controlled conditions.[11] Based on intensity of symptoms, plant growth, and death, citrus genotypes were divided into three 3 major groups (sensitive, moderately tolerant, and tolerant).[11] The sensitive genotypes showed severe leaf chlorosis, decreased growth, and death. The moderately tolerant plants showed mild symptoms, while the tolerant varieties

showed few symptoms.[11] Similar results were also reported by Sagaram and Burns (2009).[12] In another study, Hajivand et al., (2009) also assessed eighteen 18 Malaysian citrus species for susceptibility to CLas by graft inoculation.[13] Based on the severity of symptoms, the species were categorized in four 4g groups: sensitive (72–58% severity), moderate (50–41% severity), mild (25–17% severity), and tolerant group (no symptoms).

To defend themselves against insects and pathogens attacks, plants produce a wide spectrum of defense compounds such as terpenoids, alkaloids, nonprotein amino acids, benzoxazinoids, cyanogenic glucosides, and phenolic compounds.[14-17] Although the primary function of phloem is to transport nutrients from source to sink, it is also believed that the phloem is implicated in plant defense.[18] The phloem contains many cells that are implicated in the synthesis, distribution, and release of large number of defense compounds such as terpenes, alkaloids, ketones, and glucosinolates.[18]

Although many studies have been conducted on HLB disease, the mechanisms underpinning citrus tolerance to CLas has not been determined yet. Exploring this mystery is essential for development of economically important CLas-tolerant varieties. Some citrus varieties such as *P. trifoliata* which are tolerant to CLas are also tolerant to citrus tristeza virus (CTV).[19] Although the mechanism of *P. trifoliata* resistance to CTV has not been revealed yet, it seems that it is due to the failure of the CTV to spread within its host despite its ability to replicate.[20] Because several antibacterial compounds have been identified in the fruit and seed of *P. trifoliata*, it was also suggested that the phloem sap of CLas-tolerant varieties contain some antibacterial compounds.[19,21,22]

Herein, we hypothesize that the phloem sap composition of CLas-tolerant varieties is different from that of sensitive varieties. To test this hypothesis, we studied the phloem sap composition of thirteen different varieties with different degrees of tolerance to CLas.

Material and methods

Plants

Citrus plants (12 months old, about 1 m height) were kept in temperature-controlled greenhouse (28–32°C) and were watered twice a week and fertilized once a week using 20-10-20 fertilizer (Allentown, PA). The thirteen different citrus varieties that were used in this study are shown in Table 1.

Phloem sap collection

Citrus phloem sap was collected as described by Hijaz and Killiny 2014.[23] Briefly, three flush shoots (2–3 week-old, about 0.5 cm diameter) were collected from each plant from three different locations (top, middle, and bottom). Five different plants were sampled from each variety. The bark was stripped into two pieces and was manually removed from the twig. The inner part of the bark was rinsed with deionized water and dried with Kimwipes® to exclude any contamination from the xylem sap. Then the bark strips were cut into about 1-cm pieces using a sterile razor blade. To collect the phloem sap, three to five pieces of the bark tissue were vertically placed in a 0.5-ml

Table 1. Citrus plants used in this study.

Citrus variety	Degree of tolerance to CLas*
Valencia sweet orange (*C. sinensis* (L.) Osbeck)	Sensitive (1)
Madam Vinous sweet orange (*C. sinensis* (L.) Osbeck)	Sensitive (1)
Duncan grapefruit (*C. paradisi* MacFadyen)	Sensitive (1)
Ruby red grapefruit (*C. paradisi* MacFadyen)	Sensitive (1)
Sour orange (*C. aurantium* L.)	Moderately tolerant (2)
Volkamer lemon (*C. limonia* Osbeck 'Volkameriana')	Moderately tolerant (2)
C. macrophylla Wester (Alemow)	Moderately tolerant (2)
Palestine Sweet lime (*C. aurantifolia* (Christm.)	Moderately tolerant (2)
Mexican lime (*C. aurantifolia* (Christm.)	Moderately tolerant (2)
Carrizo citrange (X *Citroncirus webberi*)	Tolerant (3)
Severinia buxifolia	Tolerant (3)
Poncirus trifoliata (L.) Raf.	Inconsistent (4)
Citrus latipes	Inconsistent (4)

*Folimonova et al. (2009).[11]

eppendorf tube. A small hole was made at the bottom of the tube using a pin and the tube was inserted into a 2-ml eppendorf tube. The sample was centrifuged at 12,000 rpm for 15 minutes at room temperature and the phloem sap collected from each plant was pooled together and stored at −80°C until analysis.

Methyl chloroformate (MCF) derivatization

The phloem sap was derivatized with methyl chloroformate (MCF) and analyzed with GC-MS as described by Hijaz and Killiny (2014). Briefly, a 20-μl aliquot of the pure phloem was transferred to a 1-ml silanized GC-MS insert and mixed with 200 μl of NaOH (1M). Then the alkaline sample was mixed with 167 μl of methanol and 34 μl of pyridine, followed by the addition of 20 μl of MCF. The sample was vigorously mixed for 30 seconds. An additional 20 μl of MCF was added and the sample was mixed for another 30 seconds. A 100-μl aliquot of chloroform was added with vigorous mixing for 10 seconds, followed by 100 μl of sodium bicarbonate (50 mM) with vigorous mixing for 10 seconds. The upper layer was discarded and about 100 μl of the organic layer was transferred to a new insert. A few mg of sodium sulfate was added to dry the organic layer, and 0.5 μl (splitless injection) was injected into the GC-MS instrument running in the full scan mode (40–600 m/z). Standards of amino acids, organic acids, and fatty acids were derivatized as mentioned above and were used to determine the concentration of each metabolite in citrus phloem sap.

GC-MS analyses

Derivatized samples and standards were analyzed using Clarus 500 GC-MS system (Perkin Elmer, Waltham, MA, USA) fitted with an ZB-5MS column (cross-linked 5% Ph Me siloxane, 30 m × 0.25 mm × 0.025 μm film thickness). The flow rate for the hydrogen carrier gas was 1 ml/min. The GC temperature program was as follows: initial temperature was held at 70°C for 1 min, and then increased to 220°C at a rate of 10°C/min,

held for 1 min, increased further to 300°C at 10°C/min, held for 0 min. The injector and the detector temperatures were set at 250°C and 260°C, respectively.

MS peak identification

GC-MS chromatograms were analyzed using TurboMass software version 5.4.2 (Perkin Elmer, Waltham, MA, USA). Peaks were first identified by comparing their mass spectra with library entries (NIST mass spectra library (National Institute of Standards and Technology, Gaithersburg, MA, USA); Wiley 9th edition (John Wiley and Sons, Inc., Hoboken, NJ, USA)). Identification was further confirmed by comparing the retention time and mass spectra of the detected metabolites with those of derivatized standards.

Data processing and statistical analysis

Data was manually aligned using retention time and mass values. Principal component analysis (PCA) was performed on metabolite concentrations to discriminate among varieties using JMP version 9.0 (SAS Institute Inc.,). Post hoc pairwise comparison followed by Tukey honestly significant difference (Tukey's HSD) test was used to compare the mean concentration of each metabolite in the different varieties. The two-way hierarchical cluster analysis (HCA), based on Ward's method (with 95% confidence), followed by the heat-map using the concentration of individual metabolites (Table 2) were also performed to construct the similarity dendrogram and visualize differences in the phloem sap profile between the different varieties. Simple linear regression and step wise forward selection (SWFS) were also conducted to elucidate any relation between the detected metabolites and citrus resistance to *CLas* infection.

Results

We studied the phloem sap because it is the main source of nutrients for *Candidatus* Liberibacter asiaticus (CLas) and the Asian citrus psyllids (ACP). In addition, we focused on the amino acid composition of the phloem sap because amino acids are precursors for most of the secondary metabolites implicated in plant defense. Methyl chloroformate (MCF) derivatization was chosen because it reacts with amino and organic acids, but does not react with sugars which are abundant in the phloem sap. Thirty-three compounds were detected in the phloem sap after MCF derivatization (Table 2). These metabolites can be classified into three groups: amino acids (20), organic acids (8), and fatty acids (5).

All of the amino acids detected in the citrus phloem sap were proteinogenic amino acids except γ-aminobutyric acid (GABA). The concentration of GABA in citrus phloem sap ranged from 1 to 16 mM (Table 2). Proline was the most predominant amino acid in all varieties except for *C. latipes* and *P. trifoliata*. The major amino acid in these two varieties was asparagine. The concentration of proline ranged from 3.0 to 20.9 mM and it showed little variation among the studied varieties. The total concentration of amino acids in the citrus phloem sap ranged from 13 to 97 mM. *C. latipes* was the highest in total amino acids, whereas Valencia was the lowest

(Table 2). Seven organic acids (fumaric, succinic, malic, quinic, benzoic, citric, and salicylic acid) were identified in citrus phloem sap. Malic acid was the main organic acid (Table 2). Citric and quinic acid were also abundant and their concentration ranged from (1 to 18 mM) and (1 to 39 mM), respectively. Low amounts (< 0.1 mM) of myristic, palmitic, stearic, oleic, and linoleic were also detected in the citrus phloem sap.

PCA and cluster analysis indicated that the phloem sap of CLas-tolerant varieties was different from CLas-sensitive varieties

The principal component analysis (PCA) of the different citrus varieties generated using the concentration of all metabolites in Table 2 is shown in Fig. 1A. Principal component 1 and 2 explained about 52.9 % of the variation. Most of the tolerant cultivars, especially *C. latipes*, *S. buxifolia*, and *P. trifoliata* clustered to the right of the PCA plot. In addition, *V. lemon and* Palestine lime also clustered close to the previous varieties. This clustering indicated that the phloem sap composition of these varieties was different from the rest of the other varieties (sensitive). The loading plot (Fig. 1B) showed that these varieties were higher in most of the metabolites, especially amino acids (bottom of high right quadrant and top of the lower right quadrant). On the other hand, the rest of the varieties (sensitive) clustered to the left of the plot and they were low in most of the metabolites, especially amino acids. In agreement with the PCA analysis, the cluster analysis (CA) showed that *C. latipes*, and *S. buxifolia* were similar and clustered closely to each other (Fig. 1C). The differences in the metabolites of the phloem sap among the different citrus varieties are also visualized in the heat map (Fig. 1C).

The PCA generated using the concentration of amino acids also separated *C. latipes*, *S. buxifolia*, and *P. trifoliata* from the other varieties (Fig. 2A). As shown in the loading plot (Fig. 2B) these varieties were higher in most of the amino acids (top and lower right quadrants).

The PCA generated using the organic acids (Fig. 2C) showed that organic acid profiles of *S. buxifolia* and Madam vinous were different from other varieties. As shown in the loading plot (Fig. 2D), *S. buxifolia*, was high in quinic, citric, and succinic acid, whereas Madam vinous was high in malate. Although fatty acids are an important in defense signaling in plants, the PCA and CA generated using the concentration of fatty acids did not show any clear clustering of any of the selected varieties (Figs. 2E-F).

Tukey test confirmed PCA and CA results and revealed the differences among varieties

In agreement with the PCA result the Tukey's test showed that tolerant cultivars were high in most of the phloem sap components, especially the amino acids. For example, *C. latipes* was the highest in alanine, valine, lysine, serine, glutamic acid, aspartic acid, threonine, asparagine, glutamine, GABA, palmitic, stearic, and cysteine (Table 2). In addition, it was the second highest variety in phenylalanine and the third in tyrosine (Table 2). *P. trifoliata* was the highest in leucine, isoleucine, phenylalanine, methionine, and cysteine and was the second

Table 2. Phloem sap metabolites concentrations (mM) in different citrus varieties as analyzed by GC-MS after methylchloroformate derivatization (*n ranges between 4 and 5*).

compound	Valencia	Palestine Sweet lime	Poncirus trifoliata	Volkamer lemon	Rubey red grapefruit	Severinia buxifolia	Sour orange	Mexican lime	Carrizo citrange	Duncan grapefruit	Madam Vinous	Alemow	Citrus latipes
fumaric acid	0.44±0.03f	2.75±0.43cdef	0.74±0.17f	4.96±1.04abcd	5.70±1.63abc	4.48±1.89cde	3.97±1.37bcdef	6.21±1.03abc	2.84±1.79cdef	1.59±0.95ef	7.88±2.08a	6.55±2.99ab	1.42±0.80ef
succinic acid	0.18±0.02bc	0.31±0.06bc	0.14±0.01c	0.34±0.04bc	0.24±0.05bc	1.06±0.37a	0.17±0.02c	0.31±0.08bc	0.13±0.03c	0.11±0.03c	0.45±0.16b	0.26±0.08bc	0.21±0.04bc
malate	10.25±2.05e	42.22±11.53e	24.49±7.01e	161.77±32.35ab	147.52±46.80ab	165.90±42.45ab	147.08±20.85ab	126.96±15.98bcd	75.93±43.35cde	35.47±26.79e	205.52±34.21a	136.75±33.65abc	47.22±20.08e
benzoic acid	13.06±1.34a	1.73±0.46defg	0.56±0.16fg	4.05±0.68de	13.72±1.39a	5.33±0.76cd	8.56±0.78bc	0.25±0.04g	2.72±0.58defg	9.36±3.45b	13.90±2.26a	0.25±0.08g	1.87±0.50efg
glycine	0.06±0.01g	0.26±0.05cdefg	0.56±0.15ab	0.48±0.07bcd	0.46±0.17bcd	0.27±0.05cdefg	0.25±0.04defg	0.11±0.03g	0.51±0.05bc	0.41±0.13bcde	0.38±0.08bcdef	0.20±0.08efg	0.79±0.19a
alanine	0.23±0.04f	0.46±0.07ef	1.31±0.20bcd	1.46±0.24abc	1.45±0.44abc	1.29±0.14bcd	0.99±0.22cdef	0.90±0.34cdef	0.86±0.35cdef	0.95±0.31cdef	1.79±0.51ab	1.04±0.17cde	2.05±0.32a
valine	0.06±0.01c	0.32±0.09abc	0.44±0.14ab	0.16±0.06bc	0.12±0.02c	0.33±0.10abc	0.10±0.01c	0.12±0.02bc	0.09±0.01c	0.14±0.04bc	0.20±0.08bc	0.11±0.05bc	0.57±0.41a
leucine	0.06±0.01e	0.24±0.06bcd	0.54±0.15a	0.20±0.08cde	0.14±0.02cde	0.40±0.09ab	0.12±0.02cde	0.16±0.04cde	0.10±0.03de	0.16±0.04cde	0.21±0.09cde	0.13±0.05cde	0.26±0.04bc
GABA	1.22±0.29g	2.62±0.54ef	6.01±2.96bcde	6.99±1.70bc	7.62±1.13bc	6.75±1.64bc	6.23±1.05bcd	2.85±0.47efg	3.27±0.95defg	4.84±1.36cdef	8.57±1.21b	1.84±0.51fg	16.49±2.07a
isoleucine	0.11±0.01d	0.36±0.05bcd	0.72±0.24a	0.60±0.13a	0.59±0.06ab	0.58±0.04ab	0.52±0.08abc	0.29±0.05cd	0.32±0.08cd	0.32±0.13cd	0.73±0.08a	0.23±0.08d	0.22±0.08d
threonine	0.10±0.02b	0.24±0.09b	0.38±0.19b	0.30±0.04b	0.24±0.05b	0.31±0.08b	0.18±0.02b	0.11±0.02b	0.18±0.02b	0.22±0.10b	0.26±0.06b	0.10±0.04b	1.46±0.59a
malic acid	9.11±1.34e	28.84±7.09cde	15.93±3.52e	61.71±19.50ab	75.64±6.89a	53.55±25.21abcd	55.30±7.00abc	58.30±6.10abc	34.59±17.67bcde	19.31±13.69e	78.83±10.36a	61.76±11.28ab	27.03±7.57de
proline	2.53±0.30b	9.98±1.02ab	12.28±3.61ab	16.04±8.89ab	18.73±5.86ab	18.78±9.21ab	17.62±4.34ab	11.57±4.15ab	16.50±7.27ab	18.22±8.55ab	20.48±4.67a	10.52±3.85ab	20.86±9.82a
asparagine	2.69±0.17b	8.15±1.71b	48.64±16.36ab	7.15±6.29b	8.09±1.85b	5.12±1.26b	2.27±0.77b	0.15±0.03b	6.39±4.75b	2.18±1.21b	1.86±0.37b	3.03±1.72b	80.78±69.02a
aspartic acid	0.67±0.06c	1.45±0.20bc	3.12±1.37bc	3.80±1.03bc	3.13±0.47bc	2.79±1.09bc	1.94±0.17bc	1.30±0.06bc	1.34±0.42bc	1.23±0.42c	2.50±0.51bc	0.95±0.24c	7.28±3.01a
quinic acid	0.79±0.22bc	11.13±1.78bc	15.92±9.53bc	19.10±5.86bc	11.59±3.14bc	121.30±40.65a	5.04±1.37bc	10.92±2.34bc	6.30±3.08bc	2.35±2.68c	8.32±2.22bc	17.89±7.16bc	39.07±38.76b
citric acid	0.64±0.06b	2.86±0.95b	16.30±15.40b	13.53±6.38b	12.55±2.55b	59.20±24.29a	5.74±0.91b	5.55±5.68b	6.89±3.87b	2.20±3.14b	14.78±16.10b	7.07±3.97b	18.88±19.39b
serine	1.06±0.20de	1.49±0.34cde	4.68±2.05ab	2.96±1.05bcd	3.33±0.73bc	3.17±1.40bcd	1.31±0.16de	0.69±0.04e	1.82±0.42cde	2.12±0.66cde	1.99±0.53cde	0.52±0.24e	6.73±1.28a
glutamine	1.80±2.30b	1.13±0.19b	3.89±1.98b	1.91±0.63b	1.92±0.34b	1.41±0.66b	0.80±0.22b	0.24±0.01b	1.03±0.48b	0.91±0.36b	0.68±0.27b	0.49±0.26b	11.28±9.58a
salicylic acid	0.05±0.01ab	0.04±0.00ab	0.03±0.03ab	0.04±0.01ab	0.12±0.10a	0.09±0.06ab	0.06±0.01ab	0.04±0.00ab	0.02±0.01b	0.06±0.02ab	0.11±0.03a	0.04±0.01ab	0.03±0.00b
glutamic acid	0.17±0.01b	0.02±0.01cd	0.07±0.09bcd	0.07±0.03bcd	0.05±0.02bcd	0.32±0.15a	0.02±0.01d	0.12±0.03bcd	0.03±0.01cd	0.02±0.01d	0.06±0.01bcd	0.06±0.02bcd	0.13±0.03bc
methionine	0.01±0.00d	0.03±0.01d	0.08±0.01a	0.06±0.01abc	0.04 0.01bcd	0.06±0.01ab	0.02±0.01d	0.03±0.01d	0.02±0.01d	0.01±0.00d	0.03±0.02cd	0.02±0.02d	0.03±0.02d
cysteine	0.00±0.00b	0.01±0.01b	0.03±0.02ab	0.03±0.01b	0.02±0.01b	0.02±0.01b	0.02±0.03b	0.02±0.02b	0.01±0.01b	0.01±0.00b	0.02±0.01b	0.02±0.01b	0.05±0.01a
phenylalanine	0.05±0.00c	0.13±0.02bc	0.25±0.03a	0.10±0.04bc	0.08±0.01c	0.23±0.06a	0.06±0.01c	0.09±0.02bc	0.08±0.01c	0.08±0.02c	0.10±0.03bc	0.11±0.03bc	0.16±0.06b
myristic acid	ND	ND	Tr	Tr	Tr	Tr	ND	ND	ND	Tr	Tr	Tr	Tr
palmitic	0.03±0.00bcd	0.03±0.00bcd	0.03±0.00bcd	0.04±0.01abc	0.04±0.00abc	0.04±0.00abc	0.03±0.01cd	0.02±0.01d	0.03±0.00bcd	0.03±0.01bc	0.04±0.00ab	0.04±0.01ab	0.05±0.00a
lysine	0.10±0.01b	0.23±0.05b	0.33±0.07b	0.23±0.09b	0.34±0.15b	0.51±0.12b	0.12±0.02b	0.13±0.01b	0.09±0.03b	0.24±0.13b	0.29±0.15b	0.09±0.05b	1.27±0.51a
histidine	0.04±0.02bc	0.08±0.06bc	0.15±0.08bc	0.04±0.03c	0.06±0.04bc	0.24±0.07b	0.01±0.01c	0.01±0.01c	0.01±0.01c	0.06±0.06c	0.05±0.04bc	0.02±0.02c	0.46±0.23a
linoelic acid	Tr	ND	ND	Tr	Tr	Tr	ND	ND	ND	Tr	Tr	Tr	Tr
oleic acid	Tr	Tr	Tr	Tr	Tr	Tr	Tr	ND	Tr	Tr	Tr	Tr	Tr
stearic	0.02±0.00bcde	0.02±0.00bcde	0.03±0.00abcd	0.03±0.00abcd	0.03±0.00abc	0.03±0.00abcd	0.02±0.01de	0.02±0.00e	0.02±0.00cde	0.03±0.01bcd	0.03±0.00ab	0.03±0.00abcd	0.04±0.00a
tyrosine	0.1±0.00bcd	0.15±0.02a	0.14±0.01a	0.08±0.03bcd	0.07±0.01cd	0.13±0.02ab	0.04±0.01d	0.06±0.01d	0.05±0.00d	0.05±0.02d	0.07±0.02cd	0.03±0.01d	0.11±0.05abc
tryptophan	0.0±0.00bc	0.04±0.02bc	0.03±0.00bc	0.02±0.01bc	0.01±0.00bc	0.12±0.06a	0.01±0.00c	0.04±0.01bc	0.01±0.00bc	0.02±0.01bc	0.02±0.01bc	0.02±0.01bc	0.06±0.05b
amino acids	13.6±11.08	26.06±27.39	21.12±83.66	55.03±42.67	46.42±46.47	44.12±42.84	40.59±32.61	20.88±18.99	36.24±32.70	51.71±32.18	41.30±40.27	11.94±19.52	96.87±151.04
organic acids	24.23±24.26	48.57±47.68	21.84±49.61	129.30±103.73	110.47±119.57	203.51±245.01	80.43±78.84	89.78±81.59	74.22±53.49	36.03±34.98	106.69±124.28	85.48±93.80	124.68±88.51
fatty acids	0.06±0.07	0.07±0.07	0.08±0.08	0.10±0.09	0.08±0.09	0.08±0.09	0.07±0.07	0.06±0.05	0.07±0.07	0.10±0.08	0.09±0.09	0.07±0.09	0.11±0.11
total	37.89±35.41	74.71±75.14	43.03±133.34	184.44±146.48	156.98±166.13	247.71±287.93	121.1±111.51	110.72±100.63	110.53±86.25	87.84±67.24	148.08±164.65	97.49±113.42	221.67±239.66

Means in the same line with the same letters are not significantly different from each other (Tukey test, $p > 0.05$). nd: not detected and tr: < 0.01 mM. The identity of all compounds was confirmed using authentic standards except malate was tentatively identified using the mass spectra library.
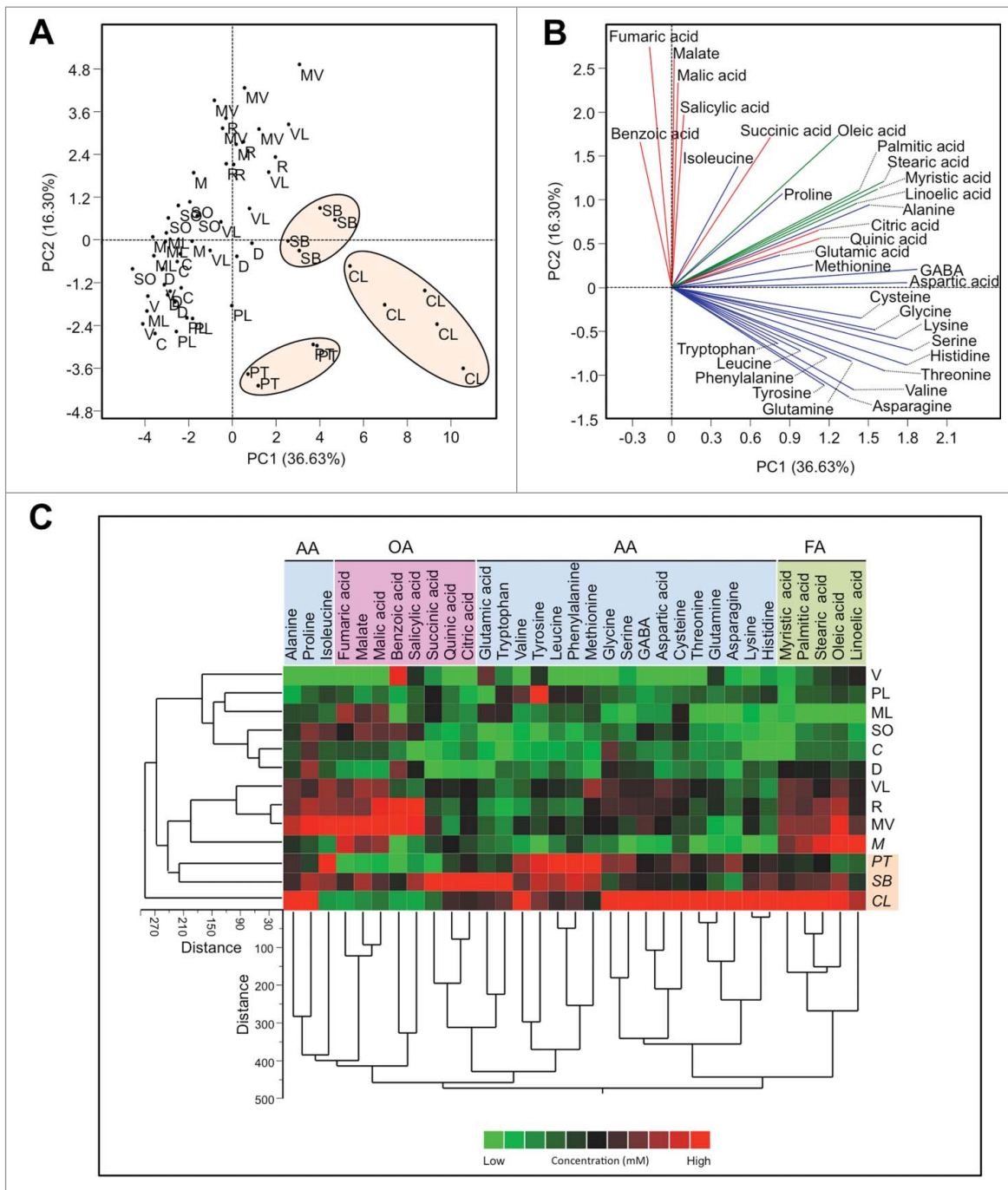
Figure 1. Principal components analysis-scatter plot (A), its associated loading plot (B) and two-way hierarchical cluster analysis and heat-map (C) showing the distribution of different citrus varieties using the concentration of detected compounds in phloem saps (*n ranges from 4 to 5*). Row represents variety and column compound concentrations. Cells are colored based on concentration in mM. AA: amino acids, OA: organic acids, FA: fatty acids. Varieties abbreviations: Valencia sweet orange (V), Madam Vinous sweet orange (MV), Duncan grapefruit (D), Ruby red grapefruit (R), Sour orange (SO), Volkamer lemon (VL), Alemow (M), Palestine sweet lime (PL), Mexican lime (ML), *Carrizo citrange* (C), *Severinia buxifolia* (SB), *Poncirus trifoliata* (PT), and *Citrus latipes* (CL).

highest variety in asparagine, glutamine, valine, glycine, and tyrosine (Table 2). *S. buxifolia* was the highest in tryptophan, leucine, succinic, citric acid, and quinic acid (Table 2). In addition, *S. buxifolia* was the second highest cultivar in phenylalanine, and the third highest variety in methionine, tyrosine, and valine (Table 2). Volkamer lemon was high in methionine and aspartic acid and the fourth in quinic acid (Table 2). Palestine lime was the highest in tyrosine and the third in valine and tryptophan (Table 2). Mexican lime was third in cysteine and

fourth in glutamic acid (Table 2). *C. macrophylla* was the third in quinic acid and the fifth in phenylalanine (Table 2). *C. citrange* was the second highest variety in quinic acid and the third in glycine (Table 2).

Interestingly, the levels of phenylalanine, tyrosine, and tryptophan as well as their total level were correlated with citrus tolerance to *C*Las (Figs. 3A-C). As shown in Fig. 3A, *P. trifoliata*, *S. buxifolia*, *C. latipes*, Palestine lime, *C. macrophylla*, and Volkmer lemon were the highest in phenylalanine. Palestine
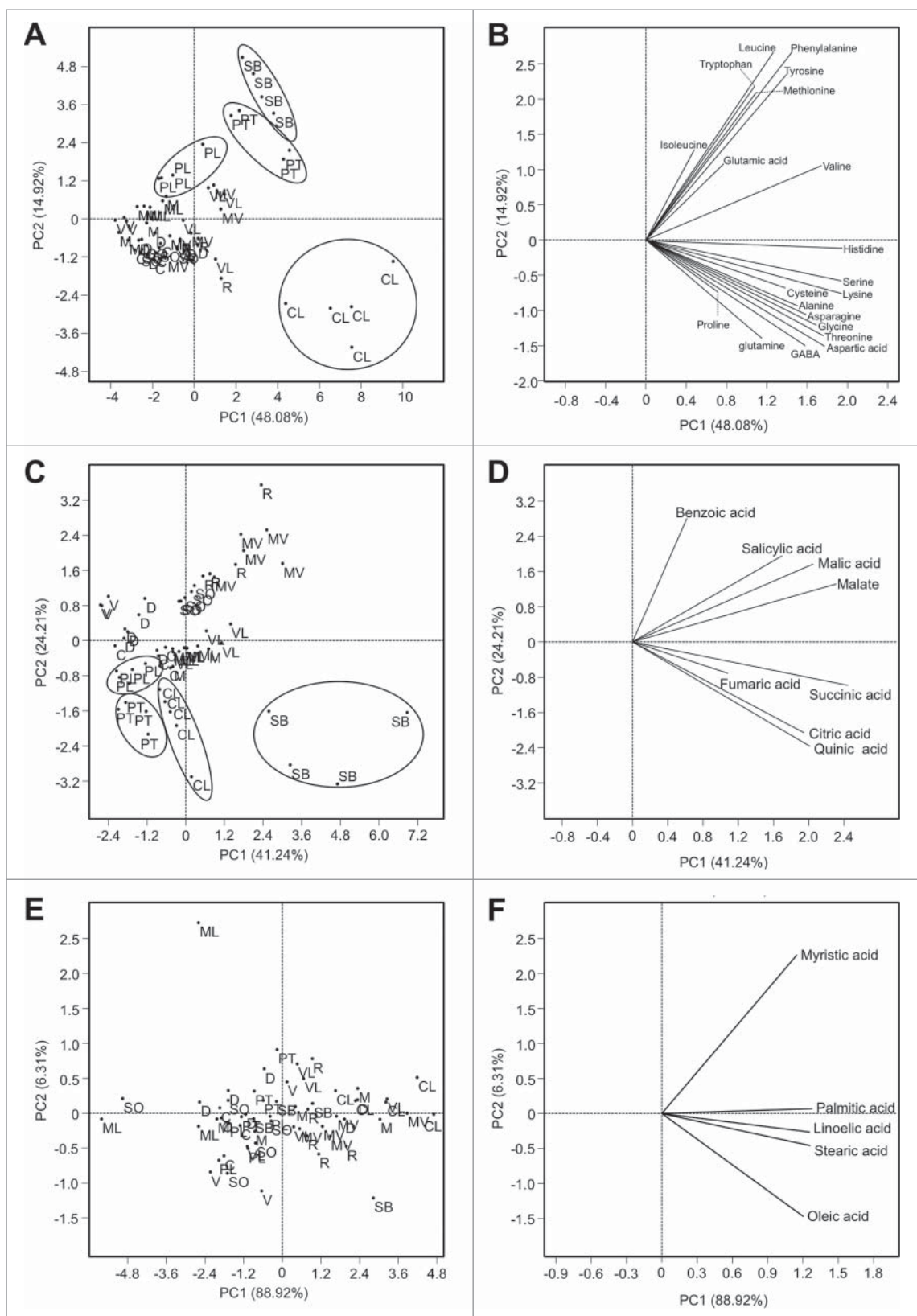
Figure 2. Principal components analysis (PCA)-scatter plot and its associated loading plot showing the distribution of different citrus varieties using the concentrations of detected compounds (*n ranges from 4 to 5*). (A) PCA-scatter plot using the concentrations of the amino acid group and its PCA-loading plot (B), (C) PCA-scatter plot using the concentration of organic acid group and its PCA-loading plot (D), (E) PCA-scatter plot using the concentration of fatty acid group and its PCA-loading plot (F).Varieties abbreviations: Valencia sweet orange (V), Madam Vinous sweet orange (MV), Duncan grapefruit (D), Ruby red grapefruit (R), Sour orange (SO), Volkamer lemon (VL), Ale-mow (M), Palestine sweet lime (PL), Mexican lime (ML), *Carrizo citrange* (C), *Severinia buxifolia* (SB), *Poncirus trifoliata* (PT), and *Citrus latipes* (CL).
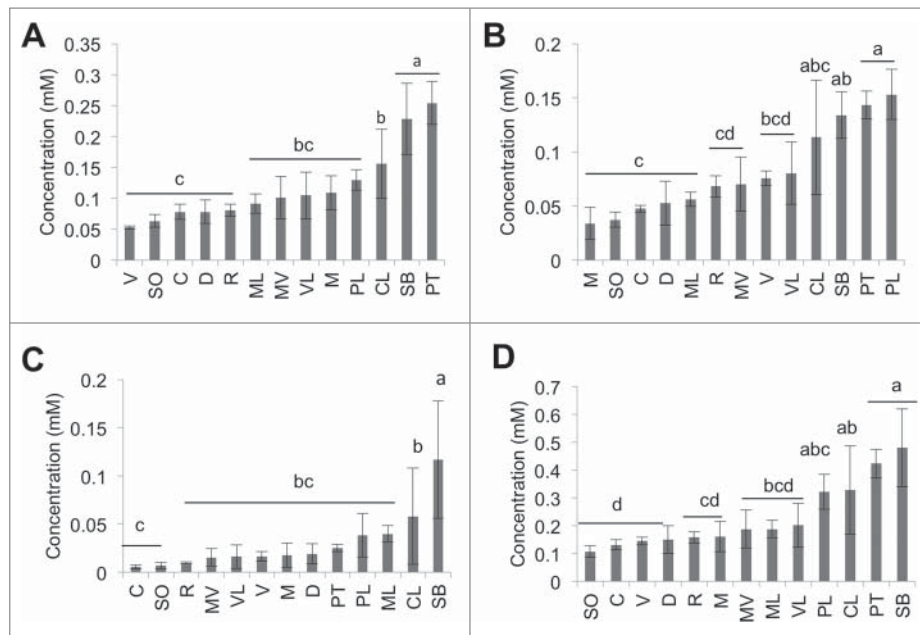
Figure 3. Average concentration of phenylalanine (A), tyrosine (B), tryptophan (C), and total of phenylalanine, tyrosine, and tryptophan together in different citrus varieties (D). (*n ranges from 4 to 5*)., Varieties abbreviations: Valencia sweet orange (V), Madam Vinous sweet orange (MV), Duncan grapefruit (D), Ruby red grapefruit (R), Sour orange (SO), Volkamer lemon (VL), Alemow (M), Palestine sweet lime (PL), Mexican lime (ML), *Carrizo citrange* (C), *Severinia buxifolia* (SB), *Poncirus trifoliata* (PT), and *Citrus latipes* (CL).

lime, *P. trifoliata*, *S. buxifolia*, *C. latipes*, and Volkamer lemon were the highest in tyrosine (Fig. 3B). *S. Buxifolia*, *C. latipes*, Mexican lime, Palestine lime, and *P. trifoliata* were the highest in tryptophan (Fig. 3C). In the same manner, *S. Buxifolia*, *P. trifoliata*, *C. latipes*, Palestine lime, and Volkamer were the highest in the total of these three amino acids (Fig. 3D). On the other hand, most of the sensitive cultivars were low in these amino acids (Fig. 3A-C).

Simple linear regression (SLR) and Stepwise forward regression (SWFR) suggest that citrus tolerance to CLas is dependent on phloem sap composition

The relationship between the phloem sap metabolites and citrus tolerance to *C*Las was investigated using simple and multiple linear regression (Table 3). The simple linear regression showed that most of the phloem sap amino acids were implicated in citrus resistance to *C*Las (Table 3). As shown in Table 3, phenylalanine was highly correlated with citrus tolerance to *C*Las (R^2: 0.4, P-value < 0.0001). Other amino acids such as valine, leucine, tyrosine, tryptophan, histidine, lysine, cysteine, methionine, serine, and glutamine were also positively correlated with citrus tolerance to *C*Las (Table 3). On the other hand, organic acids were negatively correlated with citrus tolerance to *C*Las (Table 3), however this correlation was not strong except for benzoic acid (R^2: 0.4, P-value < 0.0001). Fatty acids also did not show a strong correlation with citrus tolerance to *C*Las; R^2 was either too small or p-value was larger than 0.05 (Table 3).

Stepwise forward regression (SWFR) was also used to investigate the relation between phloem sap metabolites and varieties' tolerance to *C*Las. The best model that accounts for this relation is shown in (Table 3). In agreement with the SLR,

phenylalanine was also the most important component in the model and it explained about 50% of the variation in varieties' tolerance to *C*Las. Benzoic acid which showed a negative correlation with citrus resistance to *C*Las in SLR was the second most important component in the model and it accounted about 20% of the variation (Table 3). Serine, GABA, leucine, stearic acid, linoleic acid, and glutamic acid were also significant to the model (Table 3).

Discussion

Clustering of the tolerant and sensitive cultivars into two groups indicated that the phloem sap composition of the tolerant cultivars was different from that of susceptible cultivars. This clustering also indicated that the phloem sap composition may have an influence on citrus tolerance to *C*Las and ACP. Our results showed that most of the *C*Las-tolerant cultivars were high in amino acids, especially those implicated in plant defense to pathogens such as phenylalanine, tyrosine, and tryptophan.

Phenylalanine is the end product of the shikimate pathway which also produces tyrosine and tryptophan.[24] On the other hand, phenylalanine is the first precursor in phenylpropanoid cycle which is involved in plant defense, structural support, and survival.[25] The phenylpropanoid pathway produces many important secondary metabolites which are involved in plant defense such as flavonoids, lignin, lignans, coumarins, and hydroxycinnamic acid conjugates.[26]

Induction of phenylalanine ammonia lyase (PAL), the first key enzyme of the phenylpropanoid pathway, has been observed in tobacco, soybean, cucumber, and melon after viral and pathogen infections.[27-29] Induction of PAL and cinnamate 4-hydroxylase (C4H), the second enzyme in phenylpropanoid

Table 3. Simple linear regression (SLR) and stepwise forward regression (SWFR) results.

Simple linear regression (SLR) between the concentration of phloem sap metabolites in different citrus varieties and their degrees of tolerance to CLas.

compound	R^2	P-value	Regression formula
fumaric	0.07	0.0330	rank = 2.4672713 − 0.1033573*fumaric
succinic acid	0.01	0.5423	rank = 2.0159913 + 0.2544109*succinic acid
malate	0.05	0.0858	rank = 2.4228736 − 0.0033815*malate
benzoic acid	0.40	<0.0001	rank = 2.846556 − 0.1247415*benzoic acid
glycine	0.24	<0.0001	rank = 1.2137884 + 2.464121* L-glycine
alanine	0.08	0.02008	rank = 1.4595241 + 0.5635009* L-alanine
valine	0.33	<0.0001	rank = 1.4365366 + 3.1788655* L-valine
leucine	0.35	<0.0001	rank = 1.1479306 + 4.6403882* L-leucine
GABA	0.14	0.0018	rank = 1.5367921 + 0.0968826*GABA
isoleucine	0.01	0.8939	rank = 2.1275553 − 0.0894776* L-isoleucine
threonine	0.26	0.0001	rank = 1.6537338 + 1.4218318* L-threonine
malic acid	0.06	0.0372	rank = 2.5545697 − 0.0107363*malic acid
proline	0.01	0.4064	rank = 1.8546825 + 0.0159869* L-proline
asparagine	0.30	<0.0001	rank = 1.8293058 + 0.0201661* L-asparagine
aspartic acid	0.21	<0.0001	rank = 1.4964464 + 0.2476606* L-aspartic acid
quinic acid	0.13	<0.0024	rank = 1.8535996 + 0.0121467*quinic acid
citric acid	0.09	0.0153	rank = 1.8560199 + 0.0190329*citric acid
serine	0.29	<0.0001	rank = 1.3563804 + 0.3112015* L-serine
glutamine	0.21	<0.0001	rank = 1.8325813 + 0.1282487* L-glutamine
salicylic acid	0.10	0.0106	rank = 2.5278381 − 7.9565749*salicylic acid
glutamic acid	0.03	0.2052	rank = 1.9431001 + 1.7149994* L-glutamic acid
methionine	0.21	<0.0001	rank = 1.4207654 + 20.700626* L-methionine
cysteine	0.26	<0.0001	rank = 1.481304 + 31.896956* L-cysteine
phenylalanine	0.41	<0.0001	rank = 0.8664052 + 10.784315* L-phenylalanine
myristic acid	0.06	0.0451	rank = 1.2877006 + 151.58439* myristic
palmitic acid	0.07	0.0343	rank = 1.2694889 + 24.498395* palmitic
lysine	0.24	<0.0001	rank = 1.6619978 + 1.4715583* L-lysine
histidine	0.30	<0.0001	rank = 1.7394908 + 4.0341786*histidine
linoleic acid	0.01	0.4235	rank = 1.8792104 + 39.64309*linoleic acid
oleic acid	0.01	0.3618	rank = 1.825534 + 37.441287*oleic acid
stearic	0.05	0.0804	rank = 1.4599026 + 23.933753* stearic
tyrosine	0.18	0.0003	rank = 1.3115472 + 9.9256764* L-tyrosine
tryptophan	0.14	0.0019	rank = 1.7886113 + 10.823357* L-tryptophan

Stepwise forward regression (SWFR) between the concentration of phloem sap metabolites in different citrus varieties and their degree of tolerance to CLas.

Compound (step number)	R^2	P-value	Mallowe's Criterion (Cp)	Akaike information criterion (AICc)	Bayesian information criterion (BIC)
phenylalanine (1)	0.41	0.0000	70.419	476.516	482.749
benzoic acid (2)	0.61	0.0000	28.482	452.017	460.191
serine (3)	0.70	0.0000	8.0795	435.048	445.088
stearic acid (4)	0.73	0.0148	3.9023	430.992	442.82
fumaric (5)	0.76	0.0039	−1.917	424.284	437.818
linoleic acid (6)	0.78	0.0316	−3.884	421.664	436.818
GABA (7)	0.79	0.0815	−4.367	420.863	437.548
leucine (8)	0.80	0.0918	−4.616	420.323	438.441
glutamic acid (9)	0.81	0.1473	−4.235	420.704	440.156

pathway, was also observed in cucumber and tomato after infection with the bacterial pathogen *Pseudomonas syringae*.[30,31] Induction of PAL and C4H enzymes has been also observed in many insect-damaged plants.[32] It is also believed that the biosynthesis of phenylalanine, the first precursor in phenylpropanoid cycle, is induced after insect damage.[32]

In addition, phenylalanine is implicated in the synthesis of salicylic acid (SA) which mediates plant defense against pathogens and insect attack.[17,33] SA accumulation, in both infected and distal leaves after pathogens attack, is necessary to activate pathogenesis-related gene expression and induce the synthesis of defensive compounds in infected plants.[33] Although SA could be synthesized from several pathways, silencing and chemical inhibition of PAL genes in plants indicated that the synthesis of SA from cinnamate (produced from phenylalanine) was the most important pathway during pathogen

infection.[33,34] Like salicylic acid, the phytohormone ethylene could be derived from amino acids. Ethylene controls many aspects of the plant life cycle and plays an important role in plant response to biotic and abiotic stresses.[35] Ethylene can be derived from methionine via three key enzymatic reactions.[35] The knockout of the methionine cycle in *Arabidopsis* reduced ethylene synthesis in seedlings.[36]

Recently, Slisz et al. (2012) showed that juice from symptomatic CLas-positive sweet orange fruit was lower in sugar, and many amino acids including alanine, arginine, isoleucine, leucine, proline, threonine, and valine compared to that obtained from healthy fruits.[37] On the other hand, the concentration of phenylalanine, asparagine, and histidine were higher in CLas-positive fruits.[37] The high levels of phenylanine in symptomatic fruits, was explained by the inhibition of the phenylpropanoid biosynthesis pathway by CLas.[37] However, it is

also possible that the increased demand for phenylalanine in the phenylpropanoid pathway may increase the synthesis of phenylalanine in *C*Las-infected fruits. In agreement with the latter possibility, Görlach et al. (1995) suggested that the increased demand for phenylalanine in the phenylpropanoid pathway after elicitor treatment increased *de novo* synthesis of phenylanine biosynthesis.[38] Our results together with Slisz's observation suggested that phenylalanine may play an important role in citrus response to *C*Las.

In addition to phenylalanine, tyrosine and tryptophan were also high in *C*Las-tolerant varieties. Like phenylalanine, tyrosine and tryptophan are also precursors for many secondary metabolites which are implicated in plant defense against biotic stress.[16] Phenylalanine, tyrosine, and tryptophan are also implicated in the synthesis of plant phenolics.[16] Constitutive phenolics act as feeding deterrents for herbivores, however their role in resistance against fungi, bacteria, and nematode is controversial.[16] Although cultivars with high levels of constitutive phenolics were less attractive to herbivores, it is believed that the speed and the duration of *de novo* biosyntheses of phenolics are more important for resistance than constitutive concentrations.[16] Because the *C*Las-tolerant cultivars contain high levels of these aromatic amino acids, these cultivars may contain high levels of phenolics or they could synthesize higher amounts of phenolics compounds rapidly after ACP and *C*Las attack. The high levels of constitutive phenolics or their rapid synthesis from *de novo* may explain why some citrus varieties are more tolerant to ACP and *C*Las. However, more work is needed to test the role of citrus phenolics against *C*Las.

*C*Las-tolerant varieties were higher in amino acids which are implicated in plant defense. This result was not surprising because many plant defensive compounds such as glucosinolates are derived from amino acids.[39] In addition, some amino acid metabolic pathways are an important part in plant immune system.[40] For example, pipecolic acid which is formed from lysine, derived from aspartic acid, has been found to induce the production of SA and camalexin in Arabidopsis leaves inoculated with bacterial pathogens.[40] Although the levels of amino acids in plants are under a continual state of flux and only one time point was investigated, our results indicated that amino acids may play an important role in citrus tolerance to *C*Las. In addition, the induction of these amino acids in *C*Las-infected citrus plants (non-published data) supports our current results. Time-wise determination of amino acids levels in *C*Las-tolerant and susceptible varieties before and after infection with *C*Las could reveal more insights about the role of amino acids in citrus response against *C*Las.

Field observation and controlled studies showed that ACP prefers some citrus varieties more than others.[5-7] However, the characteristics that make some varieties resistant to ACP are not known yet.[41] Plant resistance to ACP could result from different traits such as non-preference, antibiosis, and tolerance.[42] It is also possible that non-preferred hosts could be deficient in essential nutrients. However, because most of the tolerant cultivars were, in general, rich in amino acids including the essential ones, this possibility can be eliminated.

Although SLR showed that fatty acids were not implicated in citrus tolerance to *C*Las, SWFS showed that linoleic acid and stearic acid were necessary in the model. Fatty acids are precursors for jasmonic acid which is released in response to wounding and herbivore attack (Zeier, 2013). High levels of these fatty acids in *C*Las tolerant varieties may explain why they are also tolerant to ACP.

Plant defense against bacterial pathogens and insects requires the biosynthesis of metabolites with antimicrobial or toxic activities and these metabolites could be produced constitutively (phytoanticipins) and/or induced (phytoalexins) after biotic stress.[40] Plant defense also depends on the performance of plant metabolism.[40] Because many plant defensive compounds are derived from amino acids, the metabolism of certain amino acids impacts plant resistance to pathogens.[40] Our results showed that phloem sap of *C*Las-tolerant varieties was higher in amino acids implicated in plant resistance to pathogens and insects. The high amounts of these amino acids may be responsible for the tolerance of these varieties to *C*Las and ACP; these varieties may contain higher amount of constitutive defensive compound and/or they may synthesize them in more efficient manner after infection.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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