Sec-Delivered Effector 1 (SDE1) of ‘*Candidatus Liberibacter asiaticus*’ Promotes Citrus Huanglongbing

Kelley J. Clark,¹ Zhiqian Pang,² Jessica Trinh,¹ Nian Wang,^{2,†} and Wenbo Ma^{1,†}

¹ Department of Microbiology and Plant Pathology, University of California, Riverside, CA 92521, U.S.A.

² Citrus Research and Education Center, University of Florida, Lake Alfred, FL 33850, U.S.A.

Accepted 16 September 2020.

Sec-delivered effector 1 (SDE1) from the huanglongbing (HLB)-associated bacterium ‘*Candidatus Liberibacter asiaticus*’ was previously characterized as an inhibitor of defense-related, papain-like cysteine proteases in vitro and in planta. Here, we investigated the contributions of SDE1 to HLB progression. We found that SDE1 expression in the model plant *Arabidopsis thaliana* caused severe yellowing in mature leaves, reminiscent of both ‘*Ca. L. asiaticus*’ infection symptoms and accelerated leaf senescence. Induction of senescence signatures was also observed in the SDE1-expressing *A. thaliana* lines. These signatures were apparent in older leaves but not in seedlings, suggesting an age-associated effect. Furthermore, independent lines of transgenic *Citrus paradisi* (L.) Macfadyen (Duncan grapefruit) that express SDE1 exhibited hypersusceptibility to ‘*Ca. L. asiaticus*’. Similar to *A. thaliana*, transgenic citrus expressing SDE1 showed altered expression of senescence-associated genes, but only after infection with ‘*Ca. L. asiaticus*’. These findings suggest that SDE1 is a virulence factor that contributes to HLB progression, likely by inducing premature or accelerated senescence in citrus. This work provides new insight into HLB pathogenesis.

Keywords: bacterial pathogenesis, citrus greening, HLB, pathogen effectors, senescence

Huanglongbing (HLB) is currently the most devastating disease of citrus and has created unprecedented challenges to global citrus production (Bové 2006; da Graça et al. 2016; Gottwald 2010; Wang 2019). Continued spread of the disease and lack of genetic resistance in citrus cultivars has contributed to enormous loss in the citrus industry during the last decade. In order to properly manage the disease, genetic resources must be explored to develop tolerance and resistance in citrus. To achieve this, much needs to be elucidated on the pathogen virulence mechanisms and host responses during HLB progression.

K. J. Clark and Z. Pang contributed equally to this work.

[†]Corresponding authors: W. Ma; wenbo.ma@ucr.edu; and N. Wang; nianwang@ufl.edu

Funding: National Institute of Food and Agriculture grant number 2016-70016-24833.

*The e-Xtra logo stands for “electronic extra” and indicates that a supplementary table and supplementary materials are published online.

The author(s) declare no conflict of interest.

The most prevalent HLB-associated disease agent in major citrus-producing areas (i.e., Asia and the Americas) is ‘*Candidatus Liberibacter asiaticus*’ (Gasparoto et al. 2012; Wang 2019; Gottwald 2010). ‘*Ca. L. asiaticus*’ is a phloem-colonizing bacterium transmitted by the Asian citrus psyllid, *Diaphorina citri* (Bové 2006). Upon ‘*Ca. L. asiaticus*’ transmission, symptom progression in citrus is highly variable and can take anywhere from several months to years to develop in infected trees (Bové 2006; Coletta-Filho et al. 2014; Lee et al. 2015). Disease symptoms include yellowing of leaf veins and shoots, blotchy mottle, reduced fruit production, discolored or deformed fruit, premature fruit drop, tree stunting, off-season flowering, and dieback (Bové 2006; da Graça et al. 2016; Wang and Trivedi 2013). Fruit produced from diseased trees are often not viable for commercial purposes due to their altered appearance and off-tasting flavor (Dala-Paula et al. 2019). All citrus cultivars and several citrus relatives can be infected and very few varieties have been reported to be tolerant (Folimonova et al. 2009; Ramadugu et al. 2016; Stover et al. 2015).

‘*Ca. L. asiaticus*’ is a fastidious, Gram-negative bacterium (Jagoueix et al. 1994). Gram-negative bacterial pathogens possess secretion systems which deliver virulence proteins, known as effectors, to manipulate their hosts (Ma and Guttman 2008; Toruño et al. 2016; Uhse and Djamei 2018). By modulating host physiology and immunity, effectors promote pathogen colonization and disease development (Dou and Zhou 2012; Feng and Zhou 2012; Jones and Dangl 2006). Unlike many other Gram-negative bacterial pathogens, ‘*Ca. L. asiaticus*’ does not encode a type III secretion system, which functions as an injectosome to facilitate effector translocation into host cells (Duan et al. 2009). However, it does encode the general Sec secretion system which can secrete proteins from bacterial cells into their environment in a Sec translocon-dependent manner (Natale et al. 2008).

Previous research found that ‘*Ca. L. asiaticus*’ encodes several Sec-delivered effectors (SDEs), many of which are conserved across ‘*Ca. L. asiaticus*’ isolates (Pagliaccia et al. 2017; Thapa et al. 2020) and exhibit differential expression in ‘*Ca. L. asiaticus*’-infected citrus tissues relative to psyllids (Prasad et al. 2016; Thapa et al. 2020; Yan et al. 2013). Among these “core” SDEs, SDE1 (CLIBASIA_05315) is highly expressed in citrus, indicating a plausible role in ‘*Ca. L. asiaticus*’ colonization of citrus and HLB disease progression (Clark et al. 2018; Pagliaccia et al. 2017). SDE1 was previously shown to interact with citrus papain-like cysteine proteases (PLCPs) and inhibit their enzymatic activity in vitro and in planta (Clark et al. 2018). PLCPs are secreted proteases that regulate multiple processes in plants, including defense against microbial pathogens (Misas-Villamil et al. 2016); therefore, the

Copyright © 2020 The Author(s). This is an open access article distributed under the CC BY-NC-ND 4.0 International license.

PLCP inhibition activity of SDE1 is likely a virulence mechanism of ‘*Ca. L. asiaticus*’. In addition, transient expression of a truncated SDE1 protein in *Nicotiana benthamiana* was shown to induce chlorosis, suggesting that SDE1 may contribute to HLB development (Pitino et al. 2018).

HLB symptomatic leaves with chlorosis and blotchy motting are visually similar to leaves with nutritional deficiencies or to leaves entering a senescent state. Under low nutrient conditions or stress, plants may initiate senescence as a survival strategy to reallocate nutrients to young tissues (Killingbeck

Fig. 1. Sec-delivered effector 1 (SDE1) induces yellowing of mature leaves in *Arabidopsis thaliana*. **A**, Western blotting confirming SDE1 protein accumulation in two independent transgenic *Arabidopsis thaliana* ecotype Columbia (Col-0) lines after dexamethasone (Dex) induction. Coomassie brilliant blue (CBB) staining of the protein gel was used as a loading control. Numbers label individual leaves examined for SDE1 protein levels. The yellowing symptom of the same leaves is presented in **B**. **B**, SDE1 expression resulted in yellowing symptoms approximately 1 week after Dex treatment in adult plants. **C**, SDE1-induced yellowing symptoms are more severe in older leaves. All of the leaves in the 5-week-old plants were sprayed with Dex but yellowing symptoms first appeared on older leaves. Pictures were taken 5 days after Dex treatment. **D**, Yellowing symptoms progress as plants age. Pictures were taken 14 days after Dex treatment. Plants were approximately 8 weeks old. SDE1-2 and wild-type Col-0 plants are shown as representatives. The experiment was repeated at least three times with similar results.

1996). Interestingly, ‘*Ca. L. asiaticus*’ infection has been reported to cause changes in nutrient resorption in citrus (Cao et al. 2015) and nutrient supplementation was implemented to prolong tree productivity and reduce HLB symptoms (Pustika et al. 2008; Stansly et al. 2014). In addition, upregulation of senescence-associated genes (SAGs) has been observed in ‘*Ca. L. asiaticus*’-infected citrus. For example, senescence-related gene 1 (*SRG1*) exhibited higher expression in leaves of ‘*Ca. L. asiaticus*’-infected trees (Fu et al. 2016), and senescence 1 (*SEN1*) also showed a higher expression level in fruit after ‘*Ca. L. asiaticus*’ infection (Liao and Burns 2012). Interestingly, some PLCPs are involved in senescence. In *Arabidopsis thaliana*, SAG 12 (*AtSAG12*) encodes a PLCP and has been shown to aid in nitrogen allocation during senescence (James et al. 2018). Because SDE1 can inhibit the activity of PLCPs, it is possible that SDE1 could also affect the cellular senescence process.

Here, we report that SDE1 expression in planta is linked to a leaf yellowing symptom but only in mature leaves in *A. thaliana* or in ‘*Ca. L. asiaticus*’-infected citrus. This observation is concomitant with increased expression of SAGs in both plants. Importantly, transgenic citrus plants expressing SDE1 exhibit hypersusceptibility to ‘*Ca. L. asiaticus*’. These results suggest that SDE1 contributes to ‘*Ca. L. asiaticus*’ colonization and the development of leaf yellowing symptoms, possibly by promoting premature senescence in citrus.

RESULTS

SDE1 induces yellowing in mature *A. thaliana* leaves.

To investigate a link between SDE1 function and HLB progression, we generated transgenic *A. thaliana* ecotype Columbia (Col-0) that expresses the mature SDE1 proteins (i.e., without the predicted signal peptide amino acids 1 to 23). The *SDE1* gene is under the control of a dexamethasone (Dex)-inducible promoter (Aoyama and Chua 1997). Induction of SDE1 protein expression was confirmed in two independent transgenic lines (Fig. 1A; SDE1-2 and SDE1-5). In these two lines, expression of SDE1 was observed in mature leaves of adult plants starting from 24 h after Dex application and throughout the 2-week

period the experiments were performed (Supplementary Fig. S1). The line SDE1-3 did not show SDE1 protein accumulation after Dex induction in mature leaves and was used as a control in addition to wild-type Col-0. Approximately 1 week after Dex induction, yellowing symptoms started to appear in leaves of SDE1-2 and SDE1-5 but not SDE1-3 (Fig. 1B). Strong yellowing phenotypes correlated with SDE1 protein accumulation levels, as shown in the corresponding Western blotting on individual leaves with and without Dex induction (Fig. 1A and B). The yellowing symptoms were mainly observed in mature leaves (Fig. 1C) and became more severe as plants aged (Fig. 1D). Because leaf yellowing did not develop until 1 week after SDE1 protein accumulation and the symptom severity was increased in older plants, these results indicated that the leaf yellowing phenotype might be related to senescence.

SDE1 expression initiates cellular senescence in mature leaves of *A. thaliana*.

To determine whether SDE1-induced leaf yellowing in transgenic *A. thaliana* was related to induction of cellular senescence, we measured the expression of *AtSEN1*, a widely used senescence marker gene, using quantitative reverse-transcription PCR (qRT-PCR). *AtSEN1* exhibited increased gene expression in the mature leaves of two independent SDE1-expressing lines (SDE1-2 and SDE1-5) at 24 h after Dex induction (Fig. 2A). Induction of *AtSEN1* was also observed at 30 h after Dex induction, although to a lesser extent (Fig. 2A). It should be noted that the yellowing symptoms did not appear at this time; therefore, induction of *AtSEN1* predates the yellowing phenotype development and likely indicates the initiation of a senescence process in plant cells. SDE1-3, which did not have SDE1 protein accumulation (Fig. 2B) or exhibit yellowing in mature leaves (Fig. 1B and C), did not show increased expression of *AtSEN1* (Fig. 2A). Wild-type Col-0 plants kept in the dark for 48 h were used as a positive control (Fig. 2A) because high *AtSEN1* expression levels have been observed in dark-treated leaves (Schenk et al. 2005).

Generation of reactive oxygen species (ROS) is another molecular signature associated with senescence (Rogers and

Fig. 2. *AtSEN1* is induced in mature sec-delivered effector 1 (SDE1)-expressing *Arabidopsis thaliana*. **A**, Relative gene expression of *AtSEN1* in leaves of dexamethasone (Dex)-treated SDE1-transgenic plants compared with ecotype Columbia (Col-0) (wild-type) at 24 and 30 h posttreatment was determined by quantitative reverse-transcription PCR (qRT-PCR). Leaves from dark-treated Col-0 plants were used as a positive control for the *AtSEN1* induction. Error bars represent standard deviation of two biological repeats. *P* values were calculated by a two-tailed Student's *t* test. **B**, Western blotting showing SDE1 protein accumulation in leaves used for the qRT-PCR analysis. Coomassie brilliant blue (CBB) staining of the protein gel was used as a loading control. The experiment was repeated twice with similar results.

Munné-Bosch 2016; Woo et al. 2013). We next examined ROS accumulation in the mature leaves of the transgenic *A. thaliana* plants using 3,3'-diaminobenzidine (DAB) staining. When oxidized by hydrogen peroxide (H_2O_2), DAB forms brown precipitates, which can be visualized in decolorized leaves (Daudi and O'Brien 2012). The transgenic lines SDE1-2 and SDE1-5 accumulated these precipitates, representing physical presence and localization of H_2O_2 in the leaves, while line SDE1-3 did not exhibit ROS accumulation, similar to wild-type (Col-0) plants (Fig. 3). Formation of DAB precipitates could be seen as early as 1 and 4 days after Dex treatment (Fig. 3B), before the appearance of the yellowing phenotype in leaves (Fig. 3A). Increased with time, stronger staining was observed at 10 and 14 days after Dex treatment compared with earlier time points. Precipitates were not observed or observed at a low level in leaves of Col-0 or SDE1-3 control plants (Fig. 3B)—note that H_2O_2 is represented by brown-colored punctate spots, not overall coloring of the leaves as seen in some wild-type leaves. DAB precipitates also correlated with leaves that eventually exhibited yellowing symptoms, as seen in SDE1-2 and SDE1-5 at 10 and 14 days after Dex treatment (Fig. 3A). These results indicate that ROS accumulation can be triggered by SDE1 and links to the leaf yellowing phenotype.

SDE1 does not induce yellowing or senescence signatures in *A. thaliana* seedlings.

Because the yellowing symptoms were prominent in mature leaves, we next examined whether SDE1 had any effect on *A. thaliana* seedlings. For this purpose, we germinated *A. thaliana* transgenic seedlings on Murashige and Skoog (MS)

medium plates and, at 1 week postgermination, we exposed them to Dex to induce SDE1 expression. As in the mature plants, we also examined the three independent transgenic lines SDE1-2, SDE1-3, and SDE1-5. Unlike mature plants, SDE1-3 had SDE1 protein accumulation (Fig. 4C); therefore, wild-type Col-0 seedlings served as the control. Interestingly, SDE1-expressing seedlings did not exhibit leaf yellowing (Fig. 4A), further confirming that SDE1, by itself, is not sufficient for triggering the development of this senescence-like symptom.

To assess *AtSEN1* gene expression, the seedlings were collected at 24 h after Dex treatment and subjected to qRT-PCR. In contrast to adult plants, *AtSEN1* expression was not induced in seedlings (Fig. 4B). In addition, DAB staining showed that ROS levels remained the same in the SDE1-expressing lines at 1, 4, and 10 days after Dex treatment when compared with Col-0 seedlings (Fig. 4D). These results are consistent with the hypothesis that an age-dependent factor may be needed for SDE1-triggered induction of *AtSEN1*.

SDE1 promotes '*Ca. L. asiaticus*' infection and enhances HLB symptoms in citrus.

Previously, we generated SDE1 transgenic citrus in the *Citrus paradisi* (L.) Macfadyen (Duncan grapefruit) background and showed that the overall PLCP activities were reduced in these transgenic plants (Clark et al. 2018). To determine the susceptibility of SDE1 transgenic citrus to '*Ca. L. asiaticus*', five independent SDE1-expressing citrus lines (2 years old) were graft inoculated using buds from '*Ca. L. asiaticus*'-infected Valencia sweet orange plants. As a control group, five

Fig. 3. Sec-delivered effector 1 (SDE1) induces reactive oxygen species (ROS) accumulation prior to yellowing symptoms in *Arabidopsis thaliana* leaves. **A**, Yellowing symptom development in SDE1-expressing leaves at 1, 4, 10, and 14 days after dexamethasone treatment. Wild-type Columbia (Col-0) plants were used as controls. **B**, ROS levels measured by 3,3'-diaminobenzidine staining in the leaves. Representative leaves are presented to show punctate, brown precipitates representing hydrogen peroxide accumulation. Four leaves per treatment were used in each experiment and the experiment was repeated twice with similar results. Please note the absence of SDE1 protein accumulation in SDE1-3, which was used as an additional control in this experiment.

Fig. 4. Sec-delivered effector 1 (SDE1) does not induce leaf yellowing or senescence signatures in *Arabidopsis thaliana* seedlings. **A**, SDE1-induced leaf yellowing symptoms were not observed in SDE1-transgenic *A. thaliana* seedlings. Seedlings pictured were approximately 2 weeks old and 1 week after dexamethasone (Dex) treatment. **B**, SDE1-transgenic seedlings did not exhibit increased *AtSEN1* gene expression. Relative gene expression of *AtSEN1* in SDE1-transgenic seedlings compared with ecotype Columbia (Col-0) (wild-type) at 24 h after Dex treatment was determined by quantitative reverse-transcription PCR (qRT-PCR). Approximately 20 seedlings were pooled for each qRT-PCR analysis; "n.s." indicates that data are not significantly different based on analysis of variance with $P < 0.05$. **C**, Western blotting confirmed the accumulation of SDE1 proteins in seedlings at 1 and 7 days after Dex treatment. Coomassie brilliant blue (CBB) staining of the protein gel was used as a loading control. Please note that the line SDE1-3 did accumulate SDE1 proteins in seedlings, unlike in mature plants. Wild-type Col-0 was used as the control in this experiment. **D**, SDE1 expression in seedlings did not promote ROS accumulation. Seedlings were stained with 3,3'-diaminobenzidine at 1, 4, and 10 days after Dex treatment. Four to five leaves from at least three different seedlings per treatment were used in each experiment and the experiment was repeated twice with similar results. Images of representative leaves are presented.

wild-type Duncan grapefruit plants at the same age were also graft inoculated. The expression of SDE1 in the transgenic lines was confirmed by Western blotting (Supplementary Fig. S2). Quantitative PCR (qPCR) was used to monitor ‘*Ca. L. asiaticus*’ titers monthly (Fig. 5A). Comparison of the cycle threshold (Ct) values found that *SDE1*-expressing plants were more susceptible to ‘*Ca. L. asiaticus*’ than the control plants throughout a 5-month period, and the difference was especially significant at 3 and 5 months post-inoculation (MPI). ‘*Ca. L. asiaticus*’ became detectable earlier in the transgenic plants and the bacterial titers were higher (Fig. 5A). These results strongly suggest that SDE1 promotes ‘*Ca. L. asiaticus*’ colonization and proliferation in citrus.

Line SDE1-8, which was previously shown to exhibit a largely reduced total PLCP activity, presumably due to an inhibitory effect of SDE1 (Clark et al. 2018), allowed particularly high ‘*Ca. L. asiaticus*’ titers and developed yellowing symptoms on leaves as early as at 1.5 MPI, while other plants remained asymptomatic (Fig. 5B). ‘*Ca. L. asiaticus*’ was detectable in SDE1-8 at 1 MPI and the plants died at 4 MPI. Prior to ‘*Ca. L. asiaticus*’ infection, no yellowing symptoms or other developmental abnormalities were observed in SDE1-8 (Fig. 5B). The other ‘*Ca. L. asiaticus*’-infected SDE1 transgenic lines and some of the control plants began to exhibit leaf yellowing symptoms at 2 MPI. These results suggested that additional stress resulting from ‘*Ca. L. asiaticus*’ infection was required for the accelerated disease progression observed in the *SDE1*-expressing citrus.

Fig. 5. Sec-delivered effector 1 (*SDE1*) transgenic citrus is hypersusceptible to ‘*Candidatus Liberibacter asiaticus*’. **A**, ‘*Ca. L. asiaticus*’ bacterial titer measured by quantitative PCR during a 5-month period after graft inoculation in *SDE1* transgenic citrus lines and wild-type plants as the controls. *SDE1*-expressing transgenic citrus were generated in the *Citrus paradisi* (L.) Macfadyen (Duncan grapefruit) background. Wild-type Duncan grapefruit plant of the same age were used as controls. MPI = months postinoculation. In the box plot, samples with no amplification (N/A) are given an arbitrary cycle threshold (Ct) value of 40 to allow statistical analysis. Dots represent outliers. An asterisk (*) represents significant *P* values based on two-tailed Student’s *t* test (*P* < 0.05). For both control and *SDE1* groups, *n* = 5, with the exception of 4 MPI and 5 MPI for the *SDE1* group (*n* = 4) due to the death of *SDE1*-8. In the table, ± represents the variation in Ct values of three replicates and N/A = no amplification. **B**, *SDE1*-8 exhibited leaf yellowing at 1.5 MPI with ‘*Ca. L. asiaticus*’, while control plants remained asymptomatic. Control-4 is presented here as a representative. Size bars represent 1 cm.

SDE1 expression in citrus alters the expression of SAGs during 'Ca. L. asiaticus' infection.

To profile SAG expression in citrus, we utilized NanoString nCounter Elements technologies (hereafter NanoString) to directly measure the transcript quantity of target genes. The NanoString technology eliminates cDNA synthesis, amplification, and library preparation required for RNA sequencing or qRT-PCR; thus, it reduces technical variations and can be more accurate and sensitive when detecting gene expression changes.

Gene-specific probes were designed to detect a subset of SAGs predicted from citrus (*C. sinensis* v1.1 and *C. clementina* v1.0) based on their sequence similarity to corresponding *A. thaliana* homologs (Supplementary Tables S2 and S3). The transgenic citrus lines SDE1-8 and SDE1-9 and the control plants control-4 and control-5 were used to quantify transcript levels of these putative SAGs.

Our results showed that, with 'Ca. L. asiaticus' infection or SDE1 expression alone, no significant expression changes were

Fig. 6. Senescence-associated genes (SAGs) are induced by sec-delivered effector 1 (SDE1) during 'Candidatus Liberibacter asiaticus' (CLAs) infection in citrus. **A**, Gene expression changes detected by NanoString. Columns 1 to 5 in the chart (left) represent pairwise comparisons of SDE1 transgenic citrus lines (SDE1-8 and SDE1-9) and wild-type Duncan grapefruit plants (Control-4 and Control-5) with and without 'Ca. L. asiaticus' infection at 2 months post-inoculation (MPI). All values are presented as log₂ fold-change. The bar graph (right) was generated using the data in column 5. Significant differences are indicated with an asterisk (*) if $P < 0.05$ and a caret (^) if $P < 0.1$ based on a Student's two-tailed t test. **B**, Quantitative reverse-transcription PCR (qRT-PCR) analysis of selected SAGs at 12 MPI. Data are presented as log₂ fold-change of the 'Ca. L. asiaticus'-infected SDE1 transgenic citrus (SDE1-9 and SDE1-12) versus wild-type Duncan grapefruit (control). We used SDE1-12 to replace SDE1-8, which died after 4 MPI. **C**, Relative expression of SAGs detected by NanoString at 2 MPI (A) positively correlates with results from qRT-PCR at 12 MPI (B).

observed in the SAGs (Fig. 6A, columns 1 and 2). However, we observed a general trend of increased expression in these genes when both ‘*Ca. L. asiaticus*’ infection and SDE1 expression were present (Fig. 6A, columns 3 to 5). These results are consistent with the leaf yellowing symptoms observed in line SDE1-8 only after ‘*Ca. L. asiaticus*’ infection (Fig. 5B) and suggest that a synergistic effect is needed for the onset of a premature senescence process in citrus. When comparing ‘*Ca. L. asiaticus*’-infected SDE1 transgenic citrus to noninfected SDE1 transgenic citrus (Fig. 6A, column 3), ‘*Ca. L. asiaticus*’-infected control plants (Fig. 6A, column 4), and noninfected control plants (Fig. 6A, column 5), we found several SAGs that were significantly upregulated. These genes include *SAG12-1_v1*, *orange1.lg019063m*, *orange1.lg018958m*, *orange1.lg017548m*, and *orange1.lg036910m*, which encode PLCPs that belong to various subfamilies (Fig. 6A; Supplementary Table S2). These PLCPs have been shown to participate in senescence in *A. thaliana*. In addition, *orange1.lg018950m* (*ORE1*), encoding a transcription factor that positively regulates aging-induced cell death and senescence in *A. thaliana* leaves, was also upregulated in ‘*Ca. L. asiaticus*’-infected SDE1 transgenic citrus. Although not statistically significant, *orange1.lg045260m* (*SRG1B*) also exhibited increased gene expression in ‘*Ca. L. asiaticus*’-infected SDE1 transgenic citrus (Fig. 6A). *SRG1B* encodes an oxidoreductase and was previously shown to be upregulated in ‘*Ca. L. asiaticus*’- and citrus tristeza virus-infected citrus (Fu et al. 2016).

To confirm the gene expression changes detected by NanoString and to monitor gene expression changes triggered by SDE1 accumulation at a later infection stage by ‘*Ca. L. asiaticus*’, we conducted qRT-PCR analysis of six putative citrus SAGs using samples at 1 year postinoculation. Because line SDE1-8 had died at 4 MPI, we used another SDE1 transgenic line, SDE1-12, in addition to SDE1-9 for this experiment. Similar to the data obtained at 2 MPI from NanoString, we observed increased expression of these six genes (Fig. 6B). Furthermore, results from NanoString and qRT-PCR appear to have a strong positive correlation, with an R^2 value of 0.9616 (Fig. 6C). These results indicate a clear agreement between the two methods and suggest potential long-term effects of SDE1 on citrus gene expression during ‘*Ca. L. asiaticus*’ infection.

DISCUSSION

Citrus HLB has been a prominent threat to the citrus industry and understanding the mechanisms underlying pathogenesis is pertinent for the development of control strategies. In particular, there is a significant knowledge gap regarding virulence strategies employed by ‘*Ca. L. asiaticus*’ during phloem colonization and HLB disease progression. Here, we explored the function of the ‘*Ca. L. asiaticus*’ effector, SDE1, in citrus. We demonstrated that SDE1 promotes ‘*Ca. L. asiaticus*’ colonization or proliferation and may contribute to the development of an HLB-like symptom (i.e., leaf yellowing), likely through an early or accelerated induction of host senescence.

Adult *A. thaliana* plants expressing SDE1 exhibit severe leaf yellowing reminiscent of HLB symptoms and leaf senescence (Fig. 1). This SDE1-induced yellowing becomes more severe when leaves age, suggesting the possibility that it occurs in a senescence-associated manner. Indeed, senescence signatures, including upregulation of a senescence marker gene and ROS formation, were enhanced with SDE1 expression (Figs. 2 and 3). Importantly, age dependency was part of this response because senescence indicators were absent in seedlings (Fig. 4).

The onset of leaf senescence is often associated with changes in leaf color and is known to require internal signals based on developmental stages (Lim et al. 2007; Woo et al. 2013). During late-stage senescence, major organelles are digested,

releasing a large amount of nitrogen that can be mobilized throughout the plant (Bieker and Zentgraf 2013; Ishida et al. 2014). Digestion of chloroplasts and loss of chlorophylls is behind senescence-related color changes in leaves (Lim et al. 2007). A previous report on SDE1-induced yellowing via transient expression of a truncated form of SDE1 in *N. benthamiana* found a reduction in total chlorophylls and downregulation of chlorophyll synthesis genes (Pitino et al. 2018). Although the authors suggested starch accumulation as the major contributor to the chloroplast breakdown they observed, the chloroplast breakdown in general is consistent with an effect of SDE1 expression on promoting leaf yellowing and senescence in *Arabidopsis*.

In citrus, SDE1 expression promoted ‘*Ca. L. asiaticus*’ colonization or growth. In addition, we observed increased expression of some SAGs in SDE1-expressing citrus only when the plants were infected with ‘*Ca. L. asiaticus*’ (Fig. 6). Accelerated leaf yellowing symptoms were also observed in one of the SDE1-expressing lines but also only occurred when the plants were infected with ‘*Ca. L. asiaticus*’. These results suggest that SDE1 promotes ‘*Ca. L. asiaticus*’ colonization and may contribute to HLB symptom development but cannot accomplish it by itself (Fig. 5B). SDE1 may synergistically function with other virulence factors of ‘*Ca. L. asiaticus*’ to manipulate the cellular senescence program in citrus and facilitate HLB progression. Recently, two other ‘*Ca. L. asiaticus*’ SDEs were found to be associated with necrosis when expressed in *N. benthamiana* (Li et al. 2020), indicating that multiple SDEs have the potential to manipulate host senescence. Upregulation of *SRG1*, senescence-associated WRKY transcription factors (Fu et al. 2016), and the dark-inducible *SEN1* has previously been observed in ‘*Ca. L. asiaticus*’-infected citrus (Liao and Burns 2012). We found that SDE1 expression and ‘*Ca. L. asiaticus*’ infection are both required to induce SAG expression (Fig. 6), further confirming that SDE1 accelerates or enhances a senescence-related cellular process together with another signal, such as aging or ‘*Ca. L. asiaticus*’ infection. It should be noted that these experiments were performed via overexpression of SDE1 in plant tissues (i.e., with a constitutive promoter in citrus and an inducible promoter in *A. thaliana*). Future research is necessary to confirm SDE1-induced leaf yellowing using phloem-specific promoters or bacterial-mediated delivery.

Because senescence is accompanied by remobilization or release of nutrients (Thomas 2013; Woo et al. 2013), a possible benefit of its premature initiation for ‘*Ca. L. asiaticus*’ is to rapidly bring nutrients to the bacterial cells in aging leaves or leaves dying due to pathogen infection or lack of nutrient supply as a consequence of phloem clogging or damaging. Nutrient release from leaf cells undergoing accelerated senescence could facilitate bacterial growth, presumably leading to the increased ‘*Ca. L. asiaticus*’ titers observed in SDE1 transgenic citrus (Fig. 5A). An accelerated senescence could also contribute to the development of leaf yellowing symptoms, which might be a ploy to attract insect vectors, further disseminating ‘*Ca. L. asiaticus*’ propagules (Hall et al. 2010; Paris et al. 2017; Sathe et al. 2015). Although we propose senescence as a possible mechanism underpinning HLB progression, including the characteristic leaf yellowing symptoms, it does not exclude the conventionally noted mechanism, which involves phloem clogging and starch accumulation (Etxeberria et al. 2009; Folimonova and Achor 2010; Koh et al. 2012). Further investigations are needed to elucidate whether these cellular processes could modulate HLB development in concert or in an independent manner.

SDE1 was previously found to interact with several PLCPs in citrus and can inhibit the protease activity of some of these

PLCPs (Clark et al. 2018). PLCPs can be classified into several subfamilies with members involved in a broad range of cellular processes, including senescence (Roberts et al. 2012). Some of these senescence-associated PLCPs exhibited higher expression levels in SDE1 transgenic citrus infected with ‘*Ca. L. asiaticus*’ (Fig. 6). These include members of the *SAG12* subfamily, which has been shown to aid in nitrogen allocation during senescence (James et al. 2018), and the *AALP* and *RD21* subfamilies, which were found to contribute to dark-induced senescence in *A. thaliana* (Pružinská et al. 2017). Although it may seem contradictory that SDE1 triggers the expression of some *PLCP* genes while it could also inhibit their enzymatic activity, it is important to note that PLCPs have diverse functions and individual proteins play different roles in distinct processes. Many PLCPs have specific subcellular localization and expression patterns that are required for their function (Richau et al. 2012; van der Hoorn 2008) and, at the same time, may limit their potential interaction with SDE1 or other virulence proteins of ‘*Ca. L. asiaticus*’. For example, *AtSAG12* is reported to be confined to senescence-associated vacuoles (Otegui et al. 2005). In addition, many *PLCP* genes are clearly not induced by SDE1 expression in citrus. It should also be noted that most of the *SAGs* studied here were selected based on the role of their closest homologs in *A. thaliana*; the same functions may not translate to citrus. This could explain why some of the *SAGs* we analyzed did not exhibit expression changes in SDE1 transgenic or ‘*Ca. L. asiaticus*’-infected citrus. In summary, these findings offer new insight into how a ‘*Ca. L. asiaticus*’ effector may contribute to the development of HLB symptoms. Further investigations on premature senescence as a possible cellular event that is manipulated by ‘*Ca. L. asiaticus*’ to enhance bacterial colonization and disease progression may provide novel perspectives in managing HLB.

MATERIALS AND METHODS

Generation of SDE1-expressing transgenic *Arabidopsis* and growth conditions.

Seed of *A. thaliana* Col-0 and transgenic lines were sown in soil and vernalized at 4°C for 2 days. The plants were then grown in a conditioned growth room at 22°C with a daily regime of 12 h of light and 12 h of darkness for 4 to 6 weeks. To generate SDE1-expressing lines, a DNA sequence encoding the mature SDE1-secreted protein (with the N-terminal signal peptide sequence of 1 to 23 amino acids removed) was fused with an N-terminal 3xFLAG tag and cloned into the binary vector pTA7002 with a Dex-inducible promoter (Aoyama and Chua 1997). The recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101, which was subsequently used to transform Col-0 plants using the floral dip method (Clough and Bent 1998). Plants carrying the transgene were selected on MS-hygromycin plates using a screening method described by Harrison et al. (2006).

Dex induction of SDE1 expression.

An induction solution containing 40 µM Dex (Sigma-Aldrich, St. Louis, MO, U.S.A.) solution and 0.05% Silwet L-77 (LEHLE SEEDS, Round Rock, TX, U.S.A.) was applied to *A. thaliana* leaves from 4-week-old plants or 1-week-old seedlings by spraying or swabbing with Q-tips. Several leaves across three to five different plants were Dex induced for use in the different experiments throughout this study. For experiments using *A. thaliana* seedlings, SDE1 induction was administered in one of three methods: (i) Dex solution was sprayed onto MS plates with germinated seedlings, (ii) seed were directly germinated on MS medium containing 40 µM Dex, or (iii) seed were germinated on MS medium and then

transferred to MS medium containing Dex. All methods resulted in SDE1 protein expression.

Protein extraction and Western blotting.

Leaf tissue was ground into a powder in liquid nitrogen and suspended in 2× Laemmli buffer (Laemmli 1970). Samples were boiled for 5 min and centrifuged to separate leaf debris from aqueous proteins. The soluble portion was then run on a 12% polyacrylamide gel via sodium dodecyl sulfate polyacrylamide gel electrophoresis; and the proteins were transferred from the gel to polyvinylidene difluoride membrane (GE Healthcare, Chicago, IL, U.S.A.) for Western blotting. For Western blotting, membranes were blocked in 5% nonfat skim milk solution, then incubated with anti-FLAG-HRP antibodies at a dilution of 1:2,000 (Sigma-Aldrich). Before signal development, unbound antibody was washed off membranes with 1× Tris-buffered saline (pH 7.4) with 0.1% Tween-20 buffer. Signals were detected using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, U.S.A.).

DAB staining of *A. thaliana* leaves.

DAB (Alfa Aesar, Haverhill, MA, U.S.A.) solution was prepared fresh for each experiment according to the protocol described by Daudi and O’Brien (2012). *A. thaliana* mature leaves or seedlings were collected and submerged in the DAB staining solution overnight, in the dark, with gentle shaking. The following day, leaves were destained by boiling in a bleaching solution (ethanol/acetic acid/glycerol = 3:1:1) for 10 min. DAB precipitates were imaged using a white light transilluminator (Analytik Jena, Jena, Germany). These experiment were repeated at least two times.

‘*Ca. L. asiaticus*’ infection on citrus plants and HLB pathogenicity assay.

Five independent lines of SDE1 transgenic citrus in the *C. paradisi* (L.) Macfadyen (Duncan grapefruit) background were used for HLB pathogenicity assay via a grafting method, as previously described by Albrecht et al. (2014). Wild-type Duncan grapefruit plants of the same age were used as controls. Each SDE1 transgenic line represents an independent transgenic event. Branches from each of these plants were transferred to a new rootstock 3 to 6 months prior to graft inoculation by ‘*Ca. L. asiaticus*’-positive tissues in order to preserve the original transformants. For the ‘*Ca. L. asiaticus*’ graft inoculation, buds from ‘*Ca. L. asiaticus*’-infected greenhouse-grown Valencia sweet orange plants, with obvious disease symptoms and the presence of ‘*Ca. L. asiaticus*’ confirmed by TaqMan qPCR, were grafted onto SDE1 transgenic plants with two buds for each branch. Leaf samples were collected from the graft-inoculated grapefruit plants monthly beginning immediately after grafting (i.e., 0 MPI) until 5 months postgrafting (i.e., 5 MPI). Midribs of three mature leaves were collected for each branch, which were individually processed for DNA extraction and ‘*Ca. L. asiaticus*’ quantification. TaqMan qPCR was performed using a primer-probe combination (CQULA04F-CQULAP10-CQULA04R) described by Wang et al. (2006) (Supplementary Table S1). The Ct value of each amplicon represents the ‘*Ca. L. asiaticus*’ genomic copy numbers in 100 ng of DNA.

RNA extraction from *A. thaliana* and citrus tissues.

Total RNA was extracted from *A. thaliana* or citrus midrib tissues by grinding approximately 0.1 g of tissue in liquid nitrogen followed by suspension in 1 ml of TRIzol (Ambion, Austin, TX, U.S.A.). Chloroform (200 µl) was added to the extract, followed by centrifugation at 12,000 × g for 15 min at 4°C.

The aqueous phase (containing RNA) was precipitated for 20 min at -20°C in 1 ml of isopropanol. Pellets were washed twice with 75% ethanol, then air dried before resuspension in sterile water. RNA yield and quality were measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). Total RNA was then subjected to DNase treatment using DNase I (Promega Corp., Madison, WI, U.S.A.) following the manufacturers' instructions and used to make cDNA as described below.

qRT-PCR for senescence markers.

RNA extracted from 4-week-old *A. thaliana* plants at 24 and 30 h after Dex induction was used to make cDNA. RNA from dark-treated wild-type plants was used as a control for the *AtSEN1* gene marker. Total RNA (1 μg) was DNase treated and reverse transcribed using RevertAid reverse transcription at 42°C for 1 h using oligo-dT as a primer (Thermo Scientific). The cDNA was diluted 30-fold, then subjected to qPCR analysis using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, U.S.A.) and gene-specific primers (Supplementary Table S1) on a Bio-Rad CFX96 thermocycler. Relative expression levels of *AtSEN1* were calculated by the $2^{-\Delta\Delta\text{Ct}}$ method using *AtActin-2* as the internal control.

Total RNA from citrus was DNase treated. cDNA was synthesized from 500 ng of purified RNA with the ImProm-II Reverse Transcription System (Promega Corp.) and diluted 10 times for qPCR using specific primers (Supplementary Table S1). The housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase-C was used as the internal control.

NanoString gene expression analysis.

For NanoString analysis, total RNA was extracted from the midrib tissue of citrus using TRIzol, as described above. RNA samples were assessed using the Qubit Fluorometer and Qubit RNA Broad Range assay kit (Thermo Scientific) and then used directly for NanoString analysis.

Custom probes were designed by NanoString (Seattle, WA, U.S.A.) to target a subset of SAGs in citrus (Supplementary Tables S2 and S3). Gene sequences from *C. sinensis* v1.1 and *C. clementina* v1.0 (genomes accessed via Phytozome Joint Genome Institute database) were used for probe generation. Probe working stocks were added to nCounter Elements Tag-Set along with hybridization buffer. Hybridization of target-specific probes to 50 ng of total RNA was performed for 16 h at 67°C in a thermocycler. After hybridization, sterile water was added to each sample to bring the total volume up to 30 μl before loading in the NanoString SPRINT cartridge, after which direct digital RNA counts were read by the nCounter SPRINT Profiler (NanoString). Two biological replicates, each with two technical repeats, were analyzed. Data from both biological replicates were used for statistical analysis.

Data were analyzed using nSolver software (version 4.0; NanoString). *F-BOX*, *UPL7*, and *SAND* genes were used as internal controls. Additional positive and negative controls provided by NanoString were also included for quality control. Comparisons of different samples to assess relative expression was performed in nSolver using the gene expression ratio data and presented as $\log_2$ fold-change.

Statistical analysis.

Statistical analysis was performed by either an analysis of variance with α set to 0.05 or a two-tailed *t* test ($P < 0.05$). For *t* tests to compare more than two groups, raw *P* values are presented. For statistical analysis of the NanoString data, nSolver software v4.0 was used and *P* values were generated based on two-tailed *t* tests of the log-transformed data.

ACKNOWLEDGMENTS

We thank the NanoString support staff and the staff at both the Sanford Consortium (San Diego, CA) and University of California, Riverside–School of Medicine (Riverside, CA) for their assistance; and A. De Francesco (University of California, Riverside) and the undergraduate students S. Lee, T. Forest, C. Tong, and A. Gowder (University of California, Riverside) for their technical assistance.

LITERATURE CITED

- Albrecht, U., Hall, D. G., and Bowman, K. D. 2014. Transmission efficiency of *Candidatus Liberibacter asiaticus* and progression of Huanglongbing disease in graft- and psyllid-inoculated citrus. *HortScience* 49:367-377.
- Aoyama, T., and Chua, N.-H. 1997. A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant J.* 11:605-612.
- Bieker, S., and Zentgraf, U. 2013. Plant senescence and nitrogen mobilization and signaling. In: *Senescence and Senescence-Related Disorders*. W. Zhiwei, ed. IntechOpen.
- Bové, J. M. 2006. Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. *J. Plant Pathol.* 88:7-37.
- Cao, J., Cheng, C., Yang, J., and Wang, Q. 2015. Pathogen infection drives patterns of nutrient resorption in citrus plants. *Sci. Rep.* 5:14675.
- Clark, K., Franco, J. Y., Schwizer, S., Pang, Z., Hawara, E., Liebrand, T. W. H., Pagliaccia, D., Zeng, L., Gurung, F. B., Wang, P., Shi, J., Wang, Y., Ancona, V., van der Hoorn, R. A. L., Wang, N., Coaker, G., and Ma, W. 2018. An effector from the Huanglongbing-associated pathogen targets citrus proteases. *Nat. Commun.* 9:1718.
- Clough, S. J., and Bent, A. F. 1998. Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16:735-743.
- Coletta-Filho, H. D., Daugherty, M. P., Ferreira, C., and Lopes, J. R. S. 2014. Temporal progression of '*Candidatus Liberibacter asiaticus*' infection in citrus and acquisition efficiency by *Diaphorina citri*. *Phytopathology* 104:416-421.
- da Graça, J. V., Douhan, G. W., Halbert, S. E., Keremane, M. L., Lee, R. F., Vidalakis, G., and Zhao, H. 2016. Huanglongbing: An overview of a complex pathosystem ravaging the world's citrus. *J. Integr. Plant Biol.* 58:373-387.
- Dala-Paula, B. M., Plotto, A., Bai, J., Manthey, J. A., Baldwin, E. A., Ferrarezi, R. S., and Gloria, M. B. A. 2019. Effect of Huanglongbing or greening disease on orange juice quality, a review. *Front. Plant Sci.* 9:1976.
- Daudi, A., and O'Brien, J. A. 2012. Detection of hydrogen peroxide by DAB staining in *Arabidopsis* leaves. *Bio Protoc.* 2:1-4.
- Dou, D., and Zhou, J.-M. 2012. Phytopathogen effectors subverting host immunity: Different foes, similar battleground. *Cell Host Microbe* 12:484-495.
- Duan, Y., Zhou, L., Hall, D. G., Li, W., Doddapaneni, H., Lin, H., Liu, L., Vahling, C. M., Gabriel, D. W., Williams, K. P., Dickerman, A., Sun, Y., and Gottwald, T. 2009. Complete genome sequence of citrus Huanglongbing bacterium, '*Candidatus Liberibacter asiaticus*' obtained through metagenomics. *Mol. Plant-Microbe Interact.* 22:1011-1020.
- Etcheberria, E., Gonzalez, P., Achor, D., and Albrigo, G. 2009. Anatomical distribution of abnormally high levels of starch in HLB-affected Valencia orange trees. *Physiol. Mol. Plant Pathol.* 74:76-83.
- Feng, F., and Zhou, J.-M. 2012. Plant-bacterial pathogen interactions mediated by type III effectors. *Curr. Opin. Plant Biol.* 15:469-476.
- Folimonova, S. Y., and Achor, D. S. 2010. Early events of citrus greening (Huanglongbing) disease development at the ultrastructural level. *Phytopathology* 100:949-958.
- Folimonova, S. Y., Robertson, C. J., Garnsey, S. M., Gowda, S., and Dawson, W. O. 2009. Examination of the responses of different genotypes of citrus to Huanglongbing (citrus greening) under different conditions. *Phytopathology* 99:1346-1354.
- Fu, S., Shao, J., Zhou, C., and Hartung, J. S. 2016. Transcriptome analysis of sweet orange trees infected with '*Candidatus Liberibacter asiaticus*' and two strains of Citrus Tristeza Virus. *BMC Genomics* 17:349.
- Gasparoto, M. C. G., Coletta-Filho, H. D., Bassanezi, R. B., Lopes, S. A., Lourenço, S. A., and Amorim, L. 2012. Influence of temperature on infection and establishment of '*Candidatus Liberibacter americanus*' and '*Candidatus Liberibacter asiaticus*' in citrus plants. *Plant Pathol.* 61:658-664.
- Gottwald, T. R. 2010. Current epidemiological understanding of citrus Huanglongbing. *Annu. Rev. Phytopathol.* 48:119-139.

- Hall, D. G., Sétamou, M., and Mizell, R. F. 2010. A comparison of sticky traps for monitoring Asian citrus psyllid (*Diaphorina citri* Kuwayama). *Crop Prot.* 29:1341-1346.
- Harrison, S. J., Mott, E. K., Parsley, K., Aspinall, S., Gray, J. C., and Cottage, A. 2006. A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings following floral dip transformation. *Plant Methods* 2:19.
- Ishida, H., Izumi, M., Wada, S., and Makino, A. 2014. Roles of autophagy in chloroplast recycling. *Biochim. Biophys. Acta Bioenerg.* 1837: 512-521.
- Jagoueix, S., Bove, J. M., and Garnier, M. 1994. The phloem-limited bacterium of greening disease of citrus is a member of the α subdivision of the Proteobacteria. *Int. J. Syst. Bacteriol.* 44:379-386.
- James, M., Poret, M., Masclaux-Daubresse, C., Marmagne, A., Coquet, L., Jouenne, T., Chan, P., Trouverie, J., and Etienne, P. 2018. SAG12, a major cysteine protease involved in nitrogen allocation during senescence for seed production in *Arabidopsis thaliana*. *Plant Cell Physiol.* 59: 2052-2063.
- Jones, J. D. G., and Dangl, J. L. 2006. The plant immune system. *Nature* 444:323-329.
- Killingbeck, K. T. 1996. Nutrients in senesced leaves: Keys to the search for potential resorption and resorption proficiency. *Ecology* 77:1716-1727.
- Koh, E. J., Zhou, L., Williams, D. S., Park, J., Ding, N., Duan, Y. P., and Kang, B. H. 2012. Callose deposition in the phloem plasmodesmata and inhibition of phloem transport in citrus leaves infected with "*Candidatus Liberibacter asiaticus*". *Protoplasma* 249:687-697.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Lee, J. A., Halbert, S. E., Dawson, W. O., Robertson, C. J., Keesling, J. E., and Singer, B. H. 2015. Asymptomatic spread of huanglongbing and implications for disease control. *Proc. Natl. Acad. Sci. U.S.A.* 112: 7605-7610.
- Li, H., Ying, X., Shang, L., Redfern, B., Kypraios, N., Xie, X., Xu, F., Wang, S., Zhang, J., Jian, H., Yu, H., and Lv, D. 2020. Heterologous expression of CLIBASIA_03915/CLIBASIA_04250 by *Tobacco mosaic virus* resulted in phloem necrosis in the senescent leaves of *Nicotiana benthamiana*. *Int. J. Mol. Sci.* 21:1414.
- Liao, H.-L., and Burns, J. K. 2012. Gene expression in *Citrus sinensis* fruit tissues harvested from huanglongbing-infected trees: Comparison with girdled fruit. *J. Exp. Bot.* 63:3307-3319.
- Lim, P. O., Kim, H. J., and Nam, H. G. 2007. Leaf senescence. *Annu. Rev. Plant Biol.* 58:115-136.
- Ma, W., and Guttman, D. S. 2008. Evolution of prokaryotic and eukaryotic virulence effectors. *Curr. Opin. Plant Biol.* 11:412-419.
- Misas-Villamil, J. C., van der Hoorn, R. A. L., and Doehlemann, G. 2016. Papain-like cysteine proteases as hubs in plant immunity. *New Phytol.* 212:902-907.
- Natale, P., Brüser, T., and Driessen, A. J. M. 2008. Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane—Distinct translocases and mechanisms. *Biochim. Biophys. Acta* 1778: 1735-1756.
- Otegui, M. S., Noh, Y.-S., Martínez, D. E., Vila Petroff, M. G., Staehelin, L. A., Amasino, R. M., and Guimard, J. J. 2005. Senescence-associated vacuoles with intense proteolytic activity develop in leaves of *Arabidopsis* and soybean. *Plant J.* 41:831-844.
- Pagliaccia, D., Shi, J., Pang, Z., Hawara, E., Clark, K., Thapa, S. P., De Francesco, A. D., Liu, J., Tran, T.-T., Bodaghi, S., Folimonova, S. Y., Ancona, V., Mulchandani, A., Coaker, G., Wang, N., Vidalakis, G., and Ma, W. 2017. A pathogen secreted protein as a detection marker for citrus huanglongbing. *Front. Microbiol.* 8:2041.
- Paris, T. M., Allan, S. A., Udell, B. J., and Stansly, P. A. 2017. Evidence of behavior-based utilization by the Asian citrus psyllid of a combination of UV and green or yellow wavelengths. *PLoS One* 12:e0189228.
- Pitino, M., Allen, V., and Duan, Y. 2018. LasA5315 effector induces extreme starch accumulation and chlorosis as *Ca. Liberibacter asiaticus* infection in *Nicotiana benthamiana*. *Front. Plant Sci.* 9:113.
- Prasad, S., Xu, J., Zhang, Y., and Wang, N. 2016. SEC-translocon dependent extracytoplasmic proteins of *Candidatus Liberibacter asiaticus*. *Front. Microbiol.* 7:1989.
- Pružinská, A., Shindo, T., Niessen, S., Kaschani, F., Tóth, R., Millar, A. H., and van der Hoorn, R. A. L. 2017. Major Cys protease activities are not essential for senescence in individually darkened *Arabidopsis* leaves. *BMC Plant Biol.* 17:4.
- Pustika, A. B., Subandiyah, S., Holford, P., Beattie, G. A. C., Iwanami, T., and Masaoka, Y. 2008. Interactions between plant nutrition and symptom expression in mandarin trees infected with the disease huanglongbing. *Australas. Plant Dis. Notes* 3:112-115.
- Ramadugu, C., Keremane, M. L., Halbert, S. E., Duan, Y. P., Roose, M. L., Stover, E., and Lee, R. F. 2016. Long-term field evaluation reveals huanglongbing resistance in citrus relatives. *Plant Dis.* 100:1858-1869.
- Richau, K. H., Kaschani, F., Verdoes, M., Pansuriya, T. C., Niessen, S., Stüber, K., Colby, T., Overkleeft, H. S., Bogyo, M., and Van der Hoorn, R. A. 2012. Subclassification and biochemical analysis of plant papain-like cysteine proteases displays subfamily-specific characteristics. *Plant Physiol.* 158:1583-1599.
- Roberts, I. N., Caputo, C., Criado, M. V., and Funk, C. 2012. Senescence-associated proteases in plants. *Physiol. Plant.* 145:130-139.
- Rogers, H., and Munné-Bosch, S. 2016. Production and scavenging of reactive oxygen species and redox signaling during leaf and flower senescence: Similar but different. *Plant Physiol.* 171:1560-1568.
- Sathe, T. V., Gophane, A., and Shendage, N. 2015. Colour attractivity and occurrence of some cell sap sucking pests on crop plants. *Biolife* 3: 540-546.
- Schenk, P. M., Kazan, K., Rusu, A. G., Manners, J. M., and Maclean, D. J. 2005. The SEN1 gene of *Arabidopsis* is regulated by signals that link plant defence responses and senescence. *Plant Physiol. Biochem.* 43: 997-1005.
- Stansly, P. A., Arevalo, H. A., Qureshi, J. A., Jones, M. M., Hendricks, K., Roberts, P. D., and Roka, F. M. 2014. Vector control and foliar nutrition to maintain economic sustainability of bearing citrus in Florida groves affected by huanglongbing. *Pest Manage. Sci.* 70:415-426.
- Stover, E., McCollum, G. T., Driggers, R., Lee, R., Shatters, R., Jr., Duan, Y. P., Ritenour, M., Chaparro, J. X., and Hall, D. G. 2015. Resistance and tolerance to Huanglongbing in citrus. *Acta Hortic.* 1065:899-903.
- Thapa, S. P., De Francesco, A., Trinh, J., Gurung, F. B., Pang, Z., Vidalakis, G., Wang, N., Ancona, V., Ma, W., and Coaker, G. 2020. Genome-wide analyses of *Liberibacter* species provides insights into evolution, phylogenetic relationships, and virulence factors. *Mol. Plant Pathol.* 21:716-731.
- Thomas, H. 2013. Senescence, ageing and death of the whole plant. *New Phytol.* 197:696-711.
- Toruño, T. Y., Stergiopoulos, I., and Coaker, G. 2016. Plant-pathogen effectors: Cellular probes interfering with plant defenses in spatial and temporal manners. *Annu. Rev. Phytopathol.* 54:419-441.
- Uhse, S., and Djamei, A. 2018. Effectors of plant-colonizing fungi and beyond. *PLoS Pathog.* 14:e1006992.
- van der Hoorn, R. A. L. 2008. Plant proteases: From phenotypes to molecular mechanisms. *Annu. Rev. Plant Biol.* 59:191-223.
- Wang, N. 2019. The citrus huanglongbing crisis and potential solutions. *Mol. Plant* 12:607-609.
- Wang, N., and Trivedi, P. 2013. Citrus huanglongbing: A newly relevant disease presents unprecedented challenges. *Phytopathology* 103:652-665.
- Wang, Z., Yin, Y., Hu, H., Yuan, Q., Peng, G., and Xia, Y. 2006. Development and application of molecular-based diagnosis for "*Candidatus Liberibacter asiaticus*", the causal pathogen of citrus huanglongbing. *Plant Pathol.* 55:630-638.
- Woo, H. R., Kim, H. J., Nam, H. G., and Lim, P. O. 2013. Plant leaf senescence and death—Regulation by multiple layers of control and implications for aging in general. *J. Cell Sci.* 126:4823-4833.
- Yan, Q., Sreedharan, A., Wei, S., Wang, J., Pelz-Stelinski, K., Folimonova, S., and Wang, N. 2013. Global gene expression changes in *Candidatus Liberibacter asiaticus* during the transmission in distinct hosts between plant and insect. *Mol. Plant Pathol.* 14:391-404.