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Short communication

The P0 protein encoded by cotton leafroll dwarf virus (CLRDV) inhibits local but not systemic RNA silencing

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ABSTRACT

Plants employ RNA silencing as a natural defense mechanism against viruses. As a counter-defense, viruses encode silencing suppressor proteins (SSPs) that suppress RNA silencing. Most, but not all, the P0 proteins encoded by poleroviruses have been identified as SSP. In this study, we demonstrated that cotton leafroll dwarf virus (CLRDV, genus *Polerovirus*) P0 protein suppressed local silencing that was induced by sense or inverted repeat transgenes in *Agrobacterium* co-infiltration assay in *Nicotiana benthamiana* plants. A CLRDV full-length infectious cDNA clone that is able to infect *N. benthamiana* through *Agrobacterium*-mediated inoculation also inhibited local silencing in co-infiltration assays, suggesting that the P0 protein exhibits similar RNA silencing suppression activity when expressed from the full-length viral genome. On the other hand, the P0 protein did not efficiently inhibit the spread of systemic silencing signals. Moreover, Northern blotting indicated that the P0 protein inhibits the generation of secondary but not primary small interfering RNAs. The study of CLRDV P0 suppression activity may contribute to understanding the molecular mechanisms involved in the induction of cotton blue disease by CLRDV infection.

Cotton leafroll dwarf virus (CLRDV) is the causal agent of cotton blue disease (CBD) and belongs to the *Luteoviridae* family within the genus *Polerovirus* (Corrêa et al., 2005; Distéfano et al., 2010). Important members of the genus are potato leafroll virus (PLRV, the type member), beet western yellows virus (BWYV), turnip yellows virus (TuYV, formerly known as beet western yellows virus-FL1), and cucurbit aphid-borne yellows virus (CABYV) (King et al., 2011). In all *Polerovirus* species, the genome consists of a monopartite, single-stranded, positive-sense RNA of ~5.7 kb with six recognized open reading frames (ORFs). Although CBD is the most important viral disease of cotton in South America, the complete nucleotide sequence of CLRDV genome was recently determined (Distéfano et al., 2010). Moreover, unlike in the case for other *poleroviruses*, there are no studies on the function and subcellular localization of CLRDV-encoded proteins. The 5′-proximal ORF 0 is translated from the genomic viral RNA, which codes for the P0 silencing suppressor protein (Pfeffer et al., 2002). P0 is the most divergent protein among poleroviruses (Mayo and Miller, 1999) and is also the least conserved protein of CLRDV, with amino acid sequence identity of 15–30% compared to other members of the genus. The P0 proteins from TuYV, CABYV, PLRV (European isolate), melon aphid-borne yellow virus (MABYV) and beet mild yellowing virus (BMYV) are all silencing suppressor proteins (SSPs) of local, but not systemic, RNA silencing (Han et al., 2010; Kozlowska-Makulska et al., 2010; Pfeffer et al., 2002). Interestingly, the P0 proteins display diverse levels of RNA silencing suppression activity. A recent study demonstrated that the P0 proteins of two closely related beet poleroviruses exhibited strain-specific differences in their effects on RNA silencing (Kozlowska-Makulska et al., 2010). Moreover, the P0 proteins of two isolates of BMYV and of six isolates of beet chlorosis virus (BChV) displayed no detectable RNA-silencing suppression activity. Furthermore, the P0 proteins of sugarcane yellow leaf virus (SCYLV), PLRV (Australian isolate), the unclassified beet yellow dwarf virus-GPV (BYDV-GPV) and pea enation mosaic virus-1 (PEMV-1, *Enamovirus* genus) suppressed both local and systemic RNA silencing (Fusaro et al., 2012; Liu et al., 2012; Mangwende et al., 2009).

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The P0 proteins of TuYV and CABYV were shown to interact through their F-box motif with the *Arabidopsis* S-phase kinase-associated protein 1 (SKP1) orthologs ASK1 and ASK2, which are components of the SKP1-Cullin-F-box (SCF) family of E3 ubiquitin ligases (Pazhouhandeh et al., 2006). This interaction leads to the degradation of the ARGONAUTE1 (AGO1) protein, which is the core component of the RNA silencing pathway (Baumberger et al., 2007; Bortolamiol et al., 2007). Recently, Derrien et al. (2012) demonstrated that P0-induced AGO1 degradation depends on the autophagy pathway. This finding supports a model in which a viral SCF^{P0} E3 ligase promotes AGO1 turnover through autophagy (Incarbone and Dunoyer, 2013). The F-Box-like motif present in other P0 proteins is also conserved in CLRDV P0 protein (68-**LP**xx**I**$x_{(10)}$**P**-84).

In this study we examined the silencing suppression activity of P0 from CLRDV ($P0^{CL}$) by using GFP agroinfiltration assays on *Nicotiana benthamiana* plants. The $P0^{CL}$ protein was analyzed either in its viral context or alone.

To investigate whether $P0^{CL}$ is a suppressor of RNA silencing, the ORF0 was amplified by PCR from an infectious cDNA clone of CLRDV (Delfosse et al., 2013). The PCR was performed using appropriate primers carrying non-viral extensions (italicized nucleotides) and restriction sites (underlined sequence) (5′ *CCACTAGTTTCC*ATGGTGAATTTGATCATCTGCAG 3′ and 5′ *GTGGATCCG*ATCAACTGCTTTCTCCTTCAC 3′) along with Platinum Pfx long DNA Polymerase (Invitrogen). The PCR fragment was cloned into pGEM®-T Easy Vector (Promega), sequenced and finally released with *Spe*I and *Bam*HI. This fragment was then transferred into the binary plasmid pBin61 (Voinnet et al., 2000) to produce pBin-$P0^{CL}$, which was subsequently transformed into the *Agrobacterium tumefasciens* strain C58C1 (Hamilton et al., 1996). The RNA silencing suppression tests were performed using the *N. benthamiana* line 16c, which has an integrated GFP transgene (Brigneti et al., 1998). The expression of the GFP transgene can be silenced by infiltrating a lower leaf with a binary vector designed to transiently express extra copies of the GFP transcript (pBin-GFP) (Voinnet et al., 1998). Such induced GFP silencing can be blocked by simultaneous infiltration of a vector expressing a SSP (Voinnet et al., 2000). In this way, when *N. benthamiana* 16c leaves were co-infiltrated with GFP plus $P0^{CL}$, the infiltrated patches appeared fluorescent at 5 days post-infiltration (p.i.), indicating that $P0^{CL}$ can interfere with RNA silencing (Fig. 1A, panel d). The patch co-infiltrated with GFP and pBin61 displayed silencing of the GFP signal, as evidenced by its deep red color (Fig. 1A, panel a). On the other hand, patches with GFP plus $P0^{CA}$ (CA: CABYV) and $P0^{Tu}$ (Tu: TuYV) (positive controls) (Bortolamiol et al., 2007; Pfeffer et al., 2002) became fluoresced brightly (Fig. 1A, panels b and c, respectively). However, the relative intensities of the GFP fluorescence in the different patches suggest that RNA silencing suppression activity of $P0^{CL}$ is weaker than that of $P0^{Tu}$ (a strong SSP). We further enquired whether a full-length CLRDV RNA, where the $P0^{CL}$ gene is expressed in the viral context, can elicit silencing suppression in the patch assay as it does when it is expressed alone. This is particularly relevant as all the P0's start codons are in suboptimal translation initiation context in poleroviruses (Mayo and Miller, 1999). The agroinoculation of *N. benthamiana* plants with a pBin-19-based binary construct containing a full-length CLRDV cDNA downstream a 35S promoter leads to the production of RNA transcripts, which are able to initiate a viral infection (Delfosse et al., 2013). The co-infiltration of *N. benthamiana* 16c plants with GFP plus CLRDV produced effective suppression of GFP silencing (Fig. 1A, panel e) with a fluorescence as bright as that of the patch treated with $P0^{CL}$ (Fig. 1A, panel f). Total RNA was extracted from the infiltrated leaves (as described by Bortolamiol et al., 2007) and analyzed by Northern blotting to detect the GFP mRNAs or small interfering RNAs (siRNAs). GFP transcript levels were consistent with the visual observation of $P0^{CL}$-mediated silencing suppression. At 5 days p.i., the level of GFP transcripts in the patches infiltrated with $P0^{CL}$ or CLRDV plus GFP (Fig. 1B, lanes 5 and 6, respectively) was lower than in the patches with $P0^{CA}$ or $P0^{Tu}$ (Fig. 1B, lanes 3 and 4, respectively). By contrast, the GFP transcripts in the patches treated with GFP plus pBin61 were almost undetectable (Fig. 1B, lane 2). The presence of both $P0^{CL}$ mRNAs and CLRDV genomic RNA (gRNA) was easily detected using a $P0^{CL}$ probe (Fig. 1E, lanes 5 and 6). RNA silencing is mediated through the production of siRNAs homologous to the target mRNA (Hamilton and Baulcombe, 1999). GFP-specific siRNAs (21–24 nt) were easily detected by Northern blotting in patches that had received GFP plus pBin61 (Fig. 1C, lane 2). However, these siRNAs were reduced in patches infiltrated with GFP plus $P0^{CA}$, $P0^{Tu}$, $P0^{CL}$ or CLRDV (Fig. 1C, lanes 3, 4, 5 and 6, respectively). The stabilization of functional GFP mRNA was further confirmed by the detection of GFP protein in the infiltrated 16c line through Western blot analysis (Fig. 1D). The patches co-infiltrated with GFP plus $P0^{CL}$, CLRDV or with the controls ($P0^{CA}$ or $P0^{Tu}$) exhibited increased expression levels of GFP when compared with patches of non-infiltrated 16c plants (Fig. 1D, lanes 5, 6, 3 and 4). By contrast, the patches infiltrated with GFP plus pBin61 displayed a severe reduction in GFP levels in accordance with silencing of GFP mRNA (Fig. 1D, lane 2).

We then explored the effect of $P0^{CL}$ on systemic spread of RNA silencing. For this purpose, the 16c plants were further observed at later times to monitor GFP expression or silencing in newly emerging upper leaves. A total of 80% of the 16c plants that had received GFP plus $P0^{CL}$ or CLRDV displayed vein-proximal silencing of GFP signal at 15 days p.i. in the upper leaves (Fig. 2, panels e and f). This result indicates that $P0^{CL}$ was unable to interfere with the systemic spread of RNA silencing. Interestingly, the leaves that had been infiltrated with $P0^{CL}$ and CLRDV exhibited red halos around the patches (Fig. 2, insert panel in e and f), which are indicative of short-distance cell-to-cell movement of the silencing signal. At 15 days p.i., the differences between the P0 proteins of CLRDV, TuYV and CABYV were clear. The patches that received $P0^{Tu}$ or $P0^{CA}$ remained highly fluorescent for up to 15 days (Fig. 2, panel c and d insert). By contrast, in the patches with pBin-$P0^{CL}$ or CLRDV the green fluorescence began to decrease at 8–10 days p.i. and had significantly faded at 15 days p.i. (Fig. 2, panel e and f insert). These findings could indicate a difference either in protein stability or in activity duration.

In the RNA silencing pathway, primary siRNAs are incorporated into AGO1 in the RISC complex, which mediates the amplification of the silencing signal *via* the action of a host RNA-dependant RNA polymerase. This mechanism generates a massive production of secondary siRNAs (Voinnet, 2005). To identify the level of action of $P0^{CL}$ in the silencing pathway, we investigated the effect on the production of primary and secondary siRNAs in an inverted repeat transgene assay. *N. benthamiana* leaves were infiltrated with a mixture of *A. tumefasciens* cultures that harbor binary vectors encoding a hairpin GF (hpGF) with only the 5′ half of the GFP gene (Himber et al., 2003) plus either $P0^{CL}$ or CLRDV. Similar levels of primary siRNA were detected either with or without $P0^{CL}$ or CLRDV (supplementary figure, compare lane 1 to lanes 5 and 6). This result indicates that $P0^{CL}$ interferes with a step in the RNA silencing pathway that is downstream of primary siRNA production. In a second experiment, the suppression activity of $P0^{CL}$ and its effect on secondary siRNAs were compared to those of $P0^{Tu}$, $P0^{CA}$ and P38 (the SSP of turnip crinkle virus, a *Carmovirus*). For this purpose, we performed co-infiltration experiments with GFP plus hpGF into *N. benthamiana* leaves (Fig. 3A). The patches infiltrated with GFP plus pBin61 exhibited pale fluorescence at 5 days p.i. (Fig. 3A, panel a). An efficient silencing against GFP was observed when GFP was co-infiltrated with the hairpin construct hpGF (Fig. 3A, panel b). This result was confirmed by the presence of low levels of GFP mRNA (Fig. 3B, lane 4) and high accumulation of primary GFP siRNAs (Fig. 3C, upper panel, lane 4). When $P0^{CL}$ or CLRDV were added,

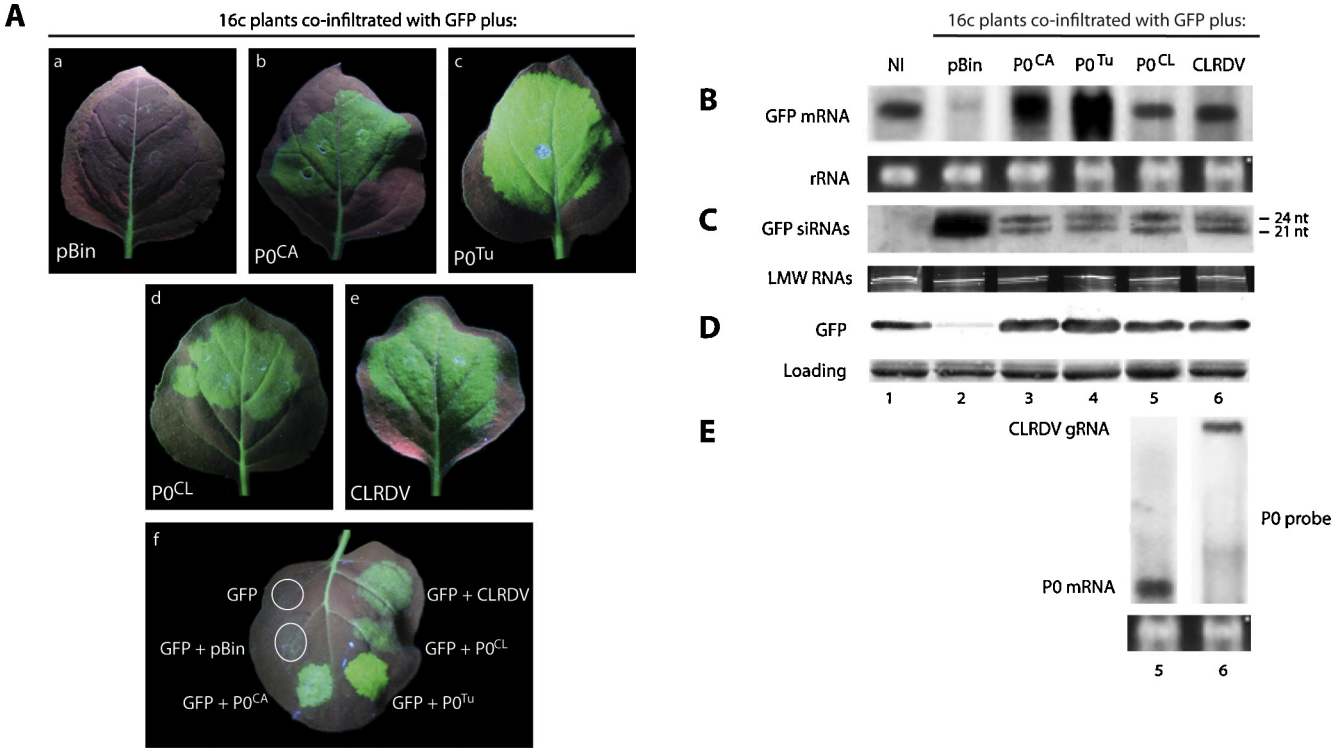

Fig. 1. CLRDV P0 protein suppresses local RNA silencing. (A) Leaves of *N. benthamiana* plants 16c line were agroinfiltrated with a mixture of agrobacteria harboring GFP plus either pBin61 (panels a and f), $P0^{CA}$ (panels b and f), $P0^{Tu}$ (panels c and f), $P0^{CL}$ (panels d and f) or CLRDV (panels e and f). Each agroinfiltration experiment was repeated three times independently. Photographs were taken with long-wavelength UV light at 5 days p.i. (B) Total RNAs (10 μg) from patches infiltrated with GFP plus pBin61, $P0^{CA}$, $P0^{Tu}$, $P0^{CL}$ or CLRDV (lanes 2, 3, 4, 5 and 6, respectively) and from non-infiltrated 16c leaves (NI, lane 1) were analyzed by Northern blotting. The RNAs were probed with a ^{32}P-labeled DNA probe specific to GFP. Ethidium bromide-stained rRNAs were used as loading controls. (C) Total RNAs (10 μg) from samples described in (B) were fractioned on 17.5% polyacrylamide gel, transferred to Hybond-XL membrane and probed with a ^{32}P-labeled DNA probe specific to GFP to analyze for the presence of GFP siRNAs. Low-molecular-weight (LMW) RNAs were used as a loading control. RNA size markers (21 and 24 nt) are indicated on the right. (D) Immunoblot analysis of GFP protein from infiltrated patches. Total protein extracts from the same samples as in (B) were analyzed using an anti-GFP monoclonal antibody (Abcam). Total proteins stained with Ponceau S after electroblotting are shown below. (E) Total RNAs from patches with GFP plus $P0^{CL}$ or CLRDV (lanes 5 and 6, respectively) were analyzed by Northern blotting and probed with a DNA fragment specific to $P0^{CL}$ to analyze the presence of P0 mRNA (lane 5) or CLRDV gRNA (lane 6).

the infiltrated leaves displayed green fluorescence and increased GFP mRNA levels (Fig. 3A, panels f and g and 3B, lanes 8 and 9) as compared to the pBin61 control (Fig. 3A, panel a and 3B, lane 4). However, these levels were weaker than those obtained with P38, $P0^{CA}$ or $P0^{Tu}$ (Fig. 3A, panels c–e and 3B, lanes 5–7) and consistent with the previous results (Fig. 1B). Moreover, the presence of $P0^{CL}$ mRNA and CLRDV gRNA was confirmed by Northern blotting (Fig. 3D, lanes 8 and 9). The 21-nt primary siRNAs of GFP produced in the presence of $P0^{CL}$ or CLRDV (Fig. 3C, upper panel, lanes 8 and 9) accumulated significantly less abundantly than with pBin61 (Fig. 3C, upper panel, lane 4). Similar results were obtained for the controls $P0^{CA}$ or $P0^{Tu}$ (Fig. 3C, upper panel, lanes 6 and 7). By contrast, the primary siRNAs produced in the presence of P38 were undetectable (Fig. 3C, upper panel, lane 5). This result is consistent with the known effect of this SSP on RNAse III-type Dicer-like (DCL) protein 2 and 4 (Deleris et al., 2006). Remarkably, a selective accumulation of the 24-nt GFP siRNAs was observed in the patch treated with $P0^{CL}$ (Fig. 3C, upper panel, lane 8). However, in our experimental conditions, this effect was not clearly observed in the patch with CLRDV (Fig. 3C, upper panel, lane 9). The secondary GFP siRNAs derived from the RDR6-directed amplification process were visualized by a "P" probe that targets a region outside of the "GF" sequence. The leaves that were infiltrated with $P0^{CL}$ or CLRDV exhibited a complete reduction in the accumulation of all size classes of secondary siRNAs (Fig. 3C, lower panel, lanes 8 and 9) as was also observed for $P0^{Tu}$ and $P0^{CL}$ (lanes 6 and 7). These data are in agreement with those previously reported showing that P0 proteins inhibited the production of all size of secondary siRNA (Baumberger et al., 2007; Bortolamiol et al., 2007).

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In this study, we have demonstrated that $P0^{CL}$ suppressed local RNA silencing in *N. benthamiana*. However, $P0^{CL}$ did not efficiently inhibit the spread of both local and systemic silencing signal. It is noteworthy to mention that $P0^{CL}$ displayed similar silencing suppression activity either when $P0^{CL}$ is expressed in the patch from the viral genome or when expressed alone. To our knowledge, the P0 proteins encoded by TuYV and BMYV ($P0^{BM}$) were the only P0 proteins studied for their RNA silencing suppression activity in their viral genomic context. $P0^{BM}$ displayed the same strong silencing suppression activity when expressed from an infectious clone or from a monocistronic construct, even though the AUG is in a suboptimal translation initiation context (Klein et al., 2014). On the other hand, the silencing suppression activity displayed by $P0^{Tu}$ is much stronger when the protein is produced alone rather than from the viral RNA (Pfeffer et al., 2002). This finding suggests that in the case of the P0 proteins, other factors apart from the efficiency of translation may be critical to the silencing suppression strength in the viral environment.

As previously described for other P0 proteins, $P0^{CL}$ interferes with secondary but not with primary siRNAs production (Bortolamiol et al., 2007; Fusaro et al., 2012). These findings indicate that $P0^{CL}$ inhibits a step in the RNA silencing pathway downstream

Fig. 2. Systemic RNA silencing suppression assay. Transgenic *N. benthamiana* 16c plants were infiltrated with mixtures harboring GFP plus one of the following vectors: pBin61 (panel b), $P0^{CA}$ (panel c), $P0^{Tu}$ (panel d), $P0^{CL}$ (panel e) or CLRDV (panel f). Non-infiltrated (NI) 16c plants (panel a) were used as a control. Pictures of whole plants were taken under UV light at 15 days p.i. The number of plants that displayed systemic silencing out of the total number of plants is indicated for each construct. The inserts show an agroinfiltrated leaf from each plant harvested at the same time.

of DCL-mediated primary siRNA production. The 21/22-nt siRNAs are the major guides of RNA degradation in RNA silencing, while the 24-nt siRNAs are involved in transcriptional gene silencing (TGS) (Incarbone and Dunoyer, 2013; Llave, 2010). A clear selective accumulation of the 24-nt GFP-siRNAs was observed in the patch with $P0^{CL}$ in the GF inverted repeat assay in *N. benthamiana*. Therefore, $P0^{CL}$ may be interfering with DCL4 and to a lesser extent, with DCL3. However, under our experimental conditions, this effect was not as clearly observed in the patch with CLRDV, where P0 is expressed in its viral context. Therefore, we cannot exclude the existence of another viral SSP, as it has been reported for other viruses (Lu et al., 2004), or an interaction between $P0^{CL}$ and another viral factor. During CLRDV infection, the 24-nt heterochromatin-associated siRNAs were quantitatively and qualitatively altered and an upregulation of *GHDcl4* as well as a downregulation of *GHDcl2* were observed (Romanel et al., 2012). Further studies are required to understand the mode of action of $P0^{CL}$. For instance, it would be informative to investigate whether $P0^{CL}$ interacts with the *Arabidopsis* S-phase kinase-associated protein 1 (SKP1) orthologs ASK1 and ASK2 (Pazhouhandeh et al., 2006) and with AGO1, as has been performed for $P0^{Tu}$ (Baumberger et al., 2007; Derrien et al., 2012).

The F-box-like domain at the N-terminal region of P0 proteins was reported to be important for their silencing suppression activity (Baumberger et al., 2007; Fusaro et al., 2012; Han et al., 2010; Pazhouhandeh et al., 2006). In addition, $P0^{CA}$, $P0^{MA}$ and $P0^{Tu}$ contain a C-terminal-proximal sequence (K/R)IYGEDGX$_3$FWR, which could represent a previously undescribed type of substrate interaction domain (Pazhouhandeh et al., 2006; Han et al., 2010). Han et al. (2010) demonstrated that the strong RNA silencing suppression activity of $P0^{MA}$ requires, besides to the F-box-like domain, several other motif-like domains. They showed that the Phe (F) and Trp (W) residues present in the C-terminal-proximal sequence [(K/R)IYGEDGX$_3$**FW**R] play a critical role in the silencing suppression function of P0. The C-terminal-proximal motif of $P0^{CL}$ carries the FW motif, while other positions in the domain vary ($P0^{CL}$: NLYGDGAX$_3$**FW**R) displaying only a 50% identity with the consensus sequence. The RNA silencing suppression activity of $P0^{CL}$ seems

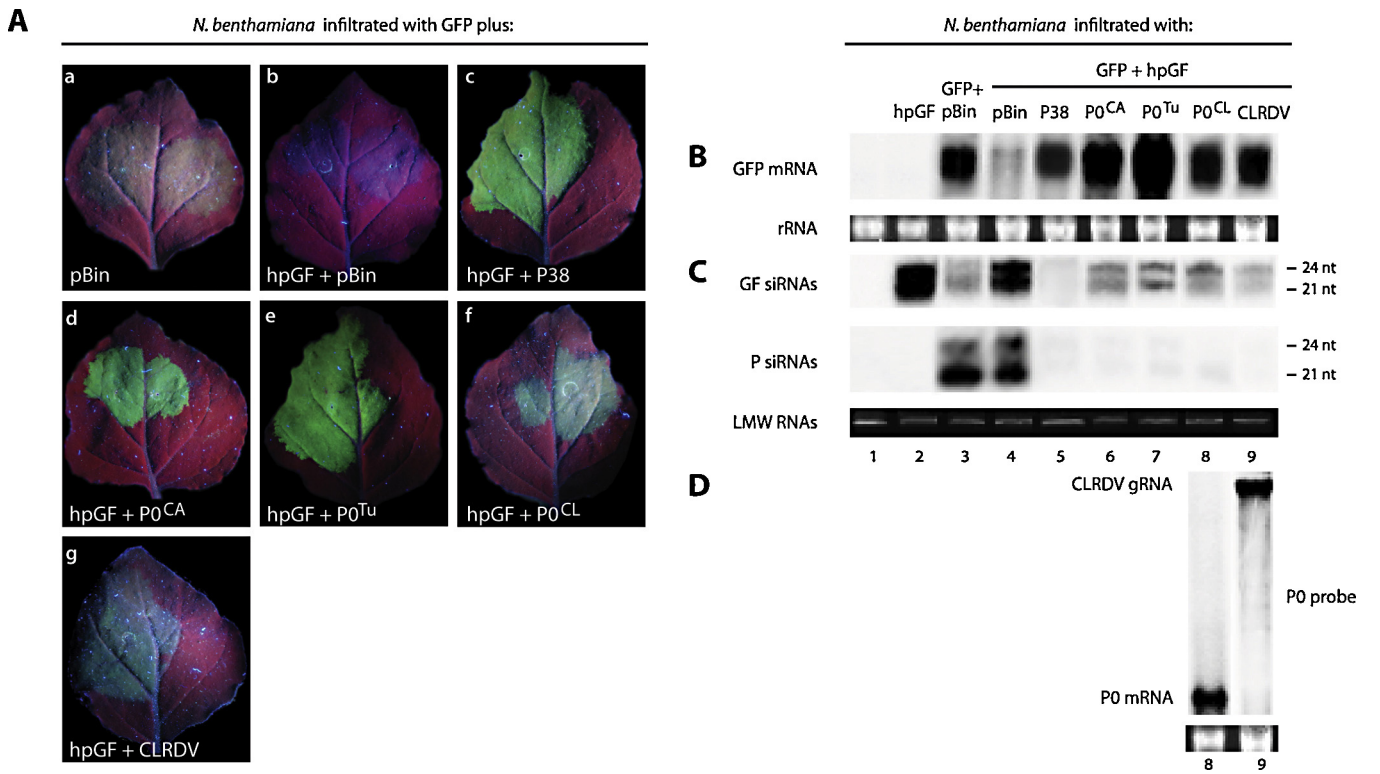

Fig. 3. CLRDV P0 protein suppresses silencing triggered by an inverted-repeat sequence. (A) *N. benthamiana* leaves were infiltrated with a mixture of agrobacteria containing GFP and hpGF plus either pBin61 (panel b), P38 (panel c), $P0^{CA}$ (panel d), $P0^{Tu}$ (panel e), $P0^{CL}$ (panel f) or CLRDV (panel g). The GFP vector plus pBin61 (panel a) was used as a control. The agroinfiltration experiment was performed three times independently. Photographs were taken at 5 days p.i. (B) Total RNAs from patches containing GFP and hpGF plus either pBin61, P38, $P0^{CA}$, $P0^{Tu}$, $P0^{CL}$ or CLRDV (lanes 4, 5, 6, 7, 8 and 9, respectively) were analyzed by Northern blotting. The RNAs were probed with a ^{32}P-labeled DNA probe specific to GF. Controls show RNA from patches with pBin-GFP plus empty vector pBin61 (lane 3), pBin-hpGF (lane 2) and from non-infiltrated *N. benthamiana* leaves (lane 1). (C) Total RNAs from samples described in (B) were fractioned as indicated in Fig. 1C and analyzed for the presence of GF primary siRNAs (^{32}P-labeled GF probe, upper panel) or secondary P siRNAs (^{32}P-labeled P probe, lower panel). (D) Total RNAs from patches with GFP and hpGF plus $P0^{CL}$ or CLRDV (lanes 8 and 9, respectively) were probed with a DNA fragment specific to $P0^{CL}$ for the presence of P0 mRNA (lane 8) or CLRDV gRNA (lane 9).

weaker than that of $P0^{Tu}$ and $P0^{CA}$. One possible explanation for this could be that the C-terminal-proximal motif directly influences the activity of the protein. Another hypothesis could be that the $P0^{CL}$ protein is unstable under our experimental conditions. However, such hypotheses could not be tested because, at present, there are no available specific antibodies targeting $P0^{CL}$.

To our knowledge, this study provides the first evidence that P0 of CLRDV is a SSP. The data herein generated provide a starting point to understanding the molecular mechanisms involved in the induction of cotton blue disease by CLRDV infection.

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