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Research article

GC–MS metabolomic differentiation of selected citrus varieties with different sensitivity to citrus huanglongbing

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ABSTRACT

Huanglongbing (HLB) is the most destructive disease of citrus worldwide. The rapid identification of tolerant varieties is considered a critical step towards controlling HLB. GC–MS metabolite profiles were used to differentiate HLB-tolerant citrus varieties '*Poncirus trifoliata*' (TR) and 'Carrizo citrange' (CAR) from HLB-sensitive varieties 'Madam Vinous sweet orange' (MV) and 'Duncan' grapefruit (DG). PCR analyses revealed that MV was the most sensitive variety followed by DG and the tolerant varieties CAR and TR. Metabolomic multivariate analysis allowed classification of the cultivars in apparent agreement with PCR results. Higher levels of the amino acids L-proline, L-serine, and L-aspartic acid, as well as the organic acids butanedioic and tetradecanoic acid, and accumulation of galactose in healthy plants were characteristic of the most sensitive variety MV when compared to all other varieties. Only galactose was significantly higher in DG when compared to the tolerant varieties TR and CAR. The tolerant varieties showed higher levels of L-glycine and mannose when compared to sensitive varieties MV and DG. Profiling of the sensitive varieties MV and DG over a 20-week period after inoculation of those with the HLB-containing material revealed strong responses of metabolites to HLB infection that differed from the response of the tolerant varieties. Significant changes of L-threonine level in the leaves from old mature flushes and L-serine, L-threonine, scyllo-inositol, hexadecanoic acid, and mannose in the leaves from young developing flushes were observed in MV. Significant changes in myo-inositol in old flushes and L-proline, indole, and xylose in new flushes were observed in DG.

1. Introduction

Citrus huanglongbing (HLB), also known as the citrus greening disease, has become one of the greatest challenges for citrus growers across the world. Although Koch postulates have not been confirmed, the disease has been associated with a phloem-limited bacterium, *Candidatus* Liberibacter spp. First detected in China in the early 1900's, HLB has now spread all over the world [1]. Two psyllid species – *Diaphorena citri* (Kuw.), the Asian citrus psyllid; and *Trioza erytreae*, the African citrus psyllid – are responsible for the tree-to-tree transmission of the disease [2]. Currently the disease has no cure. Upon development of HLB infection in a tree, leaves accumulate high amounts of starch and show a pattern of yellow and green blotches [3]; the fruit becomes smaller, lopsided, and color does not fully develop. As HLB progresses, estimated yield reductions from 30 to 100% have made groves unprofitable within 7–10 years of infection [3]. Detection can be done by visual symptoms or by polymerase chain reaction (PCR). However, incidences reported by PCR have been twice as high as those reported by visual examination [4]. Other methods such as starch detection and chlorophyll fluorescence have also been suggested for HLB detection [5].

The development of tolerant and resistant citrus varieties is being emphasized as an alternative to reduce the impact of the disease [6]. Although HLB affects all citrus varieties, certain varieties have been reported to be more susceptible than others. Folimonova et al. [7] classified 30 citrus genotypes ranging from sensitive to tolerant according to their response to HLB in terms of symptom development and bacterium titer determined by PCR. However, the internal factors responsible for HLB susceptibility in citrus are yet to be understood.

Metabolomics is a growing field of analytical chemistry that focuses on the identification of small metabolites. Initially, mainly used in pharmaceutical applications, metabolomics has become

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a powerful tool in agriculture and food science [8,9] and has been used to characterize metabolic changes in plants after biotic and abiotic stresses [10], as well as biotic contamination of foods [11]. Metabolomic techniques have been able to identify changes in the metabolite profile of different citrus varieties [12], including those affected by HLB [13,14], as well as metabolomic changes in both pathogen and host after *Arabidopsis thaliana* infection with *Pseudomonas syringae* [15]. Additionally, GC–MS based metabolic profiling has been used to identify differences between two sunflower genotypes varying in response to *Sclerotinia sclerotiorum* [16]. However, no specific metabolites relative to tolerance were reported.

The objective of this research was to determine GC–MS-based metabolomic differences between two citrus varieties that are sensitive to HLB – 'Madam Vinous' sweet orange (MV) and 'Duncan' grapefruit (DG) – and two tolerant citrus varieties 'Carrizo citrange' (CAR) and '*Poncirus* trifoliata' (TR) [7]. We also monitored metabolomic changes occurring during HLB infection of sensitive varieties as a first step towards understanding the HLB tolerance mechanism of citrus.

2. Results

2.1. PCR and symptom development

Seedlings of both MV and DG varieties demonstrated a strong visible response to the HLB infection, with MV developing more pronounced symptoms earlier than all other varieties. Plants of these varieties developed severe yellowing of young leaves and reduced growth. Symptoms began to appear around 12–14 weeks after graft-inoculation and their severity progressed with time. PCR tests conducted using samples collected from the inoculated plants and HLB-specific primers showed high levels of the HLB bacterium, with the mean values of the threshold cycle (Ct) being 22.1 $\pm$ 0.28 for MV and 23.9 $\pm$ 0.30 for DG at week 14. At week 20, Ct values for these cultivars measured 23.4 $\pm$ 0.19 and 25.1 $\pm$ 0.27, respectively. In contrast, at week 14, Ct values for CAR and TR were 30.5 $\pm$ 0.45 and 31.6 $\pm$ 0.26, respectively. At week 20, CAR and TR had Ct values of 29.3 $\pm$ 0.20 and 32.6 $\pm$ 0.33, respectively, indicating that these varieties were highly tolerant to HLB. CAR and TR seedlings continued to grow vigorously, similar to control seedlings grafted with PCR-negative twigs for the duration of this experiment.

2.2. Metabolomic differentiation of citrus varieties

Approximately 61 compounds with a signal to noise ratio of at least 3 were detected by GC–MS in each variety. Fig. 1 shows typical chromatograms of each analyzed citrus cultivar. The overall metabolite profile of each variety was used for Principal Components Analysis (PCA, Fig. 2). Sample grouping by PCA correlated with variety as indicated by well-defined cluster regions in Fig. 2A and possibly with susceptibility. Separation of the most sensitive variety MV was mostly characterized by low scores of the first principal component (PC1). A variety less susceptible than MV, DG was separated from the tolerant varieties in the second principal component (PC2). Compounds with highest absolute loading values in PC1 were amino acids such as L-proline, L-serine, L-aspartic acid, L-glycine, and L-threonine; organic acid derivatives such as tetradecanoic acid, butanedioic acid, and hexadecanoic acid trimethyl-ester; and other compounds such as inositol and mannose (Fig. 2). Fig. 3 shows the abundance of the compounds that yielded significant differences ($P < 0.05$) among varieties after ANOVA. The most sensitive variety, MV, showed significantly higher levels of L-proline, L-serine, L-aspartic acid, butanedioic acid, tetradecanoic acid, and galactose oxime. The variety DG only

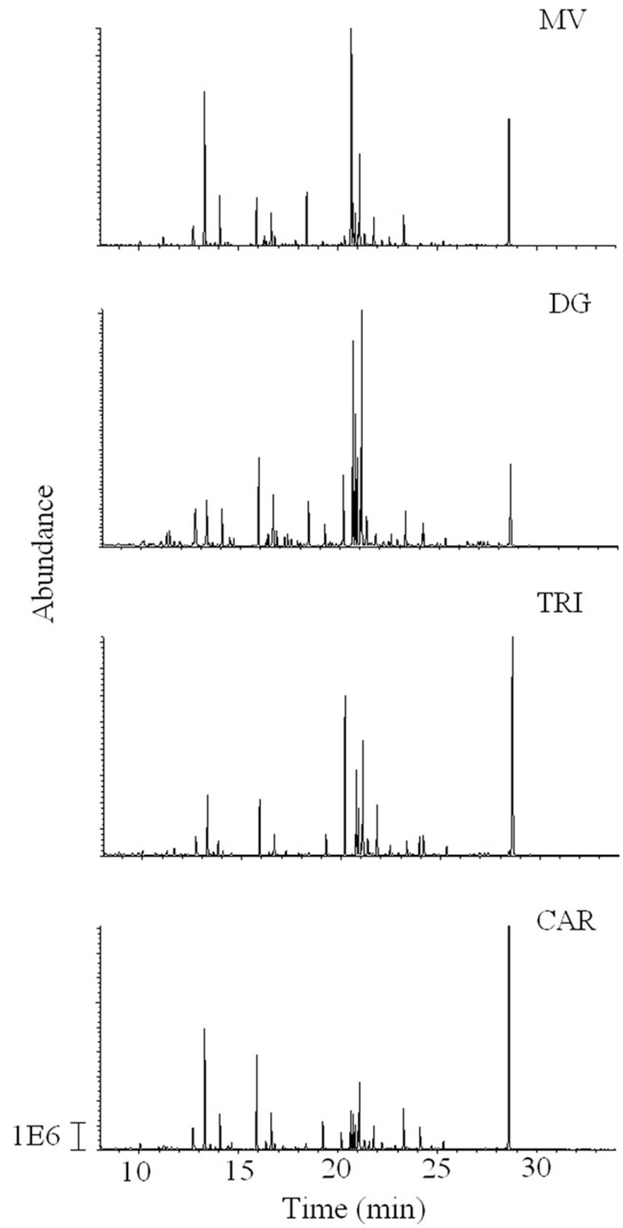

Fig. 1. Typical chromatograms of Madam Vinous' (MV), 'Duncan' grapefruit (DG), 'Trifoliate' (TR), and 'Carrizo' (CAR) orange.

presented significant differences in L-glycine, galactose, and mannose when compared with the tolerant varieties CAR and TR. L-glycine and mannose levels were significantly lower in MV and DG when compared to CAR and TR; whereas, galactose was in a significantly higher concentration in sensitive varieties when compared to the tolerant ones.

2.3. Changes in metabolite profile of sensitive varieties after inoculation with HLB

In this experiment, all varieties were preliminarily analyzed 14 weeks after inoculation. Only the susceptible varieties MV and DG showed significant differences in the metabolite profile and were selected for this part of the study. The complete metabolite profile obtained by GC–MS at 14 and 20 weeks after inoculation was used for PCA. Infected samples of MV were grouped by PCA at week 14 after inoculation (Fig. 4A), showing an improved grouping at week