Citrus reticulata CrRAP2.2 Transcriptional Factor Shares Similar Functions to the *Arabidopsis* Homolog and Increases Resistance to *Xylella fastidiosa*

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Xylella fastidiosa is a worldwide multihost pathogen that causes diseases in different crops. It is considered a new global threat and substantial efforts have been made in order to identify sources of resistance. Indeed, many genes have been associated with resistance to *X. fastidiosa*, but without functional validation. Here, we describe a *C. reticulata* gene homologous to the transcriptional factor RAP2.2 from *Arabidopsis thaliana* that increases resistance to citrus variegated chlorosis (CVC). This gene was previously detected in *C. reticulata* challenged with *X. fastidiosa*. Bioinformatics analysis together with subcellular localization and auto-activation assays indicated that RAP2.2 from *C. reticulata* (CrRAP2.2) is a transcriptional factor orthologous to AtRAP2.2. Thus, we used *A. thaliana* as a model host to evaluate the functional role of CrRAP2.2 in *X. fastidiosa* resistance. The inoculation of *X. fastidiosa* in the *A. thaliana* rap2.2 mutant resulted in a larger bacterial population, which was complemented by CrRAP2.2. In addition, symptoms of anthocyanin accumulation were higher in the mutant, whose phenotype was restored by CrRAP2.2, indicating that they have conserved functions in plant defense response. We therefore transformed *C. sinensis* with CrRAP2.2 and verified a positive correlation between CVC resistance and gene expression in transgenic lines. This is the first study using *A. thaliana* as model host that characterizes the function of a gene related to *X. fastidiosa* defense response and its application in genetic engineering to obtain citrus resistance to CVC.

Keywords: *Citrus reticulata* (mandarin), *Citrus sinensis* (sweet orange), citrus transgenic plants, CVC resistance, RAP2.2 transcriptional factor, *Xylella-Arabidopsis* interaction

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Xylella fastidiosa is a xylem-limited phytopathogen that causes diseases in many perennial crops such as citrus, grape, almond, coffee, and plum (Rapicavoli et al. 2018a). Until recently, *X. fastidiosa* was found only in the Americas; however, in 2013 it appeared in southern Italy causing olive quick decline syndrome (OQDS) in olive trees (Saponari et al. 2013). Since then, other European countries have reported the bacterium, and it has rapidly become a major concern in regard to food security. Moreover, the bacterium has been categorized as a quarantine pathogen included in the European and Mediterranean Plant Protection Organization (EPPO) A2 list (available online). Currently *X. fastidiosa* was divided into three subspecies: subsp. *fastidiosa*, *pauca*, and *multiplex* (Marcelletti and Scortichini 2016). *X. fastidiosa* subsp. *pauca* is responsible for causing coffee leaf scorch, citrus variegated chlorosis (CVC), and OQDS. In citrus, *X. fastidiosa* affects all varieties of sweet orange (*Citrus sinensis* L. Osbeck) but no other *Citrus* species, including mandarins (*Citrus reticulata* Blanco) and their hybrids (Coletta-Filho et al. 2007; Niza et al. 2015).

Many studies have assessed the genetic mechanism underlying the defense response of *C. reticulata* to *X. fastidiosa* through global gene expression analysis (de Souza et al. 2007a; Mauricio et al. 2019; Rodrigues et al. 2013). The genetic mechanism related to *C. reticulata* resistance involves the recognition of *X. fastidiosa* by specific receptors and the triggering of genes involved with cell-wall modification and activation of hormone pathways (Niza et al. 2015; Rodrigues et al. 2013). Similarly, the recognition of receptors and cell-wall modification have also been shown as mechanisms of *X. fastidiosa* resistance in a tolerant olive cultivar (Giampe-truzzi et al. 2016; Sabella et al. 2018). Gene expression analyses of citrus and grape at different timepoints of *X. fastidiosa* colonization showed that different genes are expressed in both hosts in early and late stages of infection. These differences seem to be a consequence of phytohormone signaling pathways, which are differentially regulated according to the stage of *X. fastidiosa* infection (Rapicavoli et al. 2018b; Rodrigues et al. 2013; Zaini et al. 2018). In citrus and grape resistant to *X. fastidiosa*, defense response involves the participation of salicylic acid-mediated defense pathways, which seem to be inhibited in grape and citrus susceptible to *X. fastidiosa* infection (de Souza et al. 2007b; Rapicavoli et al. 2018b; Rodrigues et al. 2013; Zaini et al. 2018). Despite abundant research on the genes related to the defense response against *X. fastidiosa* in different hosts, the functional role of such genes in the mechanism of resistance remains unknown in *X. fastidiosa*–plant host interactions.

Gene functional analysis involving sweet orange is very time-consuming due to slow growth of the perennial host, long juvenile period, and long incubation before the development of symptoms (Almeida et al. 2001; Coletta-Filho et al. 2007; Peña et al. 2001). However, studies have developed methods to overcome this issue; specifically, *Arabidopsis thaliana* is a good alternative host that can be used to accelerate functional studies regarding the genetic mechanism associated with *X. fastidiosa*–plant interactions (Pereira et al. 2019; Rogers 2012). Genes encoding putative transcription factors of the APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) family were identified as candidate genes conferring resistance of *C. reticulata* to *X. fastidiosa* infection (Rodrigues et al. 2013). However, they have not yet been functionally characterized.

Thus, in this study, we sought to analyze the molecular function of this gene, hereafter named *CrRAP2.2*. We found that *CrRAP2.2* is highly similar to the *Arabidopsis* *AtRAP2.2* that is associated with resistance to the pathogen *Botrytis cinerea* (Zhao et al. 2012). Using a combination of bioinformatics, genetic, and phenotypic analyses, we determined that ectopic expression of *CrRAP2.2* in the *Arabidopsis rap2.2* mutant complements its susceptibility to *X. fastidiosa* in early stages of infection. Furthermore, expression of *CrRAP2.2* in *C. sinensis* was positively correlated with CVC resistance. Our results suggest that *AtRAP2.2* and *CrRAP2.2* share similar functions as transcriptional factors and they act as positive regulators of immunity against *X. fastidiosa* in both genera *Arabidopsis* and *Citrus*. Thus, we used a *RAP2.2* from *C. reticulata* to transform *C. sinensis* aiming to confer resistance to *X. fastidiosa*. These two species can naturally cross and are frequently used in breeding programs (Coletta Filho et al. 2007; Mauricio et al. 2019) but other traits are carried, altering commercial features. In this case, transgenic plants carrying genes from genetically compatible species might be an alternative for development of resistant plants without loss of the high-quality commercial features and consumer acceptance.

RESULTS

Expression of *CrRAP2.2* is associated with *X. fastidiosa* defense response.

Here, we compared the expression of *RAP2.2* between susceptible species *C. sinensis*, and resistant species *C. reticulata*. Although

Fig. 1. Expression analysis of the citrus *RAP2.2* in *Citrus reticulata* and *C. sinensis* after inoculation with *Xylella fastidiosa*. Gene expression in each species relative to the respective noninoculated plants (mock set to 1). Results are shown as the mean of triplicates. Asterisks indicate statistical significance using a Student's *t* test ($P < 0.05$); dai = days after inoculation.

not statistically significant, the expression of *RAP2.2* is slightly induced 1 day after inoculation (dai) with *X. fastidiosa* in the resistant *C. reticulata*; the increase of expression of *RAP2.2* in *C. reticulata* was significant at 7 dai. Repression of *RAP2.2* was observed in the susceptible *C. sinensis* at the same timepoints (Fig. 1).

Alignment of the predicted *RAP2.2* protein from *C. reticulata* (*CrRAP2.2*) and the *RAP2.2* protein from *Arabidopsis* (*AtRAP2.2*), using BLASTP, revealed high similarity, with 46% amino acid identity over the entire sequence (Fig. 2A). Both proteins show highly conserved domains, particularly the APETALA2 domain and nuclear localization site (NLS) domain (Fig. 2A), which are also present in *RAP2.2* (SUB1) from *Oryza sativa* (Hinz et al. 2010).

To structurally compare the APETALA2 domain of both proteins with three-dimensional (3D) protein structures, we looked for the highest match in the Protein Data Bank database, which was the GCC-box binding domain (GBD) of *A. thaliana* ethylene responsive element binding factor 1 (*AtERF1*) (Allen et al. 1998). Both proteins shared a high sequence identity (72.41%) with the GBD, including all the residues responsible for DNA binding in the β -sheets (Fig. 2B).

CrRAP2.2 is a transcription factor.

Aiming to confirm that *CrRAP2.2* is a transcription factor similar to *AtRAP2.2*, we evaluated its transcriptional activity using a yeast system. We observed that the full-length activation domain (AD)::*CrRAP2.2* protein binds to and activates the *lacZ* promoter in *Saccharomyces cerevisiae* EGY48 (p8op-*lacZ*) (Fig. 3A, indicated by the blue color in colony 3). Furthermore, we evaluated the three truncated versions of AD::*CrRAP2.2* lacking amino acids (*CrRAP2.2* Δ 1-230, *CrRAP2.2* Δ 230-388, and *CrRAP2.2* Δ 153-388), corresponding to colonies 4, 5, and 6, respectively (Fig. 3A). Only *CrRAP2.2* Δ 1-230 was able to activate the *lacZ* promoter; therefore, the activation domain of the protein is located in its C-terminal region, which is in agreement with that observed for the *AtRAP2.2* transcription factor (Welsch et al. 2007).

In addition, we analyzed the subcellular localization of *CrRAP2.2*, using a 35S promoter-driven green fluorescent protein (GFP)::*CrRAP2.2* construct that was transiently expressed in *N. benthamiana* leaves, revealing that the GFP::*CrRAP2.2* protein is exclusively localized in the nucleus of epidermal cells (Fig. 3B).

AtRAP2.2 contributes to *X. fastidiosa* resistance and ectopic expression of *CrRAP2.2* complements *rap2.2*.

As *AtRAP2.2* has been implicated in plant immune response (Zhao et al. 2012) and *CrRAP2.2* seems to be an ortholog of *AtRAP2.2*, we performed a genetic complementation assay by expressing the *CrRAP2.2* protein in the *A. thaliana rap2.2* mutant to analyze its role in resistance to *X. fastidiosa*. Interestingly, we observed a significantly higher *X. fastidiosa* population (50-fold difference) in *rap2.2* compared with the Col-0 wild type (Fig. 4). However, this difference is not present 2 weeks after inoculation, suggesting that one or more other genetic mechanisms could take place at later stages of infection. Nonetheless, this observation created an opportunity to test whether the early susceptibility of *rap2.2* could be rescued by *CrRAP2.2* and whether *CrRAP2.2* could enhance the resistance response in *A. thaliana* Col-0 at later stages (2 to 3 weeks) of *X. fastidiosa* infection. To test these hypotheses, we first created two independent transgenic lines of *rap2.2* and Col-0 expressing p35S-*CrRAP2.2*, named *rap2.2/CrRAP2.2* and Col-0/*CrRAP2.2*, respectively (Supplementary Fig. S3).

We found that expression of *CrRAP2.2* in the *rap2.2* background restored the *X. fastidiosa* colonization phenotype, as the bacterial population observed 1 week after inoculation was

similar to that observed in Col-0 at the same timepoint (Fig. 4). These findings suggest that native AtRAP2.2 is involved in resistance to *X. fastidiosa* at the early stage of infection and CrRAP2.2 has a role similar to that of AtRAP2.2, indicating

that these proteins are functional orthologs. Furthermore, expression of CrRAP2.2 in both *rap2.2* and Col-0 backgrounds significantly increased the resistance of these plants toward *X. fastidiosa*, suggesting that CrRAP2.2 is a positive regulator

Fig. 2. CrRAP2.2 is highly similar to the AtRAP2.2. **A**, Protein sequence alignment of CrRAP2.2 and AtRAP2.2 showing conserved regions: the N-terminal MCGG motif (1), an unknown function (2), nuclear localization signal (NLS), and APETALA2 (AP2) domains. CrRAP2.2 and AtRAP2.2 share 100% identity in region 1, 76.19% identity in region 2, 76.92% identity in the NLS domain, and 88.33% identity in the AP2 domain. **B**, Three-dimensional protein structure alignment of the ethylene responsive element binding factor 1 (AtERF1) with AtRAP2.2 and CrRAP2.2, showing that both share the same amino acids required to bind DNA in the GCC-box binding domain (marked in red). AtERF1 structure is shown in green, AtRAP2.2 model in blue, and CrRAP2.2 model in yellow. Proteins are shown individually, merged, and merged with DNA binding regions.

of plant immunity against this bacterium. In addition, the expression of CrRAP2.2 in Col-0, which carries the endogenous AtRAP2.2, does not lead to increased resistance as compared with *rap2.2* plants (Fig. 4), indicating no synergism between the RAP2.2 protein from *Arabidopsis* and *Citrus* species. In fact, the enhanced resistance in Col-0/CrRAP2.2 plants, compared with Col-0 plants, indicates that CrRAP2.2 alone can be a strong positive regulator of immunity when constitutively expressed.

We recently demonstrated that anthocyanin accumulation is a useful phenotypic marker for *X. fastidiosa* colonization in *Arabidopsis* (Pereira et al. 2019). In agreement with the high colonization observed in *rap2.2* at the early stage of infection (Fig. 4), a significant accumulation of anthocyanin was observed in the *rap2.2* mutant compared with the wild-type plant infected with *X. fastidiosa* (Fig. 5). Furthermore, complementation of the *rap2.2* mutant with CrRAP2.2 restored the Col-0 wild-type phenotype; significantly lower anthocyanin accumulation was observed in Col-0 overexpressing CrRAP2.2 (Fig. 5). These results reinforce the association of high anthocyanin accumulation with increased *X. fastidiosa* colonization in *A. thaliana* and suggest that AtRAP2.2 is either directly or indirectly related to this phenotype.

Transgenic sweet orange plants overexpressing CrRAP2.2 increases resistance to CVC.

To study the potential role of CrRAP2.2 in conferring resistance to CVC, the A596p9ioGusi-FMV::CrRAP2.2 construction was used to transform sweet orange (Pineapple variety). We obtained six transgenic lines with variable expression levels of the transgene (Fig. 6A). The real time quantitative PCR (RT-qPCR) analysis indicated that transgenic line T142 had an expression level 64 times greater than the wild type, whereas the other lines had expression levels only 1.5 to four times greater than the wild type (Fig. 6A). Based on this result, we selected three lines with different transgene expression levels (T142, T168, and T172) to assess the severity of CVC symptoms.

Fig. 3. Transcriptional activity assay and subcellular localization of the CrRAP2.2 protein. **A**, Transcriptional activity assay of the CrRAP2.2 protein in a yeast system. Each colony number corresponds to a different construct expressed in *Saccharomyces cerevisiae* EGY48 (p8op-lacZ). The negative control is the yeast strain EGY48 (p8op-lacZ) and the positive control is the yeast strain EGY48 (p8op-lacZ) containing vector pLexApos. The size domains of CrRAP2.2 expressed in each construct is referred by the numbers 1 to 6. **B**, Subcellular localization of CrRAP2.2 in *Nicotiana benthamiana* cells. The pB7WGF2 empty vector was used to show the localization of green fluorescent protein (GFP) alone.

The first symptoms of CVC were observed approximately 9 months after inoculation in the wild type and the T168 transgenic line. This line showed the lowest CrRAP2.2 expression level (only 1.5-fold higher than the wild type) among the transgenic lines. After 12 months, symptoms started to appear in T172, which has approximately threefold higher CrRAP2.2 expression. Line T142 was symptom-free until the end of the experiment (18 months after inoculation), when the wild type and T168 plants had severe CVC symptoms (Fig. 6B and C; Supplementary Fig. S4A). There is a good correlation between CrRAP2.2 expression and the severity of CVC; the greater the expression of CrRAP2.2 (Fig. 6A) in the transgenic lines, the lower the severity of symptoms observed in *C. sinensis* plants (Fig. 6B and C).

DISCUSSION

Many genes have been identified as associated with resistance to *X. fastidiosa* (de Souza et al. 2007a; Giampetruzzi et al. 2016; Rapicavoli et al. 2018b; Rodrigues et al. 2013; Zaini et al. 2018), including a putative transcription factor of the AP2/ERF family in the early response of *C. reticulata*, a citrus species resistant to this bacterium (de Souza et al. 2007a; Rodrigues et al. 2013). In addition, this gene was also associated with early genetic response to *X. fastidiosa* in grapevine (Rapicavoli et al. 2018b). Different functions have been attributed to the AP2/ERF family of transcription factors, such as tolerance to abiotic (Xu et al. 2006; Hinz et al. 2010) and biotic stresses (Zhao et al. 2012).

Bioinformatics and experimental analyses indicated that the citrus gene CrRAP2.2 is an *Arabidopsis* RAP2.2 ortholog (Figs. 2 and 3). A higher number of *X. fastidiosa* cells was observed in the *A. thaliana rap2.2* mutant 1 week after inoculation, which was complemented with CrRAP2.2. This finding reinforces the idea that RAP2.2 plays a role in the genetic mechanism of resistance at the beginning of the infection period. Similarities

Fig. 4. Influence of RAP2.2 on *Xylella fastidiosa* colonization in *Arabidopsis thaliana* over the duration of the study. The *X. fastidiosa* population in the Col-0 wild type, *rap2.2* mutant line, *rap2.2*/CrRAP2.2 complemented genotypes, and Col-0/CrRAP2.2 lines 1 and 5 overexpressing genotypes at 1, 2, and 3 weeks after inoculation. Results are shown as the mean of six replicates. Bars indicate standard errors. Different letters on top of the bars indicate statistically significant differences among the means calculated with a one-way analysis of variance, followed by a Tukey test ($P < 0.05$). FW = fresh weight.

between *X. fastidiosa* and *B. cinerea* have also been demonstrated in grapevines (Agüero et al. 2005). Transgenic plants overexpressing polygalacturonase-inhibiting proteins that specifically inhibit fungal endo-polygalacturonases showed improved resistance to both *B. cinerea* and *X. fastidiosa* (Agüero et al. 2005). In agreement with these results, a recent study demonstrated that grapevine transgenic plants overexpressing a plant polygalacturonase inhibitory protein (PGIP) prevents Pierce's disease by inhibiting the breakdown of pectin present in primary cell walls (Dandekar et al. 2019). Therefore, the similarity of *X. fastidiosa* and *B. cinerea* is the ability to degrade cell wall and to trigger the plant immune response (Choi and Klessig 2016; Glazebrook 2005). In this context, the participation of the ethylene responsive factor RAP2.2 might be involved in the modification of cell walls, acting to impair *X. fastidiosa* colonization (Pérez-Donoso et al. 2007). In the resistant *C. reticulata*, the fortification of the cell wall due to an increase of lignin was attributed to be a genetic mechanism to avoid *X. fastidiosa* colonization, which is delayed in susceptible *C. sinensis* (Niza et al. 2015; Rodrigues et al. 2013). In olive, the greater increase in total lignin in the resistant cultivar Leccino, compared with the susceptible cultivar Cellina di Nardò, has an essential role in its tolerance against *X. fastidiosa* (Sabella et al. 2018).

The overexpression of CrRAP2.2 increased plant resistance in both *rap2.2*/CrRAP2.2 and Col-0/CrRAP2.2, verified by the lower bacterial population observed at 2 and 3 weeks after inoculation (Fig. 4). Regarding the later stages of infection (i.e., at 2 and 3 weeks) in *A. thaliana* (Pereira et al. 2019), other genetic mechanisms occur, which explains the similar bacterial population observed in the mutant and the wild type at these timepoints (Fig. 4). Different genetic defense responses have been observed in both citrus and grape at early and late stages of *X. fastidiosa* infection; specifically, different genes involved with modulation of hormone biosynthesis and signaling play a role at each of the different stages of infection. Nonetheless, the salicylic acid-mediated defense pathway seems to be essential in later stages of infection to contain bacterial spread (Rapicavoli et al. 2018b; Rodrigues et al. 2013; Zaini et al. 2018). The change in plant response could be associated with different bacterial lifestyles in the plant host, such as biofilm and

Fig. 5. Anthocyanin accumulation in *Arabidopsis thaliana* infected with *Xylella fastidiosa*. Anthocyanin was quantified in the Col-0 wild type, *rap2.2* mutant line 1, *rap2.2*/CrRAP2.2 complemented line 1, and Col-0/CrRAP2.2 overexpressing line 1 at 3 weeks after *X. fastidiosa* inoculation. Results represent anthocyanin accumulation in each ecotype, normalized by subtraction of the basal anthocyanin content values of mock plants. The data are shown as the mean of 20 leaves. Bars represent standard errors. Different letters indicate statistical significance using a one-way analysis of variance, followed by a Tukey test ($P < 0.05$). Photographs on the right show representative leaves at 3 weeks after inoculation. *Xfp* = *X. fastidiosa* subsp. *pauca*.

planktonic phases, in which different molecules can be produced and recognized by the host and can trigger plant immunity responses (Rapicavoli et al. 2018b; Rodrigues et al. 2013; Zaini et al. 2018).

Compared with the other genotypes, the *rap2.2* mutant showed a significantly higher anthocyanin accumulation in the presence of the bacterium (Fig. 5). This phenomenon seems to be correlated to the presence of RAP2.2 and its effects on the

Fig. 6. Gene expression and symptom analysis of transgenic *Citrus sinensis* lines overexpressing CrRAP2.2. **A**, The relative expression of CrRAP2.2 was calculated in *C. sinensis* transgenic lines, with the gene expression level being normalized by the expression of *CsCYC* and *CsUBI1Q*, which were used as endogenous controls. Results represent the mean of six replicates. **B**, Severity of citrus variegated chlorosis (CVC) leaf symptoms in transgenic Pineapple sweet orange and wild-type plants was evaluated 18 months after *Xylella fastidiosa* inoculation. Disease severity was scored from 0 to 6, where 0 represents no symptoms and 6 represents severe CVC symptoms. **C**, Representative leaves from wild type and transgenic lines 18 months after inoculation. Bars represent standard errors. Different letters indicate statistically significant differences among the means, which was calculated with a one-way analysis of variance followed by a Tukey test ($P < 0.05$).

cells, since absence of *AtRAP2.2* leads to a higher accumulation of anthocyanin and the phenotype can be restored by complementation using *CrRAP2.2*. In addition, two copies of *RAP2.2* leads to a lack in anthocyanin accumulation in the presence of *X. fastidiosa*. Previous studies have shown that plant immune responses repress anthocyanin biosynthesis to activate plant defenses against bacterial or fungal pathogens (McLusky et al. 1999; Schenke et al. 2011; Serrano et al. 2012).

Our improved understanding of the function of *CrRAP2.2* in *Arabidopsis* led us to transform the *C. sinensis* Pineapple variety, which is highly susceptible to *X. fastidiosa*. Interestingly, protein alignment revealed that *RAP2.2* from Pineapple variety (orange1.lg020408m from *C. sinensis* genome v1.0 [Joint Genome Institute]) lacks 63 amino acid residues at the N terminus of the protein, as compared with *CrRAP2.2*. In addition, 27 residues at this terminus are not shared by the proteins from these two species.

Transgenic lines with different expression levels of *CrRAP2.2* allowed us to verify a negative correlation with CVC severity; we found that the higher the gene expression, the lower the disease symptoms (Fig. 6). Moreover, no change in development was observed in the transgenic plants (Supplementary Fig. S4B).

This is the first work in which a gene identified to be involved in *X. fastidiosa* defense response was functionally characterized using *A. thaliana* as a model plant. *RAP2.2* has orthologous functions in *A. thaliana* and *C. reticulata* and proved interesting for developing transgenic varieties of *C. sinensis* resistant to CVC. As this gene also confers resistance to *B. cinerea* in *A. thaliana* (Zhao et al. 2012), it is possible that a broad-spectrum resistance to different pathogens might be attained in *Citrus* and other genera. This study opens new perspectives for using basic knowledge from a model plant for application in crops, accelerating the development of new varieties, especially for perennial plant species.

MATERIALS AND METHODS

X. fastidiosa inoculation and gene expression analysis in *Citrus* spp.

A gene expression analysis was performed in two distinct experiments. First, the expression of *RAP2.2* was assessed in wild-type *Citrus sinensis* L. Osbeck (Pineapple variety) and *Citrus reticulata* Blanco (Ponkan variety) in response to *X. fastidiosa*. Briefly, *X. fastidiosa* subsp. *pauca* 9a5c was grown for 7 days in periwinkle (PW) solid media (Davis et al. 1981) at 28°C. The cells were suspended in phosphate-buffered saline (PBS) to reach an optical density at 600 nm (OD_{600}) of 0.3 (approximately 1×10^8 CFU/ml) and were needle-inoculated (Almeida et al. 2001) on the stem of *C. sinensis* and *C. reticulata*. Inoculations were performed on plants 6 months after grafting on Rangpur lime (*Citrus limonia* Osbeck). The xylem was collected at 1 and 7 days after inoculation and was submitted for RNA extraction according to Rodrigues et al. (2013). The second analysis was done in *C. sinensis* transgenic plants, aiming to evaluate *CrRAP2.2* expression.

Total RNA extraction from leaves was performed using a RNeasy plant mini kit (Qiagen, Hilden, Germany). The RNA integrity for the experiments was verified by electrophoresis on 1% agarose gel and the concentration was measured by NanoDrop™ 8000 (Thermo Fisher Scientific, San Diego, CA, U.S.A.). The cDNA was synthesized in a 20 µl total volume, from 1 µg of total RNA, 500 ng of oligo(dT)₁₅, and 20 U of RNase inhibitor, according to the manufacturer instructions (GoScript Reverse Transcription System; Promega, Madison, WI, U.S.A.).

The RT-qPCR reactions were prepared in a final volume of 25 µl with 10 µl of GoTaq qPCR master mix (Promega), 120 or 150 nM of each gene-specific primer pair (Supplementary Table S1) and 3 µl of 1:20 diluted cDNA synthesized from 1 µg of total plant RNA. Amplifications were performed in an ABI 7500 Fast Real-Time PCR device (Applied Biosystems, Foster City, CA, U.S.A.), using the standard thermal profile: 95°C for 20 s followed by 40 cycles of 95°C for 3 s, and 60°C for 30 s. Each sample was analyzed in triplicate.

The primer efficiency and quantification cycle values were determined for individual RT-qPCR, using the algorithm of the Real-time PCR Miner (available online from the Stanford University). The relative mRNA level was calculated using the $2^{-\Delta\Delta CT}$ cycle threshold method. A normalization factor (NF) for each sample was calculated by the geometric mean of the relative quantity (RQ) values of the two reference genes: *UBIQ* and *CYC* for *C. sinensis* Pineapple variety (Caserta et al. 2014, 2017). Normalized RQ (NRQ) of each sample was calculated as the ratio of the sample RQ and the appropriate NF. Individual fold change values were determined by dividing the sample NRQ by the mean NRQ of samples of the calibrator. This procedure renders a mean fold change value of 1 for the set of mock plants.

In silico sequence analysis.

CrRAP2.2 was one of the 81 genes, originally identified from an expressed sequence tag (EST) of Ponkan mandarin (*Citrus reticulata* Blanco), that were up-regulated when inoculated with *X. fastidiosa* (de Souza et al. 2007a). It is identical to the coding sequence Ciclev10025816m.g from *C. clementina* genome v1.0 available at the Citrus Genome Database. The *CrRAP2.2* protein sequence was aligned with the *A. thaliana* genome available at The Arabidopsis Information Resource (TAIR) database, using BLASTP (available online from the National Institutes of Health) to search for putative orthologous sequences. The 3D models for the APETALA2 domains were obtained using the SWISS-MODEL software (available online from the Swiss Institute of Bioinformatics), which did the target-template alignment search against the SWISS-MODEL template library. The AtERF1 crystal structure (library 1gccA) (Allen et al. 1998) was used as a template to obtain the 3D models. The alignments between AtERF1/AtRAP2.2 and AtERF1/CsRAP2.2 were obtained using PYMOL software (available online from the PyMOL Schrödinger technical support).

CrRAP2.2 transcriptional activity assay.

CrRAP2.2 was amplified from the A596p9ioGusi-FMV::*CrRAP2.2* vector (discussed below) (Supplementary Fig. S1), using primers with attB adaptors (Supplementary Table S1) and *Pfu* high fidelity DNA polymerase enzyme (Promega). It was purified in 1% agarose gel, using the Wizard SV gel and PCR clean-up system kit (Promega), was quantified using a Qubit 3.0 fluorometer (Invitrogen, Carlsbad, CA, U.S.A.), and was cloned into the entry vector using a BP Clonase II enzyme mix kit (Thermo Fisher Scientific). This entry vector has a 35S-*CrRAP2.2* cassette that was transferred to the pGILDA vector (Clontech, Mountain View, CA, U.S.A.) using the LR Clonase II enzyme mix kit (Thermo Fisher Scientific), resulting in an AD fusion construct, AD::*CrRAP2.2*. This construct was transformed into *Saccharomyces cerevisiae* EGY48 (p8op-lacZ), using the frozen-EZ yeast transformation II kit (Zymo Research, Irvine, CA, U.S.A.), to test whether this construct could activate the *lacZ* promoter. The LexA::*CrRAP2.2*-expressing yeast strain was selected on SD-galactose inducing medium (BD Biosciences, San Jose, CA, U.S.A.).

containing 80 µg of X-gal per milliliter, BU salts (70% Na₂HPO₄ · 7H₂O and 30% NaH₂PO₄), and –Ura/–His drop out supplement (BD Biosciences). Additionally, yeast strains were transformed with three truncated LexA::CrRAP2.2 constructs (CrRAP2.2Δ153-389, CrRAP2.2Δ230-389, CrRAP2.2Δ1-230) and were cloned following the same methods as for the whole CrRAP2.2 protein, to verify whether transcriptional activation signatures in the AtRAP2.2 (Welsch et al. 2007) corresponded to similar locations in the CrRAP2.2 protein sequence.

CrRAP2.2 subcellular localization assay.

Using the same procedures of the Gateway system (Hartley et al. 2000), the CrRAP2.2 amplicon was cloned into the destination vector pB7WGF2 under the control of the cauliflower mosaic virus 35S promoter (Karimi et al. 2002) to make the construct p35S-GFP::CrRAP2.2. This construct was transformed into *Agrobacterium tumefaciens* C58C1 by the freeze-thaw method (Chen et al. 1994).

Subcellular localization of the GFP::CrRAP2.2 protein was determined by using a transient expression system (Walter et al. 2004). *Agrobacterium* strains carrying the constructs p35S-GFP::CrRAP2.2 and p35S-GFP (pB7WGF2 empty vector) were infiltrated into leaves of four-week-old *Nicotiana benthamiana* plants. Initially, the *N. benthamiana* plants were grown in a greenhouse for 4 weeks and were then maintained in a growth chamber at 25°C, 60% ± 5% relative humidity, and a 12-h photoperiod with a light intensity of 100 µmol m⁻² s⁻¹. The leaves were marked around the infiltration site and, 3 to 5 days later, leaves were mounted onto microscope slides with 50% glycerol. Infiltration sites were visualized under a fluorescence microscope (Nikon Eclipse Ni), using specific filters for GFP (excitation:emission, 480/510 nm). Three independent experiments were performed.

Arabidopsis genetic transformation and *X. fastidiosa* inoculation.

The same constructs made for subcellular localization were also used for *A. thaliana* transformation. Employing the floral dip method (Clough and Bent 1998), Col-0 and *rap2.2* plants were transformed with *Agrobacterium tumefaciens* C58C1 carrying p35S-GFP::CrRAP2.2 or p35S-GFP (pB7WGF2 empty vector). Transgenic lines were selected with BASTA (0.0114% glufosinate ammonium) (BayerVR, Leverkusen, Germany) supplemented with 0.005% of Silwet L-77 (Lehle Seeds, Round Rock, TX, U.S.A.).

Seeds of *Arabidopsis thaliana* (L. Heyhn.) wild-type Columbia (Col-0, *Arabidopsis* Biological Resource Center [ABRC] stock CS60000), the *rap2.2* mutant (ABRC stock SALK_010265C) with knockdown expression (Zhao et al. 2012), Col-0/CrRAP2.2 lines 1 and 5, and *rap2.2*/CrRAP2.2 lines 1 and 3 were sown on pots containing Multipant substrate (Terra do Paraíso, Holambra, Brazil) with vermiculite in a 5:2 (vol/vol) proportion. Plants were grown in a Conviron growth chamber at 22°C, 60% ± 5% relative humidity, and a 12-h photoperiod with a light intensity of 100 µmol m⁻² s⁻¹.

Plant inoculation was performed according to the procedure described by Pereira et al. (2019). Briefly, four-week old plants were used for inoculation with *X. fastidiosa* subsp. *pauciflora* 9a5c, which was cultured at 28°C for 5 days on PW solid media (Davis et al. 1981), suspended in phosphate-buffered saline (PBS) buffer to reach an OD₆₀₀ of 0.7 (approximately 10⁸ CFU/ml). To optimize inoculation efficiency, plants were not irrigated one day before and 1 day after inoculation. Four young, fully expanded rosette leaves were inoculated by dropping 5 µl of the inoculum on the midrib at the petiole-leaf junction. The petiole tissue under the drop was pricked seven to eight times using a 13 × 0.38 mm insulin needle (Becton

Dickinson, Franklin Lakes, NJ, U.S.A.). Water-inoculated plants were used as a mock control. No influence of the vector expression (pB7WGF2) on the pathogen response was verified (Supplementary Fig. S2). Plants were kept well-watered during the experiment. Three independent experiments were performed.

Evaluation of *X. fastidiosa* colonization of *A. thaliana*.

Arabidopsis rosettes were harvested at 1, 2, and 3 weeks after inoculation with *X. fastidiosa*, as described above, and were weighed to estimate the bacterial population per gram of tissue. qPCR reactions to quantify the pathogen were conducted in a total volume of 25 µl containing 12.5 µl of TaqMan Universal PCR master mix, 525 nM of the primers CVC-1 (5'-GA TGA AAA CAA TCA TGC AAA-3') and CCSM-1 (5'-GCG CAT GCC AAG TCC ATA TTT-3'), 500 nM TAQCVC probe (5'-(6FAM)AAC CGC AGC AGA AGC CGC TCA TC (TAMRA) p-3') (Oliveira et al. 2002), and 200 ng of DNA template. A negative (DNA from mock-inoculated plants) and a positive control (bacteria genomic DNA) were included in all experiments. The amplification parameters followed those described by Oliveira et al. (2002) (50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C), and capture of the fluorescent signal was obtained in an ABI PRISM 7500 sequence detection system. All samples were processed in duplicates and the experiment was repeated at least twice (biological replicates). A standard curve for *X. fastidiosa* quantification was prepared following the methods described in Pereira et al. (2019).

Anthocyanin quantification assay.

Arabidopsis plants were inoculated as described and the procedure for quantifying the accumulated anthocyanin in response to *X. fastidiosa* was conducted according to Pereira et al. (2019). A total of 20 leaves were used to analyze the anthocyanin content in each ecotype inoculated or not (mock) with *X. fastidiosa*. The values obtained from mock plants (basal anthocyanin content) were subtracted from their respective inoculated ecotypes to determine the anthocyanin accumulation.

Citrus transformation.

The CrRAP2.2 sequence identified in the *C. reticulata* EST (identical to Ciclev10025816m.g) was chemically synthesized by the DNA Cloning Service company in a cassette containing the figwort mosaic virus (FMV) promoter. The cassette was inserted into the p9i-UbiAtm-oGusi vector to create the final construct A596p9ioGusi-FMV::CrRAP2.2. The p9i-UbiAtm-oGusi vector has an optimized codon usage for plants and intron sequences inside the β-glucuronidase (GUS) reporter gene and kanamycin as a plant selection marker. The A596p9ioGusi-FMV::CrRAP2.2 vector was used to transform shoots of *C. sinensis*, using an *Agrobacterium*-based method (Supplementary Fig. S1) (Caserta et al. 2014).

Seeds of sweet orange (*C. sinensis*) Pineapple variety were germinated in half-strength MS basal medium (Murashige and Skoog 1962) and were transformed as previously detailed (Caserta et al. 2014). To confirm transformation, a small piece of each transgenic shoot was excised and was incubated in 50 µl of phosphate buffer containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronide for 16 h at 37°C to test GUS activity. In addition, genome integration of the transgene was evaluated by PCR using CrRAP2.2-specific primers (Supplementary Table S1). Ten buds from each shoot where transformation was confirmed were used for grafting onto Rangpur lime and were maintained in a mist chamber inside a greenhouse for acclimatization, to enable subsequent studies. GUS assays were

performed for all leaves of propagated plants to identify any presence of chimeras. Only GUS-positive leaves from non-chimeric plants were used in the experiments. *CrRAP2.2* expression was evaluated in confirmed transgenic plants as described above. Buds of each selected plant were multiplied into ten clones grafted onto Rangpur lime. GUS assays were performed using different parts of these new plants to detect any possible chimeras; only GUS-positive plants were used in screenings for *X. fastidiosa* resistance.

Symptom analysis of transgenic citrus plants.

Ten clones from three transgenic lines designated T142, T268, and T172, as well as ten clones from the wild type were used in symptom development assays. The presence of the bacterium was assessed by qPCR, as described above. Infected plants were assessed for the severity of CVC, were scored by three evaluators, using a diagrammatic scale (Amorim et al. 1993; Muranaka et al. 2013) and an image panel developed by Caserta et al. (2017).

Statistical analysis.

Statistical analyses were performed using Student's *t* test ($P < 0.05$) or one-way analysis of variance followed by a Tukey's test ($P < 0.05$) in GraphPad Prism 8.2.1 for Windows (GraphPad Software, San Diego, CA, U.S.A.). All the experiments were performed at least three times with similar results, in which the data points represent the mean of three to 20 technical replicates, depending on the experiment.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Citrus Genome Database: <https://www.citrusgenomedb.org>
The DNA Cloning Service website: www.dna-cloning.com
TAIR database: <https://www.Arabidopsis.org>

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