Exploring Mechanisms of Citrus Rootstock Tolerance to HLB

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Florida citrus production is greatly affected by the devastating disease Huanglongbing (HLB) associated with the bacterial pathogen Candidatus Liberibacter asiaticus (CLas). Most commercial citrus scion cultivars are highly susceptible to HLB. However, tolerance to HLB has been identified within the species Poncirus trifoliata (trifoliate orange) and some of its hybrids which are commonly used as rootstocks. Using a rootstock tolerant to HLB does not completely protect a susceptible scion from HLB-induced decline, but even after infection with CLas, trees on some hybrid rootstocks grow much better and yield much more fruit than trees on other common rootstocks. In addition, recent field surveys of commercially grown Valencia trees in central and in southwest Florida revealed considerable differences in preharvest fruit drop depending on the rootstock used. Greenhouse studies showed that CLas populations are significantly lower in roots of psyllid-inoculated tolerant rootstocks compared with susceptible ones. We are currently exploring the mechanisms of HLB tolerance in selected rootstock cultivars using molecular and metabolomics approaches. Expression patterns of genes responding to HLB and generally associated with response to abiotic and biotic stress were considerably different depending on level of tolerance and susceptibility to HLB. Metabolomic analyses also revealed significant differences between rootstocks, although tolerance did not seem to be associated with a higher abundance of protective metabolites in response to infection. Instead, specific metabolic differences between the rootstocks prior to infection appeared to be associated with the level of tolerance to HLB. A summary of results from our ongoing studies is presented.

Citrus production in Florida is greatly affected by the devastating disease Huanglongbing (HLB), which is associated with Candidatus Liberibacter asiaticus (CLas), a phloem-limited bacterium transmitted by the Asian citrus psyllid Diaphorina citri. HLB is found in most citrus producing countries worldwide, where it causes tree decline and major reduction in fruit quality and yield. Although first visible signs of infection are an irregular blotchy mottling of the leaves and chlorosis resembling zinc- or other nutritional deficiencies, recent studies suggest that fibrous root damage occurs prior to foliar disease symptom expression (Johnson et al., 2014). Current management strategies are therefore directed toward root health and include improved irrigation and nutritional therapies.

Rootstock is a major factor in determining a tree’s ability to tolerate CLas, and methods are needed that assist in the selection of superior rootstock candidates prior to the long-term evaluation in the field. Genomics methodologies, such as high-throughput DNA sequencing and gene expression/microarray analysis are valuable tools which enable plant breeders to directly study the correlation between genotype and phenotype and improve and accelerate breeding efforts (Pérez-de-Castro et al., 2012). Metabolomics has also been used as a tool for breeders, since the metabolite composition not only depends on the genotype but also on the cultivar and the plant species (Fernie and Schauer, 2008; Krasensky and Jonak, 2012).

In the following, we present an overview of our ongoing efforts to characterize the response of different citrus rootstock cultivars to CLas and to explore molecular and physiological mechanisms associated with HLB tolerance.

Rootstock Tolerance to HLB

HLB affects all citrus species and relatives with little known resistance and most commercial scion cultivars are found to be highly susceptible (McCLean and Schwartz, 1970). Many of the commercial rootstock varieties in Florida are hybrids of trifoliate orange (P. trifoliata) and early studies by McCLean and Schwarz (1970) and Miyakawa (1980) did not observe any well-defined disease symptoms in trifoliate orange trees and seedlings. Later studies by Folimonova et al. (2009) obtained inconclusive results for P. trifoliata but characterized Carrizo citrange (C. sinensis...
Fig. 1. Average Ct Candidatus Liberibacter asiaticus values. Values were detected for leaves and roots of Citrus seedlings 4 1/2 years after growing under field conditions in St. Lucie County, FL. Real-time PCR was conducted according to Albrecht and Bowman (2012a).

x P. trifoliata) as tolerant to HLB. Our observations in 2009 on field-grown citrus trees at the U.S. Horticultural Research Laboratory (USHRL) research farm (St. Lucie County, Florida) with US-897 (C. reticulata ‘Cleopatra’ x P. trifoliata ‘Flying Dragon’) scion detected very few foliar abnormalities that resembled HLB disease symptoms in CLas-infected trees (Albrecht and Bowman, 2011). Even though 85% of trees were PCR positive for CLas at this time, infected trees were healthy and exhibited a full canopy. In addition, the number of seeds and seed weight did not differ in fruit from infected trees compared with non-infected trees. Our studies investigating HLB effects on seeds and seedling growth showed that reduced seed number and weight is one of the effects of HLB in susceptible cultivars (Albrecht and Bowman, 2009). Greenhouse studies using US-897 and one of its parents, ‘Cleopatra’ mandarin, confirmed field observations and demonstrated severe growth reduction in ‘Cleopatra’ seedlings in combination with expression of severe foliar disease symptoms (Albrecht and Bowman, 2011).

Studies were expanded and included the rootstock cultivars Benecke trifoliata (P. trifoliata), Carrizo citrange, US-802 (C. grandis ‘Siamese pummelo’ x P. trifoliata ‘Gotha Road’), US-812 (C. reticulata ‘Sunki’ x P. trifoliata ‘Benecke’), US-897, US-942 (C. reticulata ‘Sunki’ x P. trifoliata ‘Flying Dragon’), and Volkamer lemon (C. volkameriana). Based on the assessment of infection incidence, speed and intensity of disease symptom development, plant growth, and leaf bacterial titer levels under greenhouse conditions, rootstock cultivars were categorized as tolerant (US-897, US-942, ‘Carrizo’), moderately tolerant (US-802, US-812, Volkamer) and susceptible (‘Cleopatra’) to HLB (Albrecht and Bowman, 2012a). Field studies with natural inoculation were initiated in 2010 and seedlings of ‘Cleopatra’ mandarin, ‘Valencia’ sweet orange, ‘Rubidoux’ trifoliate orange (P. trifoliata), ‘Carrizo’ citrange, US-802, US-812, US-897, and US-942 were planted at the USHRL research farm in St. Lucie County, FL. Except for ‘Rubidoux’ and ‘Carrizo’, which had less than 36% incidence of infection, most seedlings were infected with CLas two years after planting (Albrecht and Bowman, unpublished). By 2015 all trees were PCR positive for CLas, but whereas the trifoliate hybrids US-812, US-897, and US-942 were visually unaffected by HLB, ‘Cleopatra’ and ‘Valencia’ were severely stunted and displayed chlorosis and blotchy mottling throughout most areas in the canopy. The visual assessment of the trifoliate rootstock cultivar Rubidoux was complicated due to its deciduous nature. Comparison of leaf bacterial titer levels 4 1/2 years after planting resulted in up to 60-fold lower levels for ‘Rubidoux’ and ‘Carrizo’ compared with ‘Cleopatra’ and ‘Valencia’. Root bacterial titer levels across all rootstock cultivars were 20-fold lower on average compared with levels in leaves, and lowest CLas levels were found in roots of US-942 (Fig. 1). Similarly, a greenhouse study with controlled psyllid inoculation detected 200 to 400-fold lower bacterial titer levels in US-942 roots compared with ‘Valencia’ roots six months after inoculation (Hall et al., 2016).

**Effect of Rootstock on Commercial Citrus Production**

A study was conducted to investigate how rootstock selection affects HLB disease development under natural conditions in the field (Albrecht et al., 2012a). The study included four trials in groves ranging from two to nine years in age with sweet orange (C. sinensis) scion on 15 different rootstocks, all located in St. Lucie County, FL. Although none of the rootstocks protected the susceptible scion from HLB-induced damage, tree performance was improved and tolerance to HLB increased by some rootstock cultivars. Results from a seven-year-old field trial conducted in Polk County, FL, and involving ‘Valencia’ orange on 17 rootstock selections suggested the use of a tolerant rootstock as an effective means of ameliorating crop losses to HLB (Bowman et al., 2016). This conclusion was based on large differences between rootstock for such metrics as yield, fruit quality, and trees size. Trees on US-942, and the recently released selection US-1516 (Bowman and McCollum, 2015) had significantly higher cumulative yield compared with the standard rootstocks ‘Swingle’ citrumelo (C. paradisi x P. trifoliata), ‘Carrizo’ citrange, Kuharske (C. sinensis x P. trifoliata), and ‘Cleopatra’ mandarin.

Recent years have shown that fruit loss induced by HLB is at least partially the result of preharvest fruit drop which has increased from 8% to 15% in the early years of HLB (production season 2007–08) to 22% to 25% for production season 2014–15. In order to investigate if preharvest fruit drop can be reduced by rootstock selection, a fruit count was conducted in two rootstock trials located in Southwest Florida (Collier County) and in Central Florida (Polk County; Bowman et al., 2016). Both trials are composed of ‘Valencia’ sweet orange and were estab-
Fig. 2. Percentage of Citrus fruit drop assessed in Feb. 2016, in a ‘Valencia’
rootstock trial planted in 2002 and located in Collier County, FL. Different
letters indicate significant ($P < 0.05$) differences between trees on different
rootstocks according to Tukey’s HSD test.

Only few genes were shown to be induced in leaves of tolerant
US-897 seedlings, several hundred genes were higher expressed in
this cultivar independent of CLas infection. Among these genes,
those encoding for constitutive disease resistance protein CDR1,
involved in the activation of salicylic acid-mediated resistance to
bacterial and fungal pathogens, and for different UDP-glucosyl
transferases, important for the storage of defense-related secondary
metabolites, were notable. Other studies were conducted including
and ‘Cleopatra’ (Bowman and Albrecht, 2015). These studies
investigated expression patterns of 21 genes selected based on
their association with salicylic acid- and jasmonic acid mediated
signaling pathways and other pathogen defense mechanisms.
Expression patterns were established for leaves and for roots
collected from different scion/rootstock combinations. Principal
component analysis (PCA) using mean ∆∆Ct values (Livak and
Schmittgen, 2001) demonstrated a clear grouping of samples
based on tissue type and rootstock cultivars (data not shown).
For leaves, samples from the susceptible cultivars Cleopatra and
Valencia separated clearly from the tolerant cultivars US-897
and US-942 and the moderately tolerant cultivar US-802 (Fig.
4). Samples from the same scion cultivar did not separate based
on rootstock. No separation of non-infected and infected leaf
samples was observed for US-802, US-897 or US-942, contrary
to ‘Cleopatra’ and ‘Valencia’ leaf samples which separated
noticeably depending on the state of infection. Similar to leaves,
PCA analysis of root samples resulted in separation of samples
based on HLB tolerance (Fig. 4). Unlike the leaf samples, root
samples from US-897 and US-942 formed separate clusters. No
clear separation between non-infected and infected root samples
was observed for any of the rootstock cultivars. Separation of
root samples based on the scion cultivar used for grafting was
observed for US-802, but not for ‘Cleopatra’. The results of this
study showed that expression profiling of genes commonly
associated with disease resistance allowed separation of cultivars
based on their response to HLB. However, tolerance did not seem
to be associated with higher expression of defense-related genes
in response to infection, but rather with a higher expression of
specific genes independent of infection.

Exploring mechanisms of tolerance using metabolomics

The levels of metabolites of a biological system can be
regarded as the ultimate response to genetic or environmental
changes (Fiehn, 2002) and study of the metabolome therefore
presents an ideal tool for plant breeders. A study was conducted
to compare leaf metabolite profiles of six greenhouse-grown
rootstock cultivars with different responses to HLB (Albrecht et al.,
2016). This study used untargeted gas chromatography-
time-of-flight mass-spectrometry (GC-TOF MS) methodology
and focused not only on known metabolites, but also on the large
group of chemical unknowns. The cultivars used in the study
were ‘Carrizo’ citrange, ‘Cleopatra’ mandarin, US-802, US-812,
US-897, and US-942. We identified 650 unique metabolites of
which 195 were identified by chemical structure. The number
of metabolites found to be differentially regulated in the infected
state compared with the non-infected state varied between the
cultivars and was largest (166) in the susceptible cultivar Cleopatra,
followed by Carrizo (94), US-812 (60) and US-802 (48).
Only three metabolites each were differentially regulated in the
tolerant cultivars US-897 and US-942 in response to infection.
Metabolites responding to infection in ‘Cleopatra’ included the
arginine pathway metabolites ornithine, citrulline and proline,
which are often reported to be associated with the response of plants to different types of abiotic and biotic stress (Alcázar et al., 2010; Mollayi et al., 2015). Proline was the only metabolite found in higher abundance in four (Cleopatra, Carrizo, US-802, US-812) of the six cultivars in response to infection. Proline is known for its function as osmoprotectant and antioxidant, and increasing evidence points to its role as signaling molecule and regulator of plant development (Szabados and Savouré, 2010).

The total number of metabolites that were significantly downregulated in the six citrus cultivars in response to infection with CLas was nearly 4-fold higher than the number of metabolites that were upregulated. In the susceptible cultivar Cleopatra, these included the sugars raffinose, glucose and fructose, but only at the later stages of infection. PCA analysis clearly separated samples into three groups. These were composed of 1) ‘Cleopatra’, 2) US-812, US-897, and US-942, and 3) US-802 and ‘Carrizo’ (Fig. 5). Many unidentified metabolites were found in considerable higher concentrations in some of the tolerant cultivars, especially US-897 and US-942, compared with ‘Cleopatra’, independent of infection. These compounds may play important roles in conferring tolerance to HLB and will be very valuable for selection of superior rootstock candidates in breeding programs. In addition, lower concentrations of raffinose, fructose, and glucose were found in the HLB-tolerant cultivars independent of infection. Duan et al. (2009) suggested that CLas is parasitic rather than pathogenic, with disease symptoms arising primarily as a result of host metabolic imbalances caused by nutrient depletion or interference of transportation. Results from research on the pathogenic mechanisms of a different group of phloem-limited pathogens, the cultivable spiroplasmas, suggest that they cause disease symptoms by depleting the phloem of specific sugar molecules (Firrao et al., 2007). Thus, it is possible that tolerance to HLB is at least partially associated with a lesser availability of specific carbohydrates in the host.

In conclusion, similar to the observation on gene expression, tolerance to HLB does not seem to be associated with accumulation of higher amounts of protective metabolites in response to infection. Instead, tolerance may be associated with different concentrations of specific metabolites independent of infection. This information may be utilized in breeding programs to preselect new rootstock and scion lines most likely to have HLB tolerance in advance of field testing.

**Literature Cited**


