Role of the *Citrus sinensis* RNA deadenylase CsCAF1 in citrus canker resistance

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**SUMMARY**

Poly(A) tail shortening is a critical step in messenger RNA (mRNA) decay and control of gene expression. The carbon catabolite repressor 4 (CCR4)-associated factor 1 (CAF1) component of the CCR4-NOT deadenylation complex plays an essential role in mRNA deadenylation in most eukaryotes. However, while CAF1 has been extensively investigated in yeast and animals, its role in plants remains largely unknown. Here, we show that the *Citrus sinensis* CAF1 (CsCAF1) is a magnesium-dependent deadenylation implicated in resistance against the citrus canker bacteria *Xanthomonas citri*. CsCAF1 interacted with proteins of the CCR4-NOT complex, including CsVIP2, a NOT2 homologue, translin-associated factor X (CsTRAX) and the poly(A)-binding proteins CsPABPN and CsPABPC. CsCAF1 also interacted with PthA4, the main *X. citri* effector required for citrus canker elicitation. We also present evidence suggesting that PthA4 inhibits CsCAF1 deadenylase activity in vitro and stabilizes the mRNA encoded by the citrus canker susceptibility gene *CsLOB1*, which is transcriptionally activated by PthA4 during canker formation. Moreover, we show that an inhibitor of CsCAF1 deadenylation activity significantly enhanced canker development, despite causing a reduction in PthA4-dependent *CsLOB1* transcription. These results thus link CsCAF1 with canker development and PthA4-dependent transcription in citrus plants.

**Keywords:** CCR4-NOT-associated factor, citrus canker, *Citrus sinensis*, CsCAF1, CsLOB1, PthA4, TAL effectors, RNA deadenylase activity, *Xanthomonas citri*, *Xanthomonas aurantifolii*.

**INTRODUCTION**

Polyadenylation of the 3′ end of messenger RNAs (mRNAs) is a coordinated RNA modification process that plays fundamental roles not only in mRNA transport, stability and processing, but also in translational control (Fasken *et al.*, 2008; Millevoy and Vagner, 2010; Weill *et al.*, 2012). The reverse process, known as deadenylation of the mRNA poly(A) tail, also represents a critical translational control mechanism and it is regarded as the first step in mRNA decay (Temme *et al.*, 2014).

In most eukaryotic cells, the CCR4-NOT complex is the major multi-subunit and multi-functional protein complex that presents mRNA deadenylation activity. The CCR4-NOT complex was first identified in yeast, and its deadenylation activity is provided by two of its components, carbon catabolite repressor 4 (CCR4) and CCR4-associated factor 1 (CAF1), also known as POP2 (Bai *et al.*, 1999; Basquin *et al.*, 2012; Liu *et al.*, 1998; Tucker *et al.*, 2001). CAF1 belongs to the DEDDh subfamily of magnesium-dependent nucleases, which have an RNase D domain required for the 3′→5′ deadenylation activity (Daugeron *et al.*, 2001; Jonstrup *et al.*, 2007; Thore *et al.*, 2003). Besides its role in mRNA deadenylation and decay, the CCR4-NOT complex has been implicated in a variety of cellular processes, including micro RNA (miRNA)-mediated gene silencing, transcriptional elongation and DNA repair (Collart, 2016; Denis *et al.*, 2001; Fabian and Sonenberg, 2012; Gaillard *et al.*, 2009; Kruk *et al.*, 2011). For instance, the CCR4-NOT complex physically interacted with RNA polymerase (Pol) II and promoted transcription elongation particularly from arrested Pol II, and this interaction is in part mediated by the CCR4 and CAF1 subunits (Dutta *et al.*, 2015; Kruk *et al.*, 2011; Reese, 2013). The miRNA-mediated deadenylation, on the other hand, required CAF1 deadenylation activity and its interaction with poly(A)-binding proteins (PABPs) (Behm-Ansmant *et al.*, 2006; Fabian *et al.*, 2009; Flamand *et al.*, 2016; Piao *et al.*, 2010). Importantly, recent studies in yeast using reconstituted CCR4-NOT complex have revealed that while CCR4 is a general deadenylase that degrades PABP1-bound poly(A) tails, CAF1 is required for the selective deadenylation of transcripts not bound by PABP1 and with lower rates of translation elongation (Webster *et al.*, 2018; Yi *et al.*, 2018). Thus, as new evidence emerges, the CCR4-NOT complex has been regarded as a macromolecular structure that not only connects transcription to translation, but also determines the translational capacity of...
the cell during transcription elongation (Babbarwal et al., 2014; Gupta et al., 2016; Villanyi et al., 2014; Webster et al., 2018; Yi et al., 2018).

Orthologues of the yeast CAF1 and NOT proteins have now been identified in virtually all eukaryotes (Dai et al., 2016; Dupressoir et al., 2001; Nousch et al., 2013; Temme et al., 2004; Winkler and Balasco, 2013). Nevertheless, in contrast to yeast and animals, the role played by CAF1 proteins in plants is less clear.

The Capsicum annuum CAF1 gene was the first to be identified as a gene upregulated in response to Xanthomonas axonopodis pv. glycines infection (Lee et al., 2004). Overexpression of CAF1 in tomato plants resulted in enhanced resistance against Phytophthora infestans whereas its down-regulation enhanced susceptibility to the pepper bacterial spot pathogen X. axonopodis pv. vesicatoria (Sarowar et al., 2007). Similarly, overexpression of the Arabidopsis thaliana AtCAF1a and AtCAF1b genes led to an increase in the expression of pathogenesis-related protein (PR) genes and enhanced resistance against Pseudomonas syringae pv. tomato. Conversely, down-regulation of AtCAF1a and AtCAF1b resulted in reduced expression of PR proteins and increased susceptibility to P. syringae pv. tomato (Liang et al., 2009).

CAF1 proteins have also been implicated in the control of plant development and stress responses. Tomato plants overexpressing CAF1 showed enlarged leaf cells whereas plants silenced for CAF1 showed reduced growth (Sarowar et al., 2007). In Arabidopsis and rice, CAF1 proteins are induced by multiple stress-related hormones and types of stress including drought, cold and wounding (Chou et al., 2014; Liang et al., 2009; Walley et al., 2010). These results thus indicate that, in plants, CAF1 proteins play important roles in cell growth, stress responses and defence against microbial pathogens.

Previously, we have identified a citrus CAF1 homologue gene, CsCAF1, that was up-regulated in sweet orange (Citrus sinensis) leaves in response to infection by the citrus canker pathogen Xanthomonas axonopodis pv. aurantifolii pathotype C (Xc), a Xanthomonas citri (Xc)-related bacterium that causes canker in Mexican limes but a defence response in sweet oranges (Abe and Benedetti, 2016; Cernadas et al., 2008). Since CsCAF1 was induced in the hypersensitivity response triggered by Xa in sweet orange, we hypothesized that it might play a role in the defence against citrus canker bacteria (Cernadas et al., 2008). Since CsCAF1 was induced in the hypersensitivity response triggered by Xa in sweet orange, we hypothesized that it might play a role in the defence against citrus canker bacteria (Cernadas et al., 2008).

Here, we confirm that CsCAF1 expression correlates with the defence response induced by Xa in sweet orange leaves and show that the protein encoded by the CsCAF1 gene, CsCAF1, displays a magnesium-dependent 3′–5′ RNA deadenylase activity. In addition, we show that CsCAF1 interacted with four citrus proteins associated with the CCR4-NOT complex, and with PthA4, the main Xc transcriptional activator-like (TAL) effector required for canker formation and transcriptional activation of the citrus canker susceptibility gene Lateral Organ Boundaries 1, CsLOB1 (Abe and Benedetti, 2016; Hu et al., 2014; Pereira et al., 2014; de Souza et al., 2012). We also present evidence suggesting that PthA4 inhibits CsCAF1 deadenylase activity and stabilizes the CsLOB1 mRNA in Xc-infected leaves. Our data suggest that by targeting the CCR4-NOT complex, the effector protein PthA4 enhances transcription and translation of CsLOB1 to promote cell hypertrophy and hyperplasia in citrus. Consistent with this idea, a novel adenine analogue inhibitor of CsCAF1 significantly enhanced canker development in Xc-infected plants, suggesting that CsCAF1 restricts cell growth in citrus.

**RESULTS**

**Increased CsCAF1 expression correlates with defence against Xanthomonas infection**

Previous large-scale gene expression analysis revealed that the CsCAF1 gene (XP_006481524.1) was up-regulated in the incompatible interaction of sweet orange plants infected with Xa (Cernadas et al., 2008). To confirm these results, sweet orange leaves were infiltrated with Xa, Xc or water, as control, and the expression of CsCAF1 was monitored by quantitative RT-PCR. The expression levels of CsLOB1, a direct target of PthA4, and the citrus pathogenesis-related PR1, a marker gene for Xa and Xc infection (Abe and Benedetti, 2016; Cernadas et al., 2008; Hu et al., 2014; Pereira et al., 2014), were also evaluated. In agreement with the microarray data (Cernadas et al., 2008), CsCAF1 was preferentially induced in response to Xa infection at 24 and 48 h after bacterial inoculation (Fig. 1A). On the other hand, CsLOB1 was highly and predominantly induced by Xc infection, whereas PR1 was similarly up-regulated by both pathogens at 24 and 48 h post-infection (Fig. 1B,C). These results thus confirm our previous data (Abe and Benedetti, 2016; Cernadas et al., 2008; Pereira et al., 2014) and show that CsCAF1 expression correlates with the defence response triggered by Xa in sweet orange plants.

**CsCAF1 is a Mg-dependent deadenylase of the RNase D superfamily**

CsCAF1 is closely related to C. annuum CaCAF1 and Arabidopsis AtCAF1a (Liang et al., 2009; Sarowar et al., 2007), sharing up to 75% sequence identity with them. CsCAF1 is also 47% identical to human CAF1, known as NOT7 (Horiiuchi et al., 2009), and 34% identical to the RNase domain of yeast POP2 (Thoré et al., 2003) (Fig. 2A). Structural modelling studies suggest that CsCAF1 has the same protein fold as human NOT7 and yeast POP2, and that the consensus DEDDh motif of the RNase D superfamily, comprising residues D52, E54, D187, H253 and D258, is structurally conserved in CsCAF1, relative to the crystal structures of POP2 and NOT7 (Fig. 2B,C).
To know whether CsCAF1 would exhibit a 3′–5′ exoribonuclease activity, recombinant CsCAF1 was produced and purified by affinity and size exclusion chromatography (Fig. 3A). CsCAF1 migrated with an apparent molecular mass of ~32 kDa in denaturing polyacrylamide gels and eluted with an estimated molecular mass of 31 kDa in size exclusion chromatography (Fig. 3B), suggesting that it is a monomer in solution. Purified CsCAF1 was incubated with the single-strand poly(A) RNA probe (5′-GACUGACUAAAAAAA-3′) labelled with fluorescein (FITC) at its 5′ end (Horiuchi et al., 2009). The exoribonuclease assays were performed in the presence of metal salts, since metal ions were shown to be required for NOT7 deadenylase activity (Horiuchi et al., 2009). The results show that, like NOT7, CsCAF1 presents 3′–5′ exoribonuclease activity in the presence of magnesium, but not calcium, cobalt or zinc ions. A residual deadenylase activity was also observed in the presence of manganese (Fig. 3C).

Next, we tested the specificity of CsCAF1 towards different RNA molecules and found that CsCAF1 shows selective and stronger RNase activity towards poly(A) RNA. No RNase activity was observed with the poly(U) (5′-GACUGACUUUUUUU-3′) or poly(C) (5′-GACUGACUCCCCCC-3′) probes. Nevertheless, a weaker RNase activity was detected when the poly(G) probe (5′-GACUGACUGGGGGG-3′) was used as substrate (Fig. 3D). These results thus show that CsCAF1 preferentially degrades poly(A) tails.

Because in yeast caf1 deletion mutants exhibit sensitivity to high doses of caffeine (Hata et al., 1998; Liu et al., 1997), we decided to test whether CsCAF1 could serve as a functional homologue of the yeast protein by complementing this phenotype. However, CsCAF1 did not complement the yeast caf1 mutant regardless of the caffeine dose used (Fig. S1, see Supporting Information). The low degree of homology to POP2 (Fig. 2A) and the fact that POP2 has a distinct SEDQt active site might explain the lack of CsCAF1 complementation in yeast.

CsCAF1 interacts with PthAs and proteins associated with the CCR4-NOT complex

We found previously that the Xc effector proteins PthA3 and PthA4, required to induce cankers on citrus (Abe and Benedetti, 2016), interacted with several citrus proteins implicated in mRNA stabilization and translational control, including the CsVIP2 (VirE2-interacting protein 2), a NOT2 homologue, translin-associated factor X (CsTRAX), and the poly(A)-binding proteins CsPABPN and CsPABPC (de Souza et al., 2012). Because these proteins interact with each other and are homologous to the mammalian proteins associated with the miRISC/CCR4-NOT complex, they are thought to represent components of the citrus CCR4-NOT complex (de Souza et al., 2012). To verify this and examine whether CsCAF1 could also interact with PthAs, the recombinant CsVIP2, CsTRAX, CsPABPN, CsPABPC, PthA3 and....
Fig. 2  CsCAF1 is structurally related to plant, yeast and mammalian proteins belonging to the RNase D superfamily. (A) Protein sequence alignment performed with Clustal Omega showing that CsCAF1 is related to: C. annuum CaCAF1 (NP_001312000.1), A. thaliana AtCAF1a (QAP03728.1), H. sapiens HsNOT7 (AAP97145.1) and the RNase domain of S. cerevisiae ScPOP2 (EDN62858.1). The consensus DEDD motif, characteristic of the RNase D superfamily, is conserved in CsCAF1 (residues shown in yellow). (B) Superposition of the crystal structures of yeast POP2 (PDB code 1UOC, pink) and human NOT7 (PDB code 4GMJ_B, blue) with the structural model of CsCAF1 (green) generated by SWISS-MODEL using the human NOT7 structure as the search template. A close inspection of the active site of the proteins shows that the DEDD motif in CsCAF1, comprising residues D52, E54, D187, H253 and D258, is structurally conserved relative to POP2 and NOT7. These residues are thought to also coordinate two magnesium ions, as shown in the NOT7 structure (green spheres).
PthA4 fused to GST were purified and used in GST-pulldown assays as baits (Fig. S2, see Supporting Information). We found that, in addition to PthA3 and PthA4 (Fig. 4A), CsCAF1 interacts with CsTRAX, CsPABPN, CsPABPC and, to a lesser extent, CsVIP2 (Fig. 4B), suggesting that it is a component of the citrus CCR4-NOT complex.

PthA4 inhibits CsCAF1 deadenylase activity and stabilizes the CsLOB1 mRNA

The interaction of CsCAF1 with PthAs, CsPABPN, CsPABPC, CsTRAX and CsVIP2 (Fig. 4) led us to test whether any of these protein interactors could influence the CsCAF1 deadenylase activity in vitro. We found that when PthAs or the citrus proteins were incubated with the poly(A) probe before the addition of CsCAF1 to the reaction mixture, CsPABPN, CsTRAX and PthA4 inhibited the deadenylase activity in the first 5 min of reaction. However, when the interacting proteins were incubated with CsCAF1 before the addition of the poly(A) probe, we noticed that, in addition to CsTRAX and PthA4, CsVIP2, but not CsPABPN, also inhibited the deadenylase activity of CsCAF1 (Fig. 5). These results suggest that CsTRAX, CsVIP2 and PthA4 do not merely compete with CsCAF1 for RNA binding, as it appears to be the case with CsPABPN. Additionally, although PthA3 interacted with CsCAF1 in GST-pulldown assays (Fig. 4), this effector did not inhibit CsCAF1 deadenylase activity in vitro (Fig. 5).

CAF1 proteins are thought to promote mRNA degradation in response to a sudden increase in mRNA transcription as a way to balance the mRNA levels (Liang et al., 2009). Because CsLOB1 is highly and directly transcribed by PthA4 and rapidly accumulates during canker development (Abe and Benedetti, 2016; Hu et al., 2014; Pereira et al., 2014), we thought that CsCAF1 might target the CsLOB1 mRNA to counteract its massive production upon PthA4 induction. However, given that PthA4 inhibited CsCAF1 deadenylation activity in vitro, we hypothesized that PthA4 could also inhibit CsCAF1-dependent deadenylation of CsLOB1 to stabilize this message. This idea is consistent with the fact that PthA4 interacts with several citrus proteins implicated in mRNA stabilization (Domingues et al., 2015; de Souza et al., 2012) and that the increased expression of CsCAF1 in citrus leaves infiltrated with the citrus canker bacteria correlates with a decrease in CsLOB1 expression (Fig. 1A).

To examine this, the poly(A) tail length of the CsLOB1 mRNA in citrus leaves infiltrated with water, Xa, Xc or the Xc pthA4-deletion mutant, was analysed by the PAT assay (Salles and Strickland, 1999). We found polyadenylated CsLOB1 mRNA bands more abundantly in Xc-infected leaves compared to leaves
inoculated with Xa or the Xc pthA4-deletion mutant, both at 24 and 48 h after bacterial infiltration. On the other hand, a fragment of the CsLOB1 coding region was detected in all the leaf samples inspected, including those inoculated with the pthA4-deletion mutant (Fig. 6A–C). The detection of the CsLOB1 coding region in all examined leaves is in line with the results shown in Fig. 1 and previous expression data showing that CsLOB1 is also induced by Xa or the Xc pthA4-deletion mutant (Abe and Benedetti, 2016; Pereira et al., 2014).

The same experiment was performed to evaluate the poly(A) tail length of the mRNA encoded by the citrus CsPR1 gene, which, although highly induced by Xc and Xa (Fig. 1 and Cernadas et al., 2008), is not a direct target of PthA4 (Pereira et al., 2014). In contrast to CsLOB1, the CsPR1 mRNA poly(A) tail was strongly detected in all the leaves that had been infiltrated with the citrus canker pathogens, including the pthA4-deletion mutant (Fig. 6D–F). Together, these results show that the CsLOB1 mRNA poly(A) tail is specifically protected in Xc-infected leaves during initial canker development, suggesting that PthA4 not only increases CsLOB1 transcription but also stabilizes this message.

**A CsCAF1 inhibitor promotes canker development and inhibits PthA4-dependent transcription**

To search for small molecules that could serve as inhibitors of CsCAF1, a series of pyrazolo[4,3-d]-pyrimidine and quinazoline derivatives were synthesized and verified by nuclear magnetic resonance and mass spectrometry analysis, as reported previously (Rocco et al., 2004). The isolated molecules were tested for inhibition of CsCAF1 deadenylase activity in vitro. From a group of 72 test compounds, four compounds (41, 43, 44 and 69) were selected based on their capacity to inhibit CsCAF1 deadenylase activity at 80 µM concentration (Fig. 7A). These molecules were also tested for their ability to alter canker development in citrus leaves inoculated with Xc. Because compound 41 completely inhibited Xc growth in culture medium (Fig. 7B), it was not tested in citrus leaves and will be described elsewhere. Notably, we found that compound 69, an pyrazolo[4,3-d]-pyrimidine derivative that strongly inhibited CsCAF1 deadenylase activity in vitro, significantly enhanced canker formation in sweet orange leaves, compared to compounds 43 and 44 (Fig. 7A,C,D). Canker lesions developed in leaves treated with compound 69 were more...
enlarged and showed more epidermal rupture than those of untreated control leaves (Fig. 7D,E), indicating that compound 69 promotes cell division and growth induced by Xc.

These results led us to test whether compound 69 was stabilizing the CslOB1 mRNA. Surprisingly, we found that compound 69 did not apparently change the poly(A) tail length or stability of the CslOB1 and CspR1 transcripts, but significantly inhibited the PthA4-dependent transcription of CslOB1 without affecting CspR1 transcription (Fig. S3, see Supporting Information). These results were confirmed by qPCR analyses which also show that compound 69 induced CscAF1 expression in Xc-infected leaves, but repressed CscAF1 expression in leaves inoculated with the pthA4-deletion mutant (Fig. S3, see Supporting Information). Because CscAF1 expression was also significantly enhanced in leaves infiltrated with the pthA4-deletion mutant, the results suggest that PthA4 negatively regulates CscAF1 levels and supports the negative correlation observed between CscAF1 and PthA4-dependent CslOB1 expression.

**DISCUSSION**

CAF1 has been extensively studied in yeast and animals, but its role in plant development and response to pathogens remains
largely uncharacterized. Here we show that, similar to tomato and Arabidopsis CAF1, which play a role in pathogen defence (Lee et al., 2004; Liang et al., 2009; Sarowar et al., 2007), CsCAF1 is also implicated in the resistance against citrus canker bacteria. For instance, increased CsCAF1 expression correlated with a decrease in the expression of CsLOB1, the major canker

Fig. 7 A CsCAF1 inhibitor enhanced canker formation in citrus leaves. (A) In vitro CsCAF1 deadenylase activity assayed in the presence of pyrazolo[4,3-d]-pyrimidine and quinazoline derivatives. Compounds 41, 43, 44 and 69 inhibited, to different degrees, the CsCAF1 deadenylase activity at 80 µM concentration. Control reactions include samples with no CsCAF1 (N) or with CsCAF1 purified by affinity chromatography (P). (B) At 160 µM, compounds 43, 44 and 69 did not inhibited the growth of Xc in culture medium. (C) Chemical structures of compounds 43, 44 and 69. (D) Representative images of canker lesions developed in sweet orange leaves infected with Xc 10 days after bacterial inoculation. Cell hypertrophy and hyperplasia was significantly enhanced in leaves treated with compound 69 compared to compounds 43, 44 or water control. (E) Relative lesion area showing that canker lesions formed in leaves treated with compound 69 were significantly enlarged compared to those developed in control leaves. Values are the means of measurements performed in 48 independent canker lesions developed in four citrus leaves. Error bars represent the standard deviations and the asterisk denotes statistically significant difference at the 0.05 level, relative to control (water).
susceptibility gene induced by the Xc TAL effector PthA4 (Hu et al., 2014). Moreover, an inhibitor of CsCAF1 deadenylase activity, compound 69, significantly enhanced canker development in citrus leaves, suggesting that CsCAF1 restricts plant cell growth induced by Xc.

The mechanism by which CsCAF1 would contribute to bacterial resistance or restrict canker development is unknown; however, the fact that PthA4 inhibited CsCAF1 deadenylase activity in vitro and CsCAF1 transcription in vivo, and that polyadenylated CsLOB1 mRNA was more abundantly detected in citrus leaves infected with Xc than in leaves infected with Xc lacking pthA4 suggests that CsCAF1 might play a role in the transcription and/or stability of citrus canker susceptibility genes induced by PthA4.

We showed previously that PthA4 interacted with components of the citrus CCR4-NOT complex, including CsTRAX, CsPABPN, CsPABPC and CsVIP2 (de Souza et al., 2012). Here, we show that CsCAF1 also interacts with these proteins, indicating that it is a component of the citrus CCR4-NOT complex. It is thus possible that PthA4 could target the citrus CCR4-NOT complex to enhance transcription and translation of disease susceptibility messages. This idea is supported by the fact that the CCR4-NOT complex also interacts with Pol II to promote transcription elongation, particularly from arrested Pol II (Dutta et al., 2015; Kruk et al., 2013). Moreover, like PthA4, the Arabidopsis CsVIP2 homologue AtNOT12 binds the C-terminal domain of Pol II and enhances transcription of the CsLOB1 homologues AtLOB-1 and AtLOB-11 (Domingues et al., 2012; Wang et al., 2013). It should also be noted that PthA4 binds poly(U) RNA and is structurally related to PUF (pumelo and FBF) proteins, which recognize U-rich sequences at the 3′-end of mRNAs and are known to interact with CAF1 and recruit the CCR4-NOT complex to modulate mRNA stability and translation (Goldstrohm et al., 2006; Filipovska and Rackham, 2012; de Souza et al., 2012; Van Etten et al., 2012; Wang et al., 2018). Thus, given that the CCR4-NOT complex is a macromolecular machine that connects transcription elongation to translation (Babbarwal et al., 2014; Gupta et al., 2016; Villanyi et al., 2014; Webster et al., 2018; Yi et al., 2018), it seems reasonable to suggest that PthA4 could modulate its activity to enhance transcription and translation of citrus canker susceptibility genes.

Recombinant CsCAF1 showed specific activity towards poly(A) RNA and its deadenylase activity was dependent on magnesium ions. In this respect, CsCAF1 retains the major structural and functional properties of DEDDh nucleases. In fact, molecular modelling studies revealed that CsCAF1 is also structurally related to DEDDh nucleases that share no obvious sequence identity to it. This is the case of the human magnesium-dependent poly(A)-specific ribonuclease (PARN), which is also involved in poly(A) tail shortening (Körner and Wahle, 1997; Wu et al., 2005). According to our structural models, CsCAF1 not only shows the same type of fold as human PARN, but also shares the main amino acid residues involved in RNA recognition and poly(A) specificity that were mapped in the crystal structure of PARN in complex with poly(A) RNA (Wu et al., 2005). This indicates that CsCAF1 likely shows the same poly(A)-binding mode as human PARN (Fig. S4, see Supporting Information). Moreover, as shown for the Schizosaccharomyces pombe Pop2p protein (Jonstrup et al., 2007), the high degree of conservation of the DEDDh active site and divergent metal ion-binding residues among these enzymes suggests that CsCAF1 would also display the same mechanism of 3′-5′ exonuclease cleavage as Pop2p and PARN.

Small molecules that inhibit CAF1 deadenylase activity have recently been reported for human CAF1 (Jadhav et al., 2015; Maryati et al., 2014, 2015; Zhang et al., 2016). Because these inhibitors are not commercially available, we have synthesized a series of pyrazolo[4,3-d]-pyrimidine and quinazoline derivatives with the purpose of finding novel CAF1 inhibitors. Here we show that compound 69, an pyrazolo[4,3-d]-pyrimidine analogue, significantly inhibited CsCAF1 deadenylase activity in vitro while it promoted canker development in citrus leaves, indicating that CsCAF1 restricts cell growth in citrus. Although this result is consistent with the antitumour role played by CAF1-Tob proteins in mammalian cells (Doide et al., 2012; Hosoda et al., 2011), it contrasts with the findings that overexpression of Capsicum annum CAF1 led to enlarged leaf cells in pepper plants (Sarowar et al., 2007). In addition, compound 69 significantly increased canker formation despite decreasing CsLOB1 expression. This is, nevertheless, not a surprise since we have evidence suggesting that high CsLOB1 expression alone is not sufficient to induce citrus canker in sweet orange plants (Abe and Benedetti, 2016). Unfortunately, despite several attempts, we were unable to transiently modulate CsCAF1 expression in citrus leaves by Agrobacterium-mediated transformation (Jia and Wang, 2014). Therefore, the precise role of CsCAF1 in cell growth control, possibly involving transcription and translation of CsLOB1 or other canker susceptibility genes, will require further study.

**EXPERIMENTAL PROCEDURES**

**Protein expression and purification**

The cDNA corresponding to the CsCAF1 gene was subcloned into =pET28a for the expression of full-length 6×His-tagged CsCAF1 in Escherichia coli BL21 (DE3) cells. Bacterial cells were grown at 37 °C in LB medium containing kanamycin (50 µg/mL) to an OD600 ~0.6, after which 0.1 mM isopropylthio-D-galactoside (IPTG) was added to the culture. The cells were grown overnight at 18 °C, harvested by centrifugation and lyzed by sonication in lysis buffer containing 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 20% glycerol, 40 mM imidazole, 1.0 µg/mL lysozyme and 400 U DNase I (Sigma-Aldrich, San Luis, Missouri, USA). The suspension was centrifuged to remove cell debris and recombinant CsCAF1 was purified from the soluble...
fraction in a cobalt HiTrap chelating HP column (GE Healthcare, Chicago, Illinois, USA). The column was washed with ten column volumes of lysis buffer and CsCAF1 was eluted with the same buffer containing 200 mM imidazole. CsCAF1 fractions, analysed by SDS-PAGE, were concentrated, dialyzed against 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5% glycerol, and loaded on a Hi-load 16/60 Superdex 200 column (GE Healthcare) pre-equilibrated with the same buffer. CsCAF1 fractions eluted in a single peak were analysed by SDS-PAGE and concentrated. For estimation of CsCAF1 molecular weight, protein samples were loaded on an analytical Superdex 200 10/300 GL column, equilibrated with the same buffer and calibrated with a set of molecular weight standards (GE Healthcare). The elution volumes of the standards were used to generate a linear plot of the retention coefficients versus the logarithm of the molecular weight, which was used to estimate the molecular weight of CsCAF1.

GST and GST-tagged CsVIP2, CsTRAX, CsPABPN, CsPABPC (residues 327 to 652), PthA3 and PthA4, described previously (de Souza et al., 2012), were also expressed in E. coli BL21 cells upon induction with 0.1 mM IPTG. The cell pellets were suspended in phosphate-buffered saline (PBS) containing 150 mM NaCl, lysozyme (1.0 µg/mL) and DNase I. After sonication and centrifugation, soluble fractions of GST-tagged proteins were immobilized on glutathione resin (GE Healthcare) and unbound proteins were removed with four PBS washes. Elution was performed in PBS containing 7 mg/mL reduced glutathione. The quality of the purified proteins was analysed by SDS-PAGE.

**GST pulldown assays**

GST and the GST-tagged proteins CsVIP2, CsTRAX, CsPABPN, CsPABPC, PthA3 and PthA4 were immobilized on separate glutathione resin columns and unbound proteins were removed with four PBS washes. Purified 6×His-CsCAF1 was passed through each of the GST columns and the beads were washed four times with PBS to remove unbound proteins. Bound proteins were eluted with reduced glutathione and resolved on 12% SDS-PAGE gels. Proteins were transferred onto polyvinylidene difluoride membranes, probed with the anti-His (1:3000) and anti-GST (1:3000) and detected by chemiluminescence (Thermofisher, Waltham, Massachusetts, USA).

**Deadenylase activity assay**

To test the CsCAF1 exonuclease activity, the following RNA probes carrying a FITC tag at the 5′ position (Horiuchi et al., 2009) were used: GACUGACUUUUUUU, GACUGACUAAAAAAA, GACUGACUCCCCCC and GACUGACUGGGGGGG. Each tagged RNA was incubated at 37 °C with purified CsCAF1 (0.5 µM) in 20 mM HEPES buffer, pH 7.4, containing 150 mM NaCl and 1 mM DTT, and supplemented with 2 mM MgCl₂, CaCl₂, MnCl₂, CoCl₂, or ZnCl₂, for different time periods. The reactions were stopped by the addition of an equal volume of 37% (v/v) formamide. Ten microlitres of the reaction mixtures were loaded on denaturing DNA sequencing gels and the RNA probes were detected under UV-light using a FITC filter. For CsCAF1 inhibition assays, 80 µM of the candidate inhibitors, or dimethyl sulfoxide (DMSO) as a control, were added to the reaction mixtures.

**Yeast complementation**

The yeast pop2 deletion mutant YI3945 and corresponding wild-type strain BY4742 were obtained from the National BioResource Project (Japan). The mutant was transformed with the citrus CsCAF1 gene cloned into the pESC-URA vector (Agilent, Santa Clara, Califórnia, USA). For gene complementation assays, yeast cells were grown on 5G medium plates without leucine, in the absence or presence of 0.2, 0.5 or 2 mM caffeine at 30 °C for 2 days.

**Gene expression analysis**

Xanthomonas citri and X. aurantifolii pathotype C (Cernadas et al., 2008) were grown on LB medium without NaCl (LBON), supplemented with 100 mg/L ampicillin, for 48 h at 28 °C. Single colonies were suspended in sterile water to an OD₆₀₀ of 0.1, and the bacterial suspensions were used to infect sweet orange (C. sinensis) plants of cultivar Natal (Washington Navel) kept in the greenhouse. Citrus leaves of similar age and size were ground in liquid nitrogen and total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA). The quality and quantity of the RNA were verified by agarose gel. The RNA samples were treated with DNase I to remove traces of DNA and cDNAs were synthesized using the Maxima First Strand cDNA Synthesis kit (Fermentas, Waltham, MA, USA) according to the supplier’s instructions. The cDNA samples were diluted and tested for specificity and amplification efficiency of the probed genes relative to the actin gene used as the endogenous control (Mafra et al., 2012). Three PCRs were performed for each gene studied and three biological replicates were analysed using the SYBR Green mix and the universal conditions of amplification provided by the 7500 System (Applied Biosystems, Foster City, CA, USA). The results were analysed by the 7500 System software (Applied Biosystems) using the relative quantification mode and expressed as the mean of nine amplification curves. Primers used for RT-qPCR analysis include qPR1-F GCAAGGTTGTTGAGCCTATTAC, qPR1-R ACCAAATGCAACCCAAATT, qCAF1-F TCGTCCGGACCTGCTGTAAC, qCAF1-R AATCTCGGCTTTTGGCTCAA, qLOB1-F TTTTCACCAACGGAACCAT, qLOB1-R TGGATATGCTGACCCAGGAAGACTT, qActin-F CCCCCCTCATGCCATTCCT and qActin-R CGGCTGTTGTTGTAACATGT.

**Protein sequence alignment and molecular modelling**

Protein sequence alignments were performed with Clustal Omega using default parameters, whereas a tridimensional model of...
CsCAF1 was generated with Swiss-Model using the crystal structure of humanCAF1 protein (PDB code 4GML_B) (Petit et al., 2012) as the template model. Protein structures were aligned and visualized with PyMOL (Schrödinger, 2010).

Synthesis of CAF1 inhibitors

CsCAF1 inhibitors were synthesized based on previous protocols with minor modifications (Rocco et al., 2004). The syntheses were performed in single steps with the utilization of a thionyl chloride to obtain the 2-chloro-quinazoline, 4-chloro-quinazoline and 4-chloro-pyrazolo[4,3-d]-pyrimidine derivatives. The chlorine served as a leaving group to facilitate the nucleophilic aromatic substitution in the final synthesis step. This final step utilized the corresponding anilines and resulted in good yields. The protocols for the synthesis of compounds 43, 44 and 69 are detailed in the Supplementary Protocol S1. All compounds were dissolved in DMSO at 10 mM concentration and kept at −80 °C until use.

In vivo CsCAF1 inhibition assay

*Xanthomonas citri* was grown as above and a bacterial suspension in sterile water (OD 600 nm = 0.1) was used to infect Natal leaves by pinprick inoculations (Soprano et al., 2017). One hour after bacterial inoculation, the leaves were detached and placed in recipients containing water supplemented with 160 µM of the inhibitor molecules or DMSO as control. The leaves were kept in a plant growth room with a 12/12 h day/night light period at 25 °C. Treatment solutions were replaced every 2 days. Canker pustules were photographed 8 days after bacterial inoculation using a Nikon SMZ18 stereomicroscope, and the area of the lesions was measured using ImageJ software.

For bacterial survival assay, the *X. citri* cells in the water suspension were treated with 160 µM of the inhibitor molecules, or DMSO as control, for 1 h at 28 °C, after which they were spotted onto a LBON medium and incubated at 28 °C for 16 h.

Poly(A) tail length assay

The poly(A) tail length determination was performed using the PAT assay (Salles and Strickland, 1999). Total RNA was extracted from leaves infiltrated with water, *X. citri* or *X. aurantifolii* for 24 or 48 h, as described above. For cDNA construction, 2 µg of RNA, diluted in 5 µL diethyl pyrocarbonate (DEPC)-treated water, were mixed with 2 µL (200 ng) oligo dT- anchor (GACTCGAGTCGACATCGACACCTTTTTTTTTTTTTTTTTTTTTT) and each reaction was mixed and incubated at 12 °C for 2 h. After incubation, the reactions were warmed at 42 °C for 2 min and 1 µL of ReverseAid H minus first-strand CDNA synthesis enzyme was added and incubated at 42 °C for 1 h. The reverse transcriptase and ligase enzymes were heat inactivated by incubation at 65 °C for 20 min and the cDNA samples were used for the PCRs. Detection of the poly(A) tail length was carried out using oligos LOB1-F1 (CCACCAACCAAGCATAAAGTTCACC or PR1-F2 (GCAAGGTGTGTGGGCACATAC) in combination with oligo dT-anchor, and the amplified fragments were resolved by electrophoresis on a 2.0% agarose gel stained with ethidium bromide. Control PCRs for the amplifications of CsLOB1 and PR1 coding regions were performed with the pair of oligonucleotides LOB1-F1 and LOB1-R (AGAGGCTCCCAAAGCTGTAC), and PR1-F1 (TCCCCATGCACAGACTACC) and PR1-R (ACCCAATGGGAACCGAATT), respectively.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site: Fig. S1 Functional complementation assay of the yeast pop2 mutant showing that CsCAF1 does not complement the caffeine sensitivity phenotype of the yeast mutant in 5D medium containing 0.2 mM, 0.5 mM or 2.0 mM caffeine.

Fig. S2 Ten percent polyacrylamide SDS PAGE gel of the recombinant proteins 6×His CsCaf1, GST and GST fusions CsPABPC, CsPABPN, CsVIP2, CsTRAX, PthA3 and PthA4, purified by affinity chromatography. The arrows indicate the corresponding protein bands with the expected molecular size. The molecular mass ruler is indicated on the left.

Fig. S3 The CsCaf1 inhibitor compound 69 modulates the expression of CsfCaf1, CsLOB1 but not CsSPP1 in citrus leaves. A) PAT assay showing that compound 69 significantly inhibited the accumulation of polyadenylated CsLOB1 but not CsSPP1 transcripts in Xc infected plants only, at 48 h post- bacterial inoculation. (B) RT qPCR analyses showing that compound 69 significantly inhibited the PthA4-dependent expression of CsLOB1 but not CsSPP1.
in Xc infected leaves, corroborating the PAT assay data depicted in panel A. Conversely, compound 69 induced the expression of CsCAF1 and CsLOB1, but not CsPR1, in noninfected leaves. The expression levels of CsCAF1 was also significantly increased in leaves inoculated with the pthA4 deletion mutant, which suggests that PthA4 represses CsCAF1 in citrus leaves. This PthA4-dependent repression of CsCAF1 was inhibited by compound 69.

Fig. S4 CsCAF1 shares the same protein fold and poly(A) binding mode as human PARN. (A) Superposition of the crystal structure of human PARN (PDB code 2A1R, grey) with the structural model of CsCAF1 (green) generated by SWISS MODEL using the human NOT7 structure as the search template. CsCAF1 shows the same type of protein fold as human PARN despite sharing low sequence identity to PARN. (B) Close view of the active site of the proteins showing the conservation of the amino acid residues (sticks) involved in RNA recognition between PARN and CsCAF1. The magnesium ions suggested to participate in the hydrolyses of the RNA phosphodiester bond are shown as green spheres.