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HLB-associated Preharvest Fruit Abscission Is Mediated by Jasmonate/Ethylene Signaling Triggered by Secondary Fungal Infection

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One symptom of citrus huanglongbing (HLB) is excessive preharvest fruit drop. Recently, a higher incidence of *Lasiodiplodia theobromae* (Diplodia) was found in HLB-symptomatic orange calyx abscission zones (AZ-C) than in non-symptomatic fruit. The infection was positively correlated with the reduction in fruit detachment force (FDF), suggesting that Diplodia infection may be involved in HLB-related preharvest fruit drop. To verify the direct correlation of HLB-related fruit drop with the fungal infection, HLB-affected sweet orange trees (Hamlin) were shaken manually during the harvest season. The fruit that dropped from trees upon shaking were collected (D), and the fruit that remained (R) on trees after shaking were harvested (R). Fruit ethylene production was measured, and the titers of Diplodia and *C*Las in AZ-C of D and R fruit were analyzed. Gene expression levels related to signaling and biosynthesis of phytohormone (jasmonate, ethylene, and abscission) were evaluated by qRT-PCR. The results indicate significantly higher Diplodia titers in D than in R fruit; more than half of D fruit but none of R fruit exhibited ethylene production, which was positively correlated with Diplodia titer in D fruit. Genes related to synthesis and signaling of ethylene (ET) and jasmonates (JA) were up-regulated, while abscisic acid (ABA) was down-regulated. The results suggest that different from water stress or carbon shortage induced abscission (which is characterized by induced abscisic acid), HLB-associated preharvest fruit abscission is mediated by JA/ET signaling, which was triggered by secondary fungal infections at the calyx abscission zone.

Citrus Huanglongbing (HLB, also known as citrus greening) is one of the most devastating diseases of citrus that has spread throughout the major citrus producing regions in Asia, Africa and the Americas, causing great losses for the citrus industry worldwide (Gottwald, 2010). Citrus HLB is associated with *Candidatus liberibacter* spp., which are Gram-negative, phloem-limited bacteria (Jagoeix et al., 1994). The Asian form of the disease is currently present in the U.S., and the causal pathogen, bacterium *Candidatus* Liberibacter asiaticus (*C*Las), was first confirmed in southeast Florida in 2005, and now is prevalent in all Florida citrus-growing areas. It has been reported that since 2006, HLB has cost Florida's economy an estimated $3.63 billion in lost revenues and 6611 jobs within five years (2006/07–2010/11) by reducing orange crop and juice production (Hodges and Spreen, 2012).

Symptoms for HLB include yellowing and an asymmetrical chlorosis of leaves, referred to as "blotchy mottle" (Bové, 2006). The yellowing may first appear on a single shoot or branch, and usually spreads throughout the tree over a year. Twig dieback, tree decline, and tree mortality occur several months to years after infection. Fruit symptoms include a reduced size, asymmetrical shape and a curved central core. Color development is poor and may only 'break' on the stem end, leaving the majority of the fruit surface green. A brown stain may be present in the fruit calyx abscission zone located at the pedicel–fruit interface (Bové, 2006). Many fruit abscise prematurely at the calyx abscission zone resulting in reduced yield. Yield reduction can reach 30% to 100% depending on the proportion of the canopy affected and the age of trees during infection (Bassanezi et al., 2011; Gottwald, 2010).

Understanding the mechanism for HLB-related fruit drop would help to develop an effective control strategy. Since excessive accumulation of starch, callose depositions and phloem plugging in leaves and stems, and a loss of fibrous roots were found in HLB diseased citrus trees, it has been proposed that the water or nutrient shortage due to the root loss/phloem plugging may be responsible for the excessive preharvest fruit drop, although evidence is lacking. The enhanced nutritional programs adopted by growers to reduce tree disease symptoms, unfortunately, did not affect fruit drop (Gottwald et al., 2012), while the attempts to increase root density and alleviate the fruit drop problem have not been proven to be significantly effective.

Recently, higher incidence of *Lasiodiplodia theobromae* (formerly known as *Diplodia natalensis*; hereafter termed Diplodia), an opportunistic fungal pathogen, was found in HLB-symptomatic orange calyx abscission zones (AZ-C) than in non-symptomatic fruit from non HLB-symptomatic trees (Zhao et al., 2015). Diplodia is the causal agent of stem end rot (SER) which is not typically a field problem, but is a common postharvest disease (Brown, 1986). Following infection of the calyx, Diplodia typically remains quiescent while the fruit is attached to the tree, and the fungus does not usually start to colonize the fruit tissue until after harvest (Brown and Wilson, 1967). However, the colonization of Diplodia was found in the HLB-affected orange fruit prior to harvest, and the infection was correlated with reduction in fruit detachment force (FDF), suggesting the possible involvement of Diplodia in the excessive fruit drop (Zhao et al., 2015). However, since HLB disease is also correlated to preharvest fruit drop, it is difficult to

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separate the effects of the two diseases. More evidence is needed to establish the role that Diplodia plays in the preharvest drop of HLB-affected citrus fruit.

In this study, we further investigate the role of Diplodia in fruit drop by investigation of fruit abscission. Diplodia and *C*Las levels in fruit AZ-C, as well as fruit ethylene production were compared between the fruit that dropped upon shaking the trees (D) and the fruit retained on trees after shaking (R), with an aim to determine the relationship of Diplodia infection and fruit drop. Since abscission signals are mediated by phytohormones, and it is known that biotic stress (as in fungal infection) and abiotic stress (such as water or nutrient shortage) trigger distinctly different patterns of hormone expression, gene expression in AZ-C was compared between the two types of fruit (D versus R) regarding induced hormones, to further confirm the roles of Diplodia infection in fruit drop.

Materials and Methods

Trees used in the abscission experiment. Six-year-old 'Hamlin' orange trees [*Citrus sinensis* (L.) Osbeck], about 2.5–3.0 m tall, on 'Swingle' citrumelo [*C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.] rootstock, in a commercial grove located in southern Florida were selected for the experiment. Trees were similar in size, all had tested *C*Las positive by qPCR (using the method of Li et al., 2006), were grown under similar agro-climatic conditions, received common cultural practices and the grower's standard pest and disease management. 'Hamlin' fruit were harvested on 1 Dec. 2014, and 5 Jan. 2015, with each sampling including nine trees. The ground under the trees was cleaned and covered with plastic cloth just before manually shaking the trees. Dropped fruit from the trees upon shaking (D) were collected and then retained fruit after shaking (R) were harvested. Thirty fruit were randomly picked from each of the D and R groups for ethylene measurement and DNA isolation; sixty fruit were randomly picked from each of the two groups and the AZ-C was excised using a #4 cork borer, then immediately frozen in liquid nitrogen and stored at –80 °C for RNA isolation.

Measurement of ethylene production. Ethylene production was determined by incubating individual fruit in 1-L glass jars which were sealed for 1 h. One ml of headspace gas was withdrawn from each jar using a gas tight syringe and analyzed for ethylene by gas chromatography (Hewlett-Packard 5890, Avondale, PA) equipped with a flame ionization detector and an activated alumina column.

DNA extraction. After ethylene production measurement, the fruit abscission zone was excised using a #4 cork borer and used for DNA extraction. DNA was extracted from 100 mg plant tissue using DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). The plant tissues were initially chopped and ground into powder in liquid nitrogen with a mortar and pestle, and then the manufacturer's isolation protocol was followed for the DNA isolation.

Real-time PCR for Diplodia and CLas detection. Real-time PCR amplifications were performed in a 7500 real-time PCR system (Applied Biosystems, Foster City, CA). For *C*Las detection, primers HLBasf and HLBr and probe HLBp were used targeting 16S rRNA genes of CLas (Li et al., 2006): HLBas (F): TCGAGCGCGTATGCAATACG; HLBr (R): GCGTTATCCCGTAGAAAAAGGTAG; HLBp (Probe): 6-FAM-AGACGGGTGAGTAACGCG-MGBNFQ. For Diplodia detection, specific primers targeting Diplodia β-tubulin gene (GenBank #DQ458858.1) were designed with software Primer Express 3.0.1. TB-F: ATGGCTCCGGTGTGTAAGTGT; TB-R: TGCTACAGGTCAGCGATTGC. PCR mixtures with a total volume of 15 μL contained 7.5 µl of TaqMan PCR master mix or SYBR Green PCR Master Mix (Applied Biosystems), 250 nM each primer, 150 nM probe (for *C*Las detection), and 100 ng of template DNA. The qPCR parameters are as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 seconds, and 60 °C for 1 min, with fluorescence signal capture at each stage of 60 °C. For the SYBR® Green Real-Time PCR reaction, the default Melt Curve (disassociation) stage is continued after the 40 cycles of PCR reaction. Cycle threshold (Ct) values are analyzed using ABI 7500 Software version 2.0.6 (Applied Biosystems, Inc., Carlsbad, CA) with a manually set threshold at 0.02 and automated baseline settings.

RNA extraction and qRT-PCR analysis for gene expression level evaluation. Total RNA was extracted from frozen AZ-C by RNeasy Plant Kit (Qiagen) following the manufacturer's instructions. DNA was removed by on column DNase treatment with RNase-free DNase Set (Qiagen). cDNA synthesis was performed using SuperScript® VILO™ cDNA Synthesis Kit (life technologies). Gene-specific primers were designed using software Primer Express 3.0.1 (Applied Biosystems). The primer sequences are listed in Table 1. Real-time PCR amplifications were performed in a 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The qPCR parameters were as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min, with fluorescence signal capture at each stage of 60 °C. The default Melt Curve (disassociation) stage is continued after the 40 cycles of PCR. Cycle threshold (Ct) values are analyzed using ABI 7500 Software version 2.0.6 (Applied Biosystems, Inc., Carlsbad, CA) with a manually set threshold at 0.05 and automated baseline settings. Relative fold differences were calculated based on the comparative Ct (threshold constant) method using Actin as an endogenous control. To determine relative fold differences for each sample, the Ct value for each gene was normalized to the Ct value for Actin and was calculated relative to a calibrator using the equation $2^{-\Delta\Delta Ct}$.

Statistical Analysis. SAS Version 9.3 (SAS Institute, Cart, NC) was used for analysis of data. One way ANOVA (PROC ANOVA) at the 95% ($P = 0.05$) confidence interval was used to determine statistical significance of differences in decay rates between D and R fruit AZ-C samples, where $P < 0.05$ was considered to be statistically significant.

Results and Discussion

Diplodia levels in abscission zone of D and R fruit, and correlation with fruit ethylene production. The *C*Las and Diplodia titers in fruit AZ-C, and fruit ethylene production were compared between D and R fruit. The results of qPCR analysis showed all the fruit were *C*Las positive, with Ct values ranging from 19.8 to 31.2 (Fig. 1, A1 and B1). Although the average *C*Las Ct values trended lower in D than in R fruit for the two batches of fruit, the difference in *C*Las Ct values was statistically significant only for the fruit harvested (harvested includes from the ground or from the tree after shaking) on 1 Dec. 2014 (Fig. 1, A1, $P < 0.05$). Diplodia Ct values were distributed in a wider range (18.5–40.0) than that of *C*Las, especially for D fruit (Fig.1A2 and B2). Diplodia Ct values in D fruit were significantly lower than in R fruit for both of the harvests (Fig. 1, A2, B2, $P < 0.0001$), indicating significantly higher Diplodia levels in D than in R

Table 1. Genes and their primers used in the qRT-PCR.

Gene	Primers
ERF1	TGAGGAAGCTGCTCTGGCTTA / GCCTTCGAGCCACGGATAT
ERF4	AAGCAGCACGTGCCTACGA / TTGGGAAGTTTGTCTTGGCTTT
ERF109	CGTGTCAGGTATGCGAAATCA / TCGCTGGGCGGGAAA
ACO	AGCCTGCATTTCTGGACAAAG / CCGCAGGAAGAGCAAAGAAC
OPR1	CGCAGCCACATGCAATTTT / CAGCAATGAGAAAGCCACCAT
JMT	GCCTGGACAAATACGCAAGAG / CGATTCTCCCACTGCCCTTA
LOX3	GGTGACATGACACAACCACCAT / AGTGGGATGTGCATGTGGTTATT
JAR1	ATGCGAAGTACCTGTCTGGAATC / CCCCTGCATAGTGCCTCAAC
ST2A	CCCCTGCTTACTTCCAATCCT / TTGGTTATTTGCATAGAGCTTGTATTC
PR4	CCAGGTCACAAACAGTGGTACAG / TTGGCACACTGATCAACAATTCT
DOX1	GCATTGGAGCTTTGGAATTATCC / GCCGTCCGTATTTTGAGGAA
PGIP1	AGCCTCACTGGCCCCATAC / CGTAGCGTCTTCAGGTTTTTGA
PDF1.4	AATGGCGGAAGCAAAAGTGT / CGGCCCCGACCATGT
PUB21	ACCGGAACAAGAGGTGCATT / CGCTTCCGCCAAAACACT
PUB24	GGCTCTTCCAGGGCAACAA / GCAAAGGCTGTTTGGTGACA
CHIB1	GCGACCCGACCAATAATGG / GCCCTGGCAAGTTTTTATTTCA
CZF1	GCTTCTGACGATATCTCTGCCTTT / GCTGGGCTCATCAACATCAA
BG1	TTTTCCTCTCTCAATACCCCTTCA / CATTAGCAGCCCAAGAAGAAACA
NCED3	AAACGGAGCCAACCCACTT / CCGTCTCCGTCGAAGAAATG
HVA22E	TTGTCAGACAGCAAATTAGGCAGTA / CCTTGCCAGTGCCAGTAGGA
JAZ3	TCCTCTGGATTTATGCCTTTCTCT / GCCATTCCACGCCTTTGAT

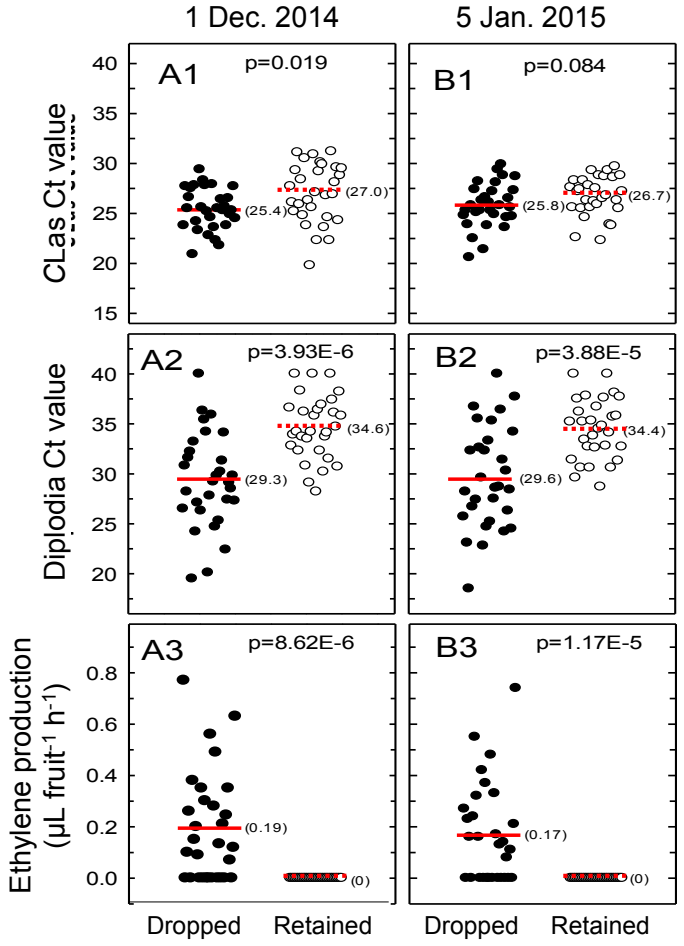

Fig. 1. CLas **(A1 and B1)** and Diplodia **(A2 and B2)** Ct values in the calyx abscission zone, and ethylene production **(A3 and B3)** of the fruit dropped upon shaking the trees (Dropped) or fruit retained after shaking the trees (Retained) of 'Hamlin' harvested on 1 Dec. 2014 and 5 Jan. 2015, n = 30. The filled black circles and the solid lines represent the fruit dropped upon shaking the trees (Dropped), while the open circles and the dotted lines represent the fruit retained after shaking the trees (Retained).

fruit. No ethylene production was detected in any of the R fruit from the two harvests under the methods used; however, ethylene production was detected in more than half of the D fruit (63% and 60% of the D fruit sampled on 1 Dec. 2014 and 5 Jan. 2015, respectively) (Fig. 1, A3 and B3). Statistical analysis indicates that ethylene production from D fruit was significantly higher than from R fruit for both harvests (Fig. 1, A3 and B3, $P < 0.0001$).

The correlation analysis was conducted between ethylene production and Ct values of Diplodia and *C*Las, respectively, for only D fruit, because no detectable ethylene was found for R fruit by the method used. The results indicated that there were negative linear correlations between ethylene production and Ct values of Diplodia and *C*Las in D fruit (Fig. 2, A1 and B1; A2 and B2), indicating positive correlations with Diplodia incidence and *C*Las. However, the correlations of ethylene production with Diplodia levels ($R^2 = 0.79 – 0.80$) were stronger than that with *C*Las ($R^2 = 0.53 – 0.66$) for both harvests (Fig. 2, A1 and B1; A2 and B2). These data suggest that fruit drop and ethylene production were more strongly connected with Diplodia levels in AZ-C than with *C*Las level.

Gene expression analysis in AZ-C of D fruit compared to R fruit. Abscission is often related to stress (biotic or abiotic), and abscission signals are mediated by phytohormones. In a general sense, hormones such as ethylene and jasmonic acid act as abscission-accelerating signals, and abscisic acid promotes abscission through the actions of ethylene. Genes related to signaling and biosynthesis of these abscission promoting hormones were analyzed by qRT-PCR. The results indicate that genes related to ethylene (ET) and jasmonate (JA) were up-regulated in dropped fruit compared to retained fruit, including ERF1, ERF4, ERF109, ACO, OPR1, JMT, LOX3, JAR1 (Fig.3). The up-regulation of JA involved key genes for JA biosynthesis (LOX, OPR), methylation of jasmonate into methyljasmonate (JMT), and the final step in the formation of the bioactive JA compound (JAR1); moreover, the key repressor of JA signaling, JAZ3 (Chini et al., 2007), was down-regulated in dropped fruit. The up-regulation of ET includes ACC oxidase genes (ACO) and several ethylene responsive factors (ERF1, ERF4, ERF109); among them, ERF1 is known to be the integrator of JA and ET signals (Lorenzo et

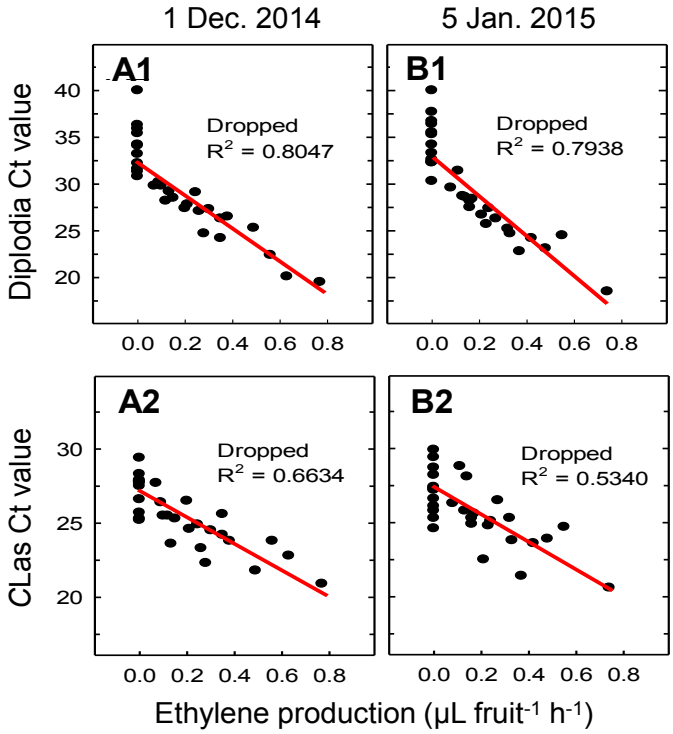

Fig. 2. The correlation between Diplodia Ct value and fruit ethylene production (**A1 and B1**), *C*Las Ct value and fruit ethylene production **(A2 and B2)** of the fruit dropped upon shaking the trees (Dropped) of 'Hamlin' sampled on 1 Dec. 2014 and 5 Jan. 2015, n = 30.

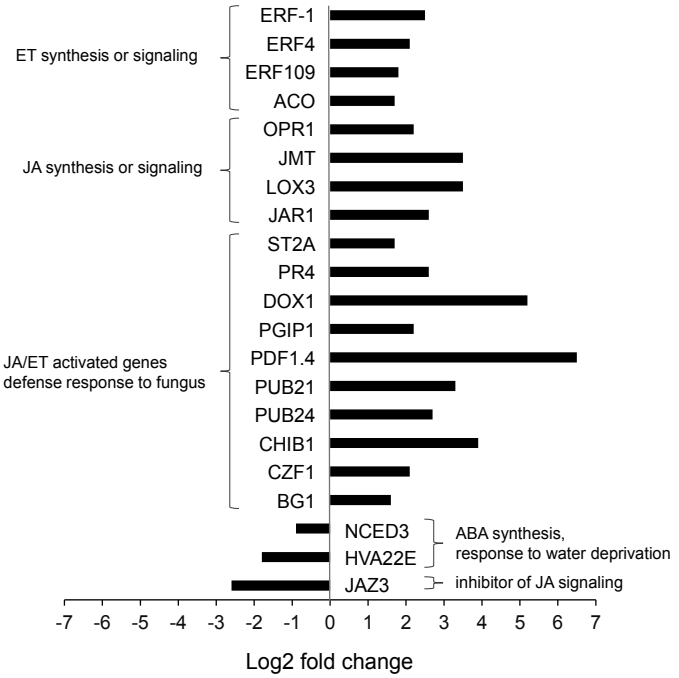

Fig. 3. Relative gene expression change in calyx abscission zone of dropped fruit vs. retained fruit.

al., 2003). ACO and ERF1 are both known to be induced by JA (Czapski and Saniewski, 1992; Lorenzo et al., 2003).

Ethylene (ET) is a key regulator of physiological processes in response to both biotic and abiotic stresses, and has long been recognized as a pivotal effector of abscission (Brown, 1997; Goren, 1993; Reid, 1985). Jasmonates (JA), including jasmonic acid and its cyclopentanone derivatives are well known as the central players of plant defense against necrotrophic fungi and herbivorous insects (Antico et al., 2012; Creelman and Mullet, 1997; Farmer et al., 2003; Vijayan et al., 1998). Classically, a combination of JA and ET signaling (JA/ET) activates plant defense response against necrotrophic pathogens (Penninckx et al., 1998; Pieterse et al., 2009). In such a plant defense response, JA and ET act synergistically, and the signaling of the two hormones have been linked through an AP-2 domain transcription factor called ethylene responsive factor 1 (ERF1) (Lorenzo et al., 2003). It has been reported that ERF1 expression is induced by fungal pathogen *Botrytis cinerea*, and constitutive expression of ERF1 in Arabidopsis confers resistance to several necrotrophic fungi (Berrocal-Lobo et al., 2002).

Consistent with the up-regulation of ET and JA synthesis and signaling, JA/ET activated defense genes that are known to be induced in response to fungal infection, were up-regulated in dropped fruit, including ST2A, PR4, DOX1, PGIP1, PDF1.4, PUB21, PUB24, CHIB1, CZF1, BG1. Among these genes, several genes are known to be induced exclusively by fungi, including plant defense PDF1.4, PGIP1 (polygalacturonase inhibiting protein 1) and BG1 (beta-1,3-glucanase 1).

The key gene for abscisic acid (ABA) biosynthesis (NCED3), and a downstream ABA-induced gene (HVA22E) were down-regulated (Fig. 3). In contrast to JA, ABA is widely recognized as the primarily hormonal signal of plant response to abiotic stress conditions, such as dehydration, cold temperatures, or nutrient shortage (Danquah et al., 2014; Gómez-Cadenas et al., 2000; Gómez-Cadenas et al., 1998; Tuteja, 2007). In citrus, ABA has been identified as the main modulator of response to water stress and carbon shortage, and consequently modulates the levels of ACC and ethylene, which activate plant organ abscission (Iglesias et al., 2007). Fruitlet abscission (June drop) caused by carbohydrate shortage (Gómez-Cadenas et al., 2000), and the abscission of fruit or leaves induced by water stress(Gómez-Cadenas et al., 2003; Gómez-Cadenas et al., 1998) were both associated with an increase in ABA levels. The hormone ABA accumulation was also found in water stressed vegetative and reproductive tissues of citrus (Agustí et al., 2007).

The data suggest that abiotic stresses could not be directly responsible for HLB-associated fruit drop, although phloem plugging and root loss were found in the HLB-affected citrus trees, which could cause carbohydrate shortage and water stress for the tree. Rather than the abiotic stress associated plant hormones, the hormone profile in the AZ-C of dropped fruit was similar to that of abscission caused by fungal infection. The infection of citrus petals with the fungus *Colletotrichum acutatum*, for example, causes a disease known as post-bloom fruit drop (PFD) characterized by necrotic brown lesions in petals and premature drop of young fruit (Zulfiqar et al., 1996). In PFD, ABA levels did not change, but PFD was associated with increased ethylene production in flowers, and increased levels of 12-oxo-PDA (a precursor for JA biosynthesis) and jasmonic acid (JA) in petals (Lahey et al., 2004).

Taken together, our results indicate that the significantly higher Diplodia levels in the calyx abscission zone of dropped

fruit compared to retained fruit, and fruit ethylene production in dropped fruit (that was positively correlated with Diplodia levels) accelerated fruit abscission. In agreement with this, gene expression data revealed an elevated JA/ET biosynthesis and signaling in the dropped fruit, as well as up-regulation of JA/ET downstream defense genes against fungal infection. The data consistently demonstrated that the excessive preharvest abscission of HLB-affected fruit was mediated by JA/ET signaling, which was triggered in response to the secondary fungal infection.

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