Two suppressors of RNA silencing encoded by cereal-infecting members of the family *Luteoviridae*

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Several members of the family *Luteoviridae* are important pathogens of cultivated plant species of the family Gramineae. In this study, we explored RNA-silencing suppressors (RSSs) encoded by two cereal-infecting luteoviruses: barley yellow dwarf virus and wheat yellow dwarf virus (BYDV and WYDV, respectively). The P0 protein of WYDV-GPV (P0<sub>GPV</sub>) and the P6 protein of BYDV-GAV (P6<sub>GAV</sub>) displayed RSS activities when expressed in agro-infiltrated leaves of *Nicotiana benthamiana*, by their local ability to inhibit post-transcriptional gene silencing of GFP. Analysis of GFP, mRNA and GFP-specific small interfering RNA indicated that both P0<sub>GPV</sub> and P6<sub>GAV</sub> are suppressors of silencing that can restrain not only local but also systemic gene silencing. This is the first report of RSS activity of the P6 protein in a member of the genus *Luteovirus*.

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Barley yellow dwarf viruses (BYDVs), belonging to the family *Luteoviridae*, are naturally transmitted by different species of cereal aphids. They have an ssRNA monopartite genome with a size that varies from 5600 to 6000 nt, depending upon the species and isolate. The host range of BYDVs is limited to the family Gramineae, including barley, wheat, oats and grasses (Gray & Gildow, 2003). Early work by Rochow and others distinguished five different strains of the virus, based on their primary aphid vector (Rochow, 1969; Rochow & Muller, 1971). The cereal-infecting members of the family *Luteoviridae* comprise species BYDV-PAV, -MAV and -PAS within the genus *Luteovirus*; cereal yellow dwarf virus-RPV (CYDV-RPV, formerly BYDV-RPV) and CYDV-RPS in the genus *Polerovirus*, as well as BYDV-SGV and -RMV, which have not yet been assigned to any genus. In China, four BYDV species, namely BYDV-GAV, -GPV, -PAV and -RMV, were reported (Zhou et al., 2009; Jin et al., 2004; Liu et al., 2007a; Zhang et al., 2009; Wu et al., 2011). In the most recent report by the International Committee on Taxonomy of Viruses (King et al., 2011), BYDV-GPV is considered an as-yet-unassigned member of the family *Luteoviridae*. However, following analysis of the complete sequence, its classification in the genus *Polerovirus* and the new name wheat yellow dwarf virus (WYDV)-GPV were proposed (Zhang et al., 2009). For clarity, in the present work the luteovirus corresponding to GenBank accession no. FM865413 will be named WYDV-GPV. WYDV-GPV is distributed mainly in the north-west of China and in Sweden (Kvarnheden, 2011). Its genome comprises 5673 nt, with six predicted ORFs (P0–P5) and three UTRs that are similar to those in members of the genus *Polerovirus* (Zhang et al., 2009) (Fig. 1a). The genome of BYDV-GAV, the most popular species found in China in recent years (Liu et al., 2007b), comprises 5685 nt (GenBank accession no. AY220739) with an organization similar to those of members of the genus *Luteovirus*, and contains six ORFs (P1–P6) and four UTRs (Jin et al., 2004) (Fig. 1b).

The phenomenon of RNA-silencing suppression was first discovered in GFP-transgenic plants infected with potato virus Y and cucumber mosaic virus (Brigneti et al., 1998). Subsequently, the list for such transgene silencing entities has extended to include many plants (Alvarado & Scholthof, 2009).Viruses have evolved a variety of proteins, known as suppressors of gene silencing, that are able to target all steps of the silencing pathway, such as viral RNA recognition, dicing, RISC assembly, RNA targeting and amplification (Burgyán & Havelda, 2011). More than 40 viral silencing suppressors in plants have been identified, and many of them are multifunctional and play important roles in virus replication, coiling, movement and pathogenesis, in addition to suppressing host RNA silencing-based antiviral immunity (Csorba et al., 2009). In the family *Luteoviridae*, ORF0 is particularly characteristic of members of the genus *Polerovirus*. It has been identified as an RNA-silencing suppressor (RSS) in many viruses, including beet mild yellowing virus (BMYM), beet western yellow virus (BWYV), cucurbit aphid-borne yellows virus (CABYV), potato leafroll virus (PLRV), sugar cane yellow leaf virus (SCYLV) and turnip yellows virus (TuYV) (Pfeffer et al., 2002; Kozlowska-Makulska, et al., 2010; Mangwende et al., 2009).

The aim of this study was to evaluate the RSS activity of different proteins encoded by WYDV-GPV and BYDV-GAV originating from China by using GFP agro-infiltration assays...
on Nicotiana benthamiana. The selected genes were P0, UP0 (5′UTR + P0), P3 (CP) and P4 (MP) for WYDV-GPV, and P1, P3 (CP) and P6 for BYDV-GAV.

The fragments carrying the genes of interest of WYDV-GPV were first PCR-amplified and cloned in pMD18-T Simple (Takara), then released with XhoI and SalI and transferred into corresponding sites of binary plasmid pGD (Goodin et al., 2002) to produce the constructs CP<sub>GPV</sub>, MP<sub>GPV</sub>, P0<sub>GPV</sub> or UP0<sub>GPV</sub>. For BYDV-GAV, the PCR-amplified genes cloned into pMD18-T Simple were inserted into pMCG161 (an intermediate vector, which contains a 35S promoter-expression gene) (http://www.chromdb.org; GenBank accession no. AY220739), then the constructs were digested with XmaI and KpnI in pMCG161 and fragments carrying the 35S promoter were inserted into the same restriction site of pBinPLUS (van Engelen et al., 1995) to produce the constructs CP<sub>GAV</sub>, P1<sub>GAV</sub> or P6<sub>GAV</sub>. The empty vectors pGD and pBinPLUS were used as negative controls. Helper component–proteinase (HC-Pro) of tobacco etch virus (TEV) was amplified and used to generate pBin HC-Pro<sub>TEV</sub> as a positive control. The construct pBin mgfp5–ER, carrying a modified GFP for efficient sorting to the endoplasmic reticulum, under control of the cauliflower mosaic virus 35S promoter (Liu et al., 2001) was used to induce RNA silencing. Primer sequences used in these experiments are listed in Table S1 (available in JGV Online).

Wild-type (WT) and GFP-transgenic line 16C N. benthamiana plants (Ruiz et al., 1998) (generously provided by Dr David Baulcombe, Department of Plant Sciences, University of Cambridge, UK) were used for the analysis. Technically, we followed the protocol developed for studies of several other viral suppressors (Johansen & Carrington, 2001; Mangwende et al., 2009; Han et al., 2010). The plasmid vector constructs were transformed into Agrobacterium tumefaciens strain C58C1 carrying the virulence helper plasmid pCH32 (Hamilton et al., 1996) and used in agroinfiltration assays (Wydro et al., 2006). In this approach, the agrobacterium strain harbouring the GFP expressing plasmid pBin mgfp5–ER (hereafter named sGFP) was mixed with the agrobacterium carrying either a test or a control construct and the mixture was co-infiltrated into leaves of WT or 16C N. benthamiana plants at the four-leaf stage. The agro-infiltrated leaves were monitored visually under UV light for GFP at 3 days post-infiltration (p.i.). No obvious green fluorescence was observed at 3 days p.i. in leaves co-infiltrated with sGFP alone and sGFP plus empty vector (pGD and pBinPLUS). This silencing of GFP was also not affected by co-expression of GFP plus CP<sub>GPV</sub>, MP<sub>GPV</sub>, UP0<sub>GPV</sub>, CP<sub>GAV</sub> and P1<sub>GAV</sub>. In contrast, when N. benthamiana leaves were co-infiltrated with sGFP plus P0<sub>GPV</sub> or sGFP plus P6<sub>GAV</sub>, the level of GFP expression increased and the infiltrated patch appeared bright fluorescent green by 3 days p.i. In the positive control, GFP silencing was prevented by co-expression of a known viral RSS, HC-Pro<sub>TEV</sub>, which promoted high GFP fluorescence (Fig. 2a).

Total RNA [extracted according to Senda et al. (2004)] was analysed in Northern blots, using a radioactively labelled GFP-specific probe to detect the GFP mRNAs. The experiment revealed that the steady-state levels of GFP mRNA were much higher in tissues infiltrated with sGFP plus P0<sub>GPV</sub> or sGFP plus P6<sub>GAV</sub> than in tissues infiltrated with sGFP alone, sGFP plus empty vector or any other combination, at 5 days p.i. In a positive control, co-infiltration of sGFP plus HC-Pro<sub>TEV</sub> also promoted accumulation of higher amounts of the GFP transcript (Fig. 2b). Moreover, we also detected elevated levels of P0<sub>GPV</sub> or P6<sub>GAV</sub> mRNA from leaves infiltrated with sGFP plus P0<sub>GPV</sub> or P6<sub>GAV</sub> by Northern blot (Fig. 2d). Therefore, expression of P0<sub>GPV</sub> or P6<sub>GAV</sub>, as well as of HC-Pro<sub>TEV</sub>, contributed to the stabilization of GFP mRNA that led to elevated GFP fluorescence.

A hallmark of RNA silencing is the presence of small interfering RNA molecules (siRNAs) in the silenced tissue (Hamilton & Baulcombe, 1999; Hamilton et al., 2002). To test whether P0<sub>GPV</sub> or P6<sub>GAV</sub> increased GFP gene expression through suppression of RNA silencing, GFP-specific siRNAs have been analysed in all treatments as described in Fig. 2(a), following enrichment of low-molecular-mass RNA with PEG8000 and electrophoresis on acrylamide gels (Goto et al., 2007). As shown in Fig. 2(c), GFP siRNAs of 21 and 24 nt were reduced in leaves infiltrated with sGFP plus P0<sub>GPV</sub> or with sGFP plus P6<sub>GAV</sub>.

![Fig. 1. Genomic organization of (a) WYDV-GPV (GenBank accession no. FM865413) and (b) BYDV-GAV (GenBank accession no. AY220739). Grey boxes indicate the major ORFs. Numbers indicate the coordinates where each ORF starts and ends.](image-url)
These data suggest that WYDV ORF0-encoded P0 protein or BYDV ORF6-encoded P6 protein possessed an activity that was capable of suppressing sGFP-induced local silencing under our experimental conditions. Further evidence in support of this conclusion came from Western blot analyses, which showed that expression of P0\textsuperscript{GPV} or P6\textsuperscript{GAV} protein correlated directly with their silencing activity. Increased expression of GFP was detected in patches infiltrated with a construct expressing GFP plus P0\textsuperscript{GPV} or GFP plus P6\textsuperscript{GAV}, as well as GFP plus HC-Pro\textsuperscript{TEV}.

Fig. 2. Local RSS assays for proteins encoded by WYDV-GPV and BYDV-GAV in WT N. benthamiana leaves. (a) Agro-infiltration of WT N. benthamiana leaves with sGFP plus HC-Pro\textsuperscript{TEV}, empty vector pGD, empty vector pBinPLUS, P0\textsuperscript{GPV}, UP0\textsuperscript{GPV}, C\textsuperscript{GPV}, M\textsuperscript{GPV}, P6\textsuperscript{GAV}, P1\textsuperscript{GAV} and C\textsuperscript{GAV}; photographs were taken 3 days p.i. under UV illumination. (b) Northern blot analysis of mRNAs extracted from infiltrated leaf tissue. Blots were hybridized with GFP-specific probes and ethidium bromide-stained total RNA was used as loading control. (c) RNA blot analysis of GFP siRNA in infiltrated leaf tissue. The gel was loaded with 10 μg low-molecular-mass RNA and probed with a random-primed GFP probe. The processing was divided into two batches. Positions of the 21 and 24 nt RNA markers are shown on the left. The bottom panel shows ethidium bromide-stained tRNA and 5S rRNA (lanes 1–8 and lanes 9–11 belong to different batches). (d) Northern blot analysis of P0\textsuperscript{GPV} and P6\textsuperscript{GAV} mRNA in infiltrated leaf tissue. Blots were hybridized with P0\textsuperscript{GPV}- and P6\textsuperscript{GAV}-specific probes, and ethidium bromide-stained total RNA was used as a loading control. (e) Western blot analysis of the accumulation of GFP in infiltrated leaf tissues after 5 days p.i.; SDS-PAGE and Western blotting were performed as described previously (Xiong et al., 2009), using GFP antibodies purchased from Epitomics.
(positive control). In contrast, reductions in GFP levels were observed in leaves co-infiltrated with sGFP plus empty vector (negative control) and the other combinations, such as CP<sub>GPV</sub>, MB<sub>GPV</sub>, UP0<sub>GPV</sub>, CP<sub>GAV</sub> or P1<sub>GAV</sub> (Fig. 2e). UP0, where ORF0 is preceded by the 5'UTR of GPV, had no detectable suppression effect compared with P0 in our assay system.

To explore the action of systemic post-transcriptional gene silencing (PTGS) further, we investigated the effect of P0<sub>GPV</sub> and P6<sub>GAV</sub> by co-infiltration experiments with sGFP into transgenic <i>N. benthamiana</i> line 16C leaves and monitored GFP expression or silencing in systemic leaves under UV light. In approximately 95% of plants infiltrated with sGFP or with sGFP plus empty vector, the upper non-infiltrated leaves started to lose GFP fluorescence in major veins as early as 8 days p.i. Then, at 15 days p.i., green fluorescence in the whole plants, including the newly emerging leaves, disappeared completely. However, suppression of systemic silencing was observed in 60 or 65% of plants co-infiltrated with sGFP plus P6<sub>GAV</sub> or sGFP plus P0<sub>GPV</sub>, respectively, whilst no change in fluorescence intensity was detected in non-infiltrated leaves. It was also observed that non-infiltrated leaves in 58% of plants co-infiltrated with sGFP plus HC-Pro<sub>TEV</sub> retained green fluorescence for &gt;15 days p.i. (Fig. 3a). Northern blot analyses showed high accumulation of GFP mRNA (Fig. 3b) and negligible accumulation of GFP-specific siRNAs (Fig. 3c) in systemic leaves infiltrated with sGFP plus HC-Pro<sub>TEV</sub>, with sGFP plus P0<sub>GPV</sub> or sGFP plus P6<sub>GAV</sub>, providing further evidence that P0<sub>GPV</sub> and P6<sub>GAV</sub> could suppress systemic RNA silencing triggered by sGFP. Transgenic <i>N. benthamiana</i> line 16C plants that were not agro-infiltrated with sGFP, used as control, accumulated...
high levels of GFP mRNA (Fig. 3b) and low amounts of GFP-specific siRNAs (Fig. 3c). On the other hand, leaves infiltrated with sGFP alone, with sGFP plus pGD or with sGFP plus pBinPLUS showed reduced accumulation of GFP mRNA (Fig. 3b) and accumulation of siRNA (Fig. 3c).

Our results indicate that the P0GPV and P6GAV proteins exhibited both local and systemic activities in suppressing RNA silencing in N. benthamiana. This is the first report of suppressor proteins encoded by cereal-infecting members of the family Luteoviridae, although P0 proteins for other luteoviruses have previously been shown to have suppressor activity. In several dicot-infecting poleroviruses (CABYV, BWVV, TuYV, PLRV and BMYV), the P0 proteins have been shown to suppress RNA silencing locally, but not systematically (Han et al., 2010; Kozlowska-Makulska et al., 2010; Pfeffer et al., 2002). Mangwende et al. (2009) reported that the P0 protein of SCYLV suppressed not only local PTGS, but also systemic RNA silencing, in N. benthamiana. Our results indicate that this may also be the case for the cereal-infecting WYDV.

For the genus Luteovirus, little information about RSSs has been available previously. The genome organization of BYDV-GAV is similar to that of BYDV-MAV and BYDV-PAV (Jin et al., 2004) and ORF6 is the most variable ORF in the genus Luteovirus. The predicted product of ORF6 of BYDV-GAV is a protein of 4.3 kDa with unknown function. In this study, the P6 protein of BYDV-GAV was demonstrated to mediate RNA-silencing suppression, being able to suppress both local and systemic RNA silencing in planta. This is the first report of RSS activity of the P6 protein in the genus Luteovirus. To better understand the molecular mechanisms of silencing suppression in cereal-infecting members of the family Luteoviridae, future efforts will be aimed at investigating the mechanisms by which P0GPV or P6GAV proteins interfere with the gene-silencing pathways.

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References


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