Amino Acid Sequence Motifs Essential for P0-Mediated Suppression of RNA Silencing in an Isolate of *Potato leafroll virus* from Inner Mongolia

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Poliovirus P0 suppressors of host gene silencing contain a consensus F-box-like motif with Leu/Pro (L/P) requirements for suppressor activity. The Inner Mongolian *Potato leafroll virus* (PLRV) P0 protein (P0^{PL-IM}) has an unusual F-box-like motif that contains a Trp/Gly (W/G) sequence and an additional GW/WG-like motif (G139/W140/G141) that is lacking in other P0 proteins. We used *Agrobacterium* infiltration-mediated RNA silencing assays to establish that P0^{PL-IM} has a strong suppressor activity. Mutagenesis experiments demonstrated that the P0^{PL-IM} F-box-like motif encompasses amino acids 76-LPRHLHYECLEWGLLCG THP-95, and that the suppressor activity is abolished by L76A, W87A, or G88A substitution. The suppressor activity is also weakened substantially by mutations within the G139/W140/G141 region and is eliminated by a mutation (F220R) in a C-terminal conserved sequence of P0^{PL-IM}. As has been observed with other P0 proteins, P0^{PL-IM} suppression is correlated with reduced accumulation of the host AGO1-silencing complex protein. However, P0^{PL-IM} fails to bind SKP1, which functions in a proteasome pathway that may be involved in AGO1 degradation. These results suggest that P0^{PL-IM} may suppress RNA silencing by using an alternative pathway to target AGO1 for degradation. Our results help improve our understanding of the molecular mechanisms involved in PLRV infection.

RNA silencing is an immune mechanism used by hosts to combat viral infections at the nucleic acid level. RNA silencing was first discovered in plants (Lindbo and Dougherty 2005; Napoli et al. 1990), and has subsequently been shown to occur in a variety of eukaryotic organisms, including mammals (Li et al. 2013; Maillard et al. 2013). RNA silencing is initiated when double-stranded RNA (dsRNA) triggers Dicer-like enzymatic degradation of RNA transcripts to produce homologous short (21 to 24 nucleotides [nt]) RNAs, called small interfering RNAs (siRNAs) (Baulcombe 2004; Hamilton and Baulcombe 1999). One of the two strands in an siRNA duplex is recruited by an ARGONAUTE (AGO) RNA-binding and slicer protein (Bartel 2004; Elbashir et al. 2001) to form an RNA-induced

silencing complex (RISC) that targets viral genomic RNAs or messenger RNAs (mRNAs) for degradation (Baumberger and Baulcombe 2005; Ding and Voinnet 2007; Mi et al. 2008; Qi et al. 2005).

To overcome host RNA silencing, numerous viral-encoded suppressor of RNA silencing (VSR) proteins have evolved (Silhavy and Burguán 2004; Voinnet 2005; Wang et al. 2012). These include more than 35 suppressor families, which have no obvious sequence similarity, that are multifunctional and can act at more than one step in the silencing pathway (Burguán and Havelda 2011; Csorba et al. 2009). The most common mechanism of VSR action is to mediate sequestration of ds-siRNA (Csorba et al. 2007; Ding and Voinnet 2007; Lakatos et al. 2006; Mérai et al. 2006). The best-characterized VSRS employing this strategy are the tombusvirus P19 protein, which binds siRNA duplexes (Lakatos et al. 2006) and the *Turnip crinkle virus* (TCV) P38 coat protein (CP), which has two suppressor functions, one of which is binding viral dsRNAs of various sizes (Burguán and Havelda 2011; Deleris et al. 2006; Mérai et al. 2006). Overall, these findings show that RNA binding activities are a central feature of many VSR proteins.

Other suppressor proteins interact with RISC proteins encoded by the host (Csorba et al. 2009; Wang et al. 2012). A classic example of this mechanism is the *Cucumber mosaic virus* (CMV) 2b protein, which binds physically with the host AGO1/AGO4 proteins, at the PAZ domains of these proteins, to specifically inhibit RNA cleavage activities of the proteins (Duan et al. 2012; González et al. 2010; Goto et al. 2007; Hamera et al. 2012; Zhang et al. 2006). Other recent studies have revealed another strategy in which VSR proteins inhibit RISC activities by mimicking glycine/tryptophan (GW/WG) proteins known to interact directly with AGO proteins (Azevedo et al. 2010; Burguán and Havelda 2011; Giner et al. 2010; Jin and Zhu 2010; Szabó et al. 2012). A prime example of this mechanism is the TCV P38 CP, which contains two essential GW repeats required for binding to *Arabidopsis thaliana* AGO1 (Azevedo et al. 2010; Burguán and Havelda 2011). P38 binding is thought to interfere with siRNA loading into AGO1 by interacting preferentially with nonloaded AGO1 without affecting siRNA-loaded AGO1 RISC activities (Azevedo et al. 2010). The *Sweet potato mild mottle potyvirus* (SPMMV) suppressor (P1) has three N-terminal WG/GW motifs. Site-directed mutations demonstrate an absolute requirement for each of the motifs for binding to both unloaded AGO1 and siRNA-loaded AGO1 (Burguán and Havelda 2011; Giner et al. 2010). Interestingly, the P1 protein of the related *Sweet potato feathery mottle virus* (SPFMV) is a close homologue of SPMMV P1 but has a nonfunctional AGO-binding domain that

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does not elicit a silencing-suppressor activity. However, remodeling of the protein by introduction of two comparable WG/GW motifs from SPMMV converts SPFMV P1 to a silencing suppressor that is able to bind an AGO (Szabó et al. 2012).

The P0 proteins encoded by the 5'-proximal open reading frames (ORF) of several poleroviruses have been shown to suppress RNA silencing. These poleroviruses, which belong to the family *Luteoviridae*, include *Beet mild yellowing virus* (BMV), *Cucurbit aphid-borne yellows virus* (CABYV), *Turnip yellows virus* (syn. *Beet western yellows virus*), *Sugarcane yellow leaf virus* (ScYLV), *Melon aphid-borne yellows virus* (MABYV), *Wheat yellow dwarf virus* GPV isolate, and *Cotton leafroll dwarf virus* (Delfosse et al. 2013; Han et al. 2010; Kozłowska-Makulska et al. 2010; Liu et al. 2012; Mangwende et al. 2009; Pfeffer et al. 2002). The amino acid sequence identities of these polerovirus P0 proteins are very low, with an average identity value of 37.06%, and their silencing-suppressor activities vary among different virus isolates. The P0 proteins of most BMV isolates and the P0 of a *Cereal yellow dwarf virus-RPV* (CYDV-RPV) isolate from Australia exhibit RNA silencing-suppressor activities (Fusaro et al. 2012; Kozłowska-Makulska et al. 2010) but the P0 proteins of two related BMV isolates (BMV-N32 and -26), several *Beet chlorosis virus* isolates from Europe, and a CYDV-RPV isolate from New York lack suppressor functions (Guilley et al. 2009; Kozłowska-Makulska et al. 2010). However, despite substantial differences in the sequences and silencing activities of these P0 proteins, they all contain conserved F-box-like domains (Pazhouhandeh et al. 2006).

Recently, several isolates related to *Potato leafroll virus* (PLRV), the type member of the *Polerovirus* genus, have been described (King et al. 2011). Among these, the amino acid sequence identities of the P0^{PL} proteins of Iranian PLRV isolates are more closely related to those of the European PLRV isolates than to those of the other isolates (Zarghani et al. 2012). Although the P0 protein (P0^{PL-NL}) from a Dutch PLRV isolate is a weak suppressor of RNA silencing (Pfeffer et al. 2002), an Australian PLRV P0 protein (P0^{PL-Au}) is a stronger suppressor (Fusaro et al. 2012).

The action mechanisms of the P0 proteins are not as well understood as those of the tombusvirus P19, TCV P38, and CMV 2b VSRs, as mentioned above. The P0 protein of BWYV (P0^{BW}) has been suggested to suppress RNA silencing by inhibiting secondary siRNA production and by targeting AGO1 for degradation (Baumberger et al. 2007; Csorba et al. 2010). However, P0^{BW} appears to carry out these functions without direct AGO1 binding (Burgan and Havelda 2011). P0^{BW} and the P0 protein of CABYV (P0^{CA}) are thought to target AGO1 by means of their F-box-like motifs interacting with proteins belonging to the SCF family of E3-ligase S-phase kinase-related protein-1 (SKP1) components in *Nicotiana benthamiana* (Pazhouhandeh 2007). However, degradation of AGO1 is insensitive to proteasome inhibitors, suggesting that the proteasome may not be directly involved in AGO1 degradation (Baumberger et al. 2007; Bortolamiol et al. 2007; Pazhouhandeh et al. 2006). Recently, it has been reported that P0-induced AGO1 degradation depends on the autophagy pathway (Derrien et al. 2012). In addition to the F-box-like domain, the C-terminal conserved P0 sequence has an important role in suppression of RNA silencing (Han et al. 2010) because mutations of aromatic amino acids in the P0 C-terminal sequence of the polerovirus MABYV (P0^{MA}) also destroy suppressor activities.

In this study, we demonstrated that the P0 protein from an Inner Mongolian PLRV isolate (P0^{PL-IM}) is a strong suppressor of RNA silencing. We have dissected the sequence of an unusual

F-box-like motif in P0^{PL-IM} and further shown that, unlike other P0 proteins, the GW sequences within the F-box-like motif are required for suppression of RNA silencing. We also found that residues in a unique GW/WG-like motif (G139/W140/G141) affect suppressor activity, and a single-residue F220 in a C-terminal conserved sequence is essential for suppression. Additional results revealed that expression of the wild-type P0^{PL-IM} (wtP0^{PL-IM}) results in decreased accumulation of AGO1 but that none of the dysfunctional P0^{PL-IM} mutants that we constructed affects AGO1 accumulation. However, unlike most P0 proteins, P0^{PL-IM} fails to interact with SKP1, which participates in the proteasome degradation pathway. Therefore, we propose that the degradation of AGO1 may be carried out by an alternative mechanism.

RESULTS

P0^{PL-IM} is a strong RNA silencing suppressor.

Given that the PLRV P0^{PL-NL} protein is a weak suppressor of RNA silencing (Pfeffer et al. 2002), we tested the ability of wtP0^{PL-IM} to suppress silencing by using *Agrobacterium* transient expression systems similar to those previously described (Johansen and Carrington 2001; Ruiz et al. 1998). In our assays, transformed *Agrobacterium tumefaciens* GV3101 strains harboring either control or suppressor protein genes in the binary vector, pGD, were mixed individually with a GV3101 strain containing pGDG for expression of green fluorescent protein (GFP) (Goodin et al. 2002) and then co-infiltrated into *N. benthamiana* leaves. The suppressor activity of the wtP0^{PL-IM} protein expressed from pGD (pGDP0^{PL-IM}) was evaluated in the infiltrated leaves in comparison with those of the *Tomato bushy stunt virus* (TBSV) P19 protein (pGDP19) and of P0^{CA} (pGDP0^{CA}), which were used as positive controls. The plasmid pGD, which does not express GFP, served as a negative control.

Leaves *Agrobacterium*-infiltrated (agroinfiltrated) with wtP0^{PL-IM} expressed a strong GFP fluorescence by 5 days postinfiltration (dpi) that was comparable with the green fluorescence emanating from the leaves infiltrated with P19 and P0^{CA}, respectively (Fig. 1A). These results suggest that P0^{PL-IM} and P0^{CA} are as strong suppressors of RNA silencing as TBSV P19. In contrast, GFP fluorescence was negligible in leaves co-infiltrated with *agrobacteria* harboring pGDG and the empty vector pGD (Fig. 1A). To provide quantitative assessments, Western blot analyses were used to confirm GFP accumulation in sectors co-infiltrated with pGDG or with wtP0^{PL-IM}, P19, or pGD and the results agreed with the fluorescence levels observed in the infiltrated leaves (data not shown).

The LP motif L76/P77 but not L59/P60 residues in the F-box-like motif of wtP0^{PL-IM} is required for suppression of RNA silencing.

Inspection of the P0^{PL-IM} sequence revealed that P0^{PL-IM} has an unusual F-box-like motif residing between residues 59 and 95 that appears to consist of a partial duplication of the consensus F-box-like motif [LPxx(L/I)_{x10-13}P] found in other P0 proteins and in plant SFC ubiquitin E3 ligase subunits (Pazhouhandeh et al. 2006; Risseuw et al. 2003). The first region (59-LPLLNCGRGRISTSGQLQLP-77 [bold indicates landmarks of important motifs]) corresponds to the F-box-like motifs of other polerovirus P0 proteins, except for residue 63, which is an N instead of the consensus L or I residues (Fig. 1B). The second partial overlapping region (76-LPRHLHYECLEWGLLCGTHP-95) has an F-box-like motif similar to the consensus F box but harbors a WG sequence reminiscent of the GW/WG motifs found to interact with AGO1 in the CMV 2b and TCV P38 GW/WG residues involved in AGO1 interactions.

To determine which of the LP residues are essential for $P0^{PL-IM}$ suppressor activity, we constructed $P0^{PL-IM}$ -L59A/P60A and -L76A/P77A mutant proteins and compared the suppressor activities of these mutants with $WT P0^{PL-IM}$. *N. benthamiana* leaves were co-infiltrated with *Agrobacterium* derivatives carrying pGDG and $WT P0^{PL-IM}$ or the two $P0^{PL-IM}$ mutants. P19 and $P0^{CA}$ were used as positive controls and the empty binary vector pGD was used as a negative control. At 5 dpi, GFP fluorescence in leaves that had been co-infiltrated with pGDG and P0 carrying the L59A/P60A mutations was very similar to that observed in leaves infiltrated with $WT P0^{PL-IM}$ or with P19 and $P0^{CA}$ (Fig. 1A). In contrast, GFP fluorescence was not detected in leaves that were co-infiltrated with pGDG and the L76A/P77A double mutant or the pGD negative control (Fig. 1A). We also constructed single L76A and P77A mutations and found that the L76A mutant but not the P77A mutant lost the suppressor activity (Fig. 1A). These results indicate that a functional F-box-like sequence critical for the suppressor function of $P0^{PL-IM}$ resides between residues L76 and P95, and that the sequence spanning L59 to P77 does not exhibit F-box-like functions.

Critical residues in two $WT P0^{PL-IM}$ WG/GW-like motifs are required for the suppressor activity.

Our sequence comparisons revealed WG/GW-like candidate residues (W87/G88) within the $WT P0^{PL-IM}$ F-box-like motif that are not present in other consensus P0 sequences (Fig. 2A). An additional F-box-like sequence downstream of this region encompasses amino acids G139, W140, and G141 (Fig. 2A). To investigate the significance of the W/G residues within the $P0^{PL-IM}$ F-box-like motif for silencing-suppressor activity, we generated single and double W87/G88 mutations by substitutions with alanine residues (Fig. 2A). To increase the uniformity of the experiments, we infiltrated the tips of the leaves with pGDG and the control pGD, the left basal side of the leaves with pGDG and $WT P0^{PL-IM}$, and the right basal side with pGDG and the mutant $P0^{PL-IM}$ derivatives (Fig. 2B, first three upper panels). The results at 5 dpi showed that all sectors infiltrated with the mutant derivatives and with the pGD vector failed to exhibit fluorescence but sectors infiltrated with $WT P0^{PL-IM}$ exhibited fluorescence comparable with the p19 control infiltration (Fig. 2B, bottom right panel). An additional double mutant with tryptophan/glycine exchanges was constructed (Fig.

Fig. 1. Suppression of local silencing by $P0^{PL-IM}$ and mutations of the $P0^{PL-IM}$ F-box-like motif to test suppression of RNA silencing. **A**, Assessment of suppression of gene silencing in *Nicotiana benthamiana* leaves by co-infiltration of an *Agrobacterium* strain containing pGDG for expression of green fluorescent protein, plus a strain containing the control pGD plasmid, or strains containing pGD plasmids for expression of $P0^{CA}$, P19, $P0^{PL-IM}$, L59A/P60A, L76A/P77A, L76A, or P77A. Photographs were taken of leaves at 5 days postinfiltration under long-wavelength UV light. Designations of the test suppressor proteins are below the leaf photographs. **B**, Alignment of the conserved sequences of P0 F-box-like domains from six polioviruses. Two regions (amino acids 59 to 77 and 76 to 95) in $P0^{PL-IM}$, that are marked by blue and red colors, respectively, have homology to F-box-like domains. All the conserved residues are bold in black. DNAMAN program alignments of the conserved amino acid sequences from GenBank show CABYV (X76931), BMV (NC003491), BWV (NC004756), CYDV (NC004751), MABV (NC010809), and PLRV (KC456052). Alanine substitutions (bold a's) were introduced to generate the L59A/P60A, L76A/P77A, L76A, and P77A $P0^{PL-IM}$ mutants.

2A) but this mutant was also dysfunctional in silencing suppression (Fig. 2B, upper right panel). Hence, both of the F-box-like GW residues are required for P0^{PL-IM} silencing suppression and the W/G residues cannot be exchanged. Thus, our experiments reveal that the W87/G88 residues are critical for

suppressor activity and further confirm the importance of the F-box-like domain 76-LPRHLHYECLEWGLLCGTHP-95 for P0^{PL-IM} suppressor activity.

In the same set of experiments, alanine substitutions for the G139/W140/G141 residues were constructed and additional

interchanges of the tryptophan and glycine residues were incorporated into P0^{PL-IM} (Fig. 2A). These mutants and the *wt*P0^{PL-IM} were used in co-infiltration experiments to compare their suppressor activities. GFP fluorescence of the relevant leaf sectors indicated that the G139A and G141A mutants had suppressor activities similar to that of *wt*P0^{PL-IM} but that the W140A mutant had greatly reduced GFP fluorescence and, hence, weakened suppressor activity (Fig. 2B). The double mutant G139A/W140A totally disrupted the suppressor activity, whereas the G139A/G141A mutant retained strong suppressor activity (Fig. 2B). To further evaluate the G/W/G functions, tryptophan and glycine exchanges were tested for suppressor activity. Leaves infiltrated with the G139W/W140G mutant had somewhat weaker visual fluorescence than leaves co-infiltrated with *wt*P0^{PL-IM} but the activity of this mutant seemed to be higher than that of the corresponding W140A and G139A/W140A mutants (Fig. 2B). The GFP accumulation levels in each mutant infiltration experiment were further characterized by Western blotting (Fig. 2C) and, in each case, the GFP accumulation reflected the fluorescence shown in the relevant leaf sectors. The results demonstrate that the G139/W140/G141 signature residues are also essential for suppressor activity of P0^{PL-IM}.

Defective silencing mutants result in increased siRNA accumulation.

To further evaluate the above results, we selected *wt*P0^{PL-IM} and representative mutants by tagging a 3× flag epitope at their C termini, and used these derivatives for co-infiltrations followed by molecular detection. *N. benthamiana* leaves were co-infiltrated with pGDG and flag-tagged *wt*P0^{PL-IM} or its L59A/P60A, L76A/P77A, W87A, W140A, and F220R mutant derivatives. Co-infiltration of pGDG with P0^{CA} and P19 served as positive controls. An F220R P0^{PL-IM} mutant corresponding to a conserved motif near the P0^{PL-IM} C-terminal region was also tested because an F220R mutation in the analogous region of MABYV has been shown to destroy P0 suppressor activity (Han et al. 2010). At 5 dpi, GFP fluorescence had disappeared or declined to very low levels when pGDG was co-infiltrated with flag-tagged L76A/P77A, W87A, W140A, and F220R mutants and the negative pGD control (Fig. 3A). In contrast, GFP fluorescence in leaves infiltrated with the *wt*P0^{PL-IM} and the L59A/P60A derivatives was strong compared with the positive P0^{CA} and P19 controls, indicating that the flag tag did not compromise silencing-suppressor activities (Fig. 3A).

Total mRNA and protein were then extracted from regions co-infiltrated with the above combinations of constructs. Northern blot analyses revealed that the levels of GFP mRNA were much higher in leaves that expressed GFP plus *wt*P0^{PL-IM}, L59A/P60A, P0^{CA}, or P19 than in leaves agroinfiltrated with pGD, L76A/P77A, W87A, W140A, or F220R (Fig. 3B). Additional Western blot analyses showed that GFP protein accumulation levels (Fig. 3C) were consistent with GFP fluorescence levels shown in Figure 3A. The levels of GFP protein accumu-

lations in leaves co-infiltrated with pGDG and L59A/P60A were similar to those infiltrated with the *wt*P0^{PL-IM} protein and the P0^{CA} and P19 positive controls (Fig. 3C). However, reductions in GFP protein levels were observed in leaves co-infiltrated with combinations of pGDG and L76A/P77A, W87A, W140A, F220R, or the pGD negative control (Fig. 3C). Hence, the results of the flag-tagged *wt*P0^{PL-IM} proteins (Fig. 3A) are entirely consistent with the results of the untagged *wt*P0^{PL-IM} derivatives (Figs. 1A and 2B and C).

To test further whether the *wt*P0^{PL-IM} and its mutants affect GFP expression through suppression of RNA silencing, we analyzed the levels of GFP-specific siRNAs in all of the treatments described in Figure 3A. The results clearly revealed that 21- to 24-nt GFP siRNAs accumulated to high levels in leaves infiltrated with pGDG plus pGD, L76A/P77A, W87A, W140A, or F220R (Fig. 3D). The levels of the GFP siRNAs were also consistently lower in leaves infiltrated with pGDG plus *wt*P0^{PL-IM} or L59A/P60A (Fig. 3D). These results indicate that the L59A/P60A mutant that retains the suppressor activity and *wt*P0^{PL-IM} effectively block accumulation of GFP siRNAs, whereas expression of the silencing-defective mutants fails to affect the levels of GFP siRNA accumulation.

To ensure that the null mutants were expressed in the infiltrated *N. benthamiana* leaves, accumulations of the flag-tagged *wt*P0^{PL-IM} as well as L59A/P60A, L76A/P77A, W87A, and W140A mutant derivatives were examined in the presence or absence of TBSV P19. At 5 days after co-infiltration with P19 and pGDG, leaves expressing flag-tagged *wt*P0^{PL-IM} and the P0^{PL-IM} derivatives exhibited GFP expression and all P0^{PL-IM} mutant proteins accumulated to high levels (Fig. 4A). In contrast, in the absence of P19, only leaves co-infiltrated with pGDG and *wt*P0^{PL-IM} or the functional L59A/P60 mutant exhibited accumulations of these proteins as well as GFP fluorescence, and leaves infiltrated with pGDG and the rest of P0 mutants showed only trace amounts of the dysfunctional mutant proteins as well as absence of GFP expression (Fig. 4B). These results indicate that the mutant proteins were expressed to the same extent as *wt*P0^{PL-IM} when silencing was suppressed by P19 and that the inability of the L76A/P77A, W87A, W140A, and F220R mutants resulted from degradation of both the dysfunctional P0^{PL-IM} mutants and GFP by 5 dpi.

P0^{PL-IM} silencing activity is correlated with reduced AGO1 accumulation.

In order to test the effects of P0^{PL-IM} on AGO1 accumulation, a 6× myc-tagged version of *Arabidopsis thaliana* AGO1 (6myc-AtAGO1, a gift from H. Guo) was introduced into *N. benthamiana* leaves by infiltration with the 3× flag-tagged *wt*P0^{PL-IM} or *wt*P0^{PL-IM} mutant derivatives or the pGD control vector. At 4 dpi, extracts from co-infiltrated leaves were analyzed by Western blotting. No effects on 6myc-AtAGO1 accumulation were observed when the silencing-suppression-defective mutants (L76A/P77A, W87A, W140A, and F220R) were used, even though the mutant proteins accumulated to normal

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Fig. 2. Mutations in the WG/GW-like motifs of P0^{PL-IM} for suppression tests of RNA silencing. **A**, Positions of WG/GW signatures within the P0^{PL-IM} sequence are highlighted in bold uppercase letters and the substitution mutations are indicated by bold lowercase letters representing alanine (a), tryptophan (w), or glycine (g) residues. The silencing phenotype of the infiltrated leaves is indicated on the right. **B**, Co-infiltration of *Nicotiana benthamiana* leaves to test P0^{PL-IM} mutants. The first 11 leaves were infiltrated at the top of the leaf with *Agrobacterium* harboring pGDG plus the control pGD plasmid. As expected, the infiltrated regions fail to fluorescence. The bottom left side of the leaves was infiltrated with pGDG plus pGDPO^{PL-IM} for expression of green fluorescent protein (GFP) and P0^{PL-IM}. The bottom right side of these leaves shows pGDG plus pGD plasmids for expression of GFP and the mutant proteins W87A/G88A, W87A, G88A, W87G/G88W, G139A/W140A, G139A, W140A, G139W/W140G, G139A/G141A, G141A, or W140G/G141W. Individual infiltrated derivatives are illustrated on the leaves and double mutants are separated by a vertical line. The bottom right leaf shows a P19 positive suppressor control. Photographs were taken of leaves at 5 days postinfiltration under long-wavelength UV light. **C**, Western blot analyses using GFP polyclonal antiserum (α-GFP). Total proteins were extracted from the agro-infiltrated regions for sodium dodecyl sulfate polyacrylamide gel electrophoresis. The loading control was Rubisco stained with Coomassie blue.

levels when P19 was included to avoid silencing of the exogenous transcripts during the transient assays (Fig. 5). However, it was striking that co-expression of $WTPO^{PL-IM}$ and the functional L59A/P60A mutant induced a substantial reduction in the levels of 6myc-AtAGO1 (Fig. 5). This demonstrates that one of the PO^{PL-IM} silencing-suppressor functions was to trigger destabilization of AGO1.

PO^{PL-IM} fails to interact with *N. benthamiana* SKP1 (NbSKP1) in yeast and in plants.

As shown above, PO^{PL-IM} contains an F-box-like motif that is required for silencing-suppression activity. Moreover, the L76/P77 residues represent the two most highly conserved amino acids in the consensus sequence of plant F-box motifs [LPxx(L/I) x_{10-13} P], and the L76A/P77A mutant has negligible suppressor activity. To investigate the activity of PO^{PL-IM} in more detail, a yeast two-hybrid system was used to probe interactions between PO^{PL-IM} and NbSKP1. SKP1 functions in a proteasome pathway that may be involved in AGO1 degradation. The ORF of $WTPO^{PL-IM}$ and NbSKP1 were amplified and cloned into pGBKT7 and pGADT7, respectively. The resulting plasmid pGBK- PO^{PL-IM} was transformed into the yeast strain Y187 and pGAD-NbSKP1 was transformed into the yeast strain AH109 to implement a powerful yeast two-hybrid mating strategy. The combinations pGBKT7/pGADT7, pGBKT7/pGAD-NbSKP1, pGBK- PO^{PL-IM} /pGADT7, and pGBK- PO^{MA} /pGADT7 were used as negative controls, and pGBK- PO^{MA} /pGAD-NbSKP1 provided a positive control. Mixing of the Y187 and AH109 transformants resulted in vigorous growth on agar plates containing synthetic dropout (SD) media lacking leucine and tryptophan (SD/-Leu/-Trp) (Fig. 6A). However, only the diploid yeast strains derived from mating of the positive-control combination pGBK- PO^{MA} in Y187 and pGAD-NbSKP1 in AH109 could grow on the SD media lacking leucine, tryptophan, adenine, and histidine (SD/-Leu/-Trp/-Ade/-His) that contained X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) and expressed β -galactosidase activity (Fig. 6A). The results demonstrate that PO^{MA} and PO^{PL-IM} differ in that PO^{PL-IM} does not interact with NbSKP1 in yeast. PO^{PL-IM} also differs from PO^{PL-NL} , which binds NbSKP1 with a low affinity (Pazhouhandeh 2007).

To use an alternative approach to test interactions between PO^{PL-IM} and NbSKP1, we performed a bimolecular fluorescence complementation (BiFC) assay (Walter et al. 2004). The cDNAs that encode PO^{PL-IM} and NbSKP1 were cloned into the BiFC transformation vectors pSPYNE-35S and pSPYCE-35S to generate plasmids PO^{PL-IM} -YN, PO^{PL-IM} -YC, NbSKP1-YN, and NbSKP1-YC. Leaves of *N. benthamiana* co-infiltrated with *Agrobacterium tumefaciens* EHA105 strains harboring the PO^{PL-IM} -YN+NbSKP1-YC and NbSKP1-YN+ PO^{PL-IM} -YC combinations were used to test possible PO^{PL-IM} interactions with NbSKP1. The PO^{ScYLV} -YN+NbSKP1-YC and the γ b-YN+ γ b-YC combinations served as positive controls, and the PO^{ScYLV} -YN+YC combination provided a negative control. No fluorescence was observed in *N. benthamiana* leaf epidermal cells co-infiltrated with PO^{PL-IM} -YN+NbSKP1-YC or NbSKP1-

YN+ PO^{PL-IM} -YC (Fig. 6B). Likewise, fluorescence could not be visualized in the negative control combination, although yellow fluorescent protein (YFP) fluorescence was observed in the positive controls (Fig. 6B). In addition, “silencing on the spot” *Agrobacterium* assays (Johansen and Carrington 2001) revealed that the P0 constructs tagged for BiFC (PO^{PL-IM} -YN and PO^{PL-IM} -YC) are functional because they retained suppressor activities (Supplementary Fig. S1). Therefore, our data demonstrate that PO^{PL-IM} fails to interact with NbSKP1 in either yeast or plants.

DISCUSSION

Our agroinfiltration results have demonstrated that PO^{PL-IM} is a strong suppressor of gene silencing and our mutagenesis experiments show that the PO^{PL-IM} F-box-like domain is required for RNA silencing suppression, as is also the case with previous reports of other P0 proteins (Fusaro et al. 2012; Han et al. 2010; Pazhouhandeh et al. 2006). However, alignment of the amino acid sequences of several P0 proteins reveals that PO^{PL-IM} is unusual because it has two partially overlapping LP motifs. The sequence 59-LPLLNCCKRGRISTSGQLQP-77 appears to be an imperfect F-box-like sequence because it lacks a conserved L or I residue at position 63 (Fig. 1B). The sequence 76-LPRHLHYECLEWGLLCGTHP-95 contains an L80 residue and, hence, has a sequence composition consistent with consensus sequences in the F-box-like motifs of other P0 proteins (Fig. 1B). The comparisons also show that the PO^{PL-IM} region encompassing residues 76 to 95 can be distinguished from other P0 F-box-like motifs by the presence of the W87G88 sequence.

Substitutions of the conserved LP residues with A residues at positions 76 and 77 abolished the suppressor functions, although the same substitutions at residues 59 and 60 did not affect suppressor activity. Interestingly, *Pea enation mosaic virus-1* P0 (PO^{PE}), the type member of the *Enamovirus* genus in the *Luteoviridae* family, contains a silencing-suppressor protein with an F-box-like domain at positions 124 to 143 amino acids (Fusaro et al. 2012). In the case of PO^{PE} , mutations resulting in changes of amino acids L124, P125, and P143 to A residues abolished suppressor activities, whereas the L124 and P125 double mutations to A residues, plus a single mutation of P143 to an A residue, failed to suppress RNA silencing (Fusaro et al. 2012). In contrast, the PO^{PL-IM} L76A mutation but not the P77A mutation destroyed the suppressor activity (Fig. 1A). These results show that PO^{PL-IM} residues 76-LPRHLHYECLEWGLLCGTHP-95 compose a functional F-box-like motif, and that the L76/P77 residues are essential for the silencing-suppressor activity.

It has been proposed that some silencing suppressors mimic proteins that contain GW/WG repeats that sequester AGO proteins and suppress RNA silencing by preventing siRNA loading into AGO complexes (Jin and Zhu 2010). In our experiments, both of the W87/G88 residues were critical for suppressor function because neither of the amino acids could be substituted for A residues, nor could the G and W residues be interchanged

Fig. 3. Abilities of positive and negative controls and representative PO^{PL-IM} mutants to suppress gene silencing in *Nicotiana benthamiana* leaves. **A**, Agroinfiltration of leaves with pGDG plus 1, the control pGD plasmid or pGD plasmids for expression of 3xflag tagged 2, PO^{PL-IM} ; 3, L59A/P60A; 4, L76A/P77A; 5, W140A; 6, W140A; or 7, F220R PO^{PL-IM} mutants; and 8, PO^{CA} ; or 9, P19. Double mutants are separated by a diagonal line. Photographs were taken of leaves at 5 days postinfiltration under long-wavelength UV light. **B**, Northern blot analysis of high-molecular-weight RNA extracted from the agroinfiltrated regions. Blots were hybridized with probes specific for green fluorescent protein (GFP) mRNA. Ethidium-bromide-stained 28S rRNA was used as an RNA loading control. **C**, Western blot analysis using GFP polyclonal antiserum. Total proteins were extracted from the agro-infiltrated regions for sodium dodecyl sulfate polyacrylamide gel electrophoresis. The loading control was Rubisco stained with Coomassie blue. **D**, Northern blot analysis of siRNAs hybridized with a population of heterogeneous GFP probes that had been labeled with α - ^{32}P UTP. Ethidium-bromide-stained transfer RNA (tRNA) was used as an RNA loading control. Numbers of the bottom of B, C, and D correspond to extracts from the infiltrated leaves.

without loss of RNA silencing-suppressor activity (Fig. 2B). Interestingly, no discernible effects on silencing were observed when either or both of the two G residues in the G139/W140/G141 motif were substituted with A residues, or when mutants in which the GWG residues were replaced by WGG or GGW; however, replacement of GWG with GAG reduced suppressor activity substantially compared with the activity of $P0^{PL-IM}$ (Fig. 2B). These results suggest that both of the GW/WG-like sequences have characteristics typical of GW/WG motifs and, possibly, may participate in $P0^{PL-IM}$ -binding activities with other proteins. Moreover, it is possible that $P0^{PL-IM}$ could be multifunctional and participate in more than one suppressor-related activity. The location of W87/G88 within the boundary of the $P0^{PL-IM}$ F-box-like domain (76-LPRHLHYECLEWGL LCGTHP-95) is unusual for P0 proteins, and it is possible that W87/G88 residues may impact $P0^{PL-IM}$ protein silencing by eliciting conformational changes in the protein or by confer-

ring interactions with other proteins. Alignments comparing $P0^{PL-IM}$ with the P0 proteins of different PLRV isolates reveal that the 76-LPRHLHYECLEWGL LCGTHP-95 and G139/W140/G141 motifs, as well as the F220 residue, are highly conserved. In addition, the more distantly related $P0^{PL}$ protein, which is essential for virus accumulation (Sadowy et al. 2001), also shares conservation with the P0 suppressor proteins in these three regions. Thus, all three regions appear to have been selected during evolution to enable viruses encoding P0-like proteins to infect plants efficiently.

AGO1, as a key component of RISC, not only binds to siRNAs but also has RNA slicer activity (Ding 2010; Hutvagner and Simard 2008; Vaucheret 2008). Our research has identified amino acid substitution mutants that have lost the ability to destabilize AGO1, and these mutants affect the F-box-like domain (L76A/P77A and W87A), the WG-like motif (W140A), and the C-terminal conserved sequence (F220R)

Fig. 4. Accumulation of 3x flag-tagged $P0^{PL-IM}$ and $P0^{PL-IM}$ mutant derivatives in infiltrated tissue at 5 days postinfiltration in the presence or absence of the P19 protein. Western blot analysis using GFP polyclonal antiserum. Total proteins were extracted from the agro-infiltrated regions for sodium dodecyl sulfate polyacrylamide gel electrophoresis. The loading control was Rubisco stained with Coomassie blue. GFP = green fluorescent protein. **A**, Plus P19. **B**, Minus P19.

amino acids. Our finding that P0^{PL-IM} reduces AGO1 protein accumulation supports previous reports that P0^{BW} and P0^{PE} can lead to AGO protein degradation (Baumberger et al. 2007; Bortolamiol et al. 2007; Csorba et al. 2010; Fusaro et al. 2012). Given that a direct physical interaction between P0^{BW/CA} and AGO1 was reported in in vitro pull-down and BiFC analyses (Bortolamiol et al. 2007), we tested potential interactions between P0^{PL-IM} and AGO1 by co-immunoprecipitation and BiFC analyses. However, our failure to co-immunoprecipitate 3× flag-tagged P0^{PL-IM}–6× myc-tagged AGO1 complexes with affinity beads is consistent with a previous report that the P0^{BW} protein is not present in RISC fractions that contain the AGO1 complex (Csorba et al. 2010). In addition, our BiFC analyses clearly suggest that P0^{PL-IM} fails to interact with AtAGO1 (Supplementary Fig. S2). This implies that P0 may affect the stability of AGO1 indirectly, perhaps through targeting another protein that might normally be able to bind AGO1 or stabilize the protein.

We also have provided evidence suggesting that AGO1 is not degraded by the normal SKP1 proteasome pathway (Baumberger et al. 2007; Csorba et al. 2010). The ASK1 protein and its close homolog ASK2 (the most important component of the SCF complex) are the most abundant of the 21 ASK proteins in *Arabidopsis thaliana*. Each of them can interact with multiple F-box domains (Farrás et al. 2001; Gagne et al. 2002; Risseuw et al. 2003). The efficiency with which P0^{PL-NL} interacts with NbSKP1 is much lower than those of the P0^{CA} or P0^{BW} protein interactions with ASK1, ASK2, or NbSKP1 (Pazhouhandeh 2007). In contrast, our yeast two-hybrid and BiFC results indicate that P0^{PL-IM} does not interact with NbSKP1 (Fig. 6). This implies that the destabilizing effects of P0 on AGO1 accumulation may not require ubiquitination by an SCF-type E3 ligase. This possibility is consistent with previous reports that polyubiquitination and P0^{BW}-mediated AGO1 degradation may be uncoupled (Baumberger et al. 2007;

Csorba et al. 2010). Moreover, it has been suggested recently that AGO1 can be degraded by autophagy without forming a P0^{BW} complex with SCF E3 ligase, and that P0 is able to promote AGO1 degradation by autophagy (Derrien et al. 2012). However, we cannot eliminate the possibility that P0^{PL-IM} may interact with other SKP-related proteins, or that P0^{PL-IM} interacts with SKP1 during viral infection.

Finally, to the best of our knowledge, our studies with P0^{PL-IM} are the first report of a polerovirus P0 in which GW/WG-like motifs are required for suppression of RNA silencing. All mutations of the L76/P77, W87, W140, and F220 positions affect the ability of P0^{PL-IM} to function as an RNA silencing suppressor and to reduce AGO1 accumulation. Although L76A/P77A and W87A affect the F-box-like motif, W140A affects the GW/WG-like motif and F220R interferes with a C-terminal conserved sequence. The mechanisms whereby these mutations affect suppressor functions are not yet clear. A simple possibility is that conformation changes caused by the mutations might decrease P0 activity but we believe it is more likely that the mutations result in interference with a major function targeting AGO1 accumulation.

In summary, our results underscore the diversity of mechanisms for polerovirus P0 proteins to function as RNA silencing suppressors, and contribute to our understanding of viral suppression of RNA silencing and the molecular mechanisms involved in the PLRV infection. However, much more remains to be determined about whether or how these different motifs or amino acids interact collaboratively or independently to suppress RNA silencing. Additional experiments will need to address whether P0^{PL-IM} interacts physically with other AGOs, other SKP-like proteins, or other proteins that affect the silencing machinery. Moreover, the crystal structure of P0^{PL-IM} may provide a more detailed mechanistic explanation as to how these important motifs and conserved amino acids affect suppression of RNA silencing.

Fig. 5. Effects of P0^{PL-IM} and mutant derivatives on AGO1 accumulation. *Nicotiana benthamiana* leaves were co-infiltrated with 6× myc-tagged AtAGO1, plus one of the following: lane 1, pGD without a 6× myc-tagged AtAGO1; lane 2, pGD or pGD plasmids with 3× flag-tagged; lane 3, P0^{PL-IM}; lane 4, L59A/P60A; lane 5, L76A/P77A; lane 6, W87A; lane 7, W140A; or lane 8, F220R. Western blot analysis using GFP polyclonal antiserum. Total proteins were extracted from the agro-infiltrated regions for sodium dodecyl sulfate polyacrylamide gel electrophoresis. The loading control was Rubisco stained with Coomassie blue. Western blots of total protein extracts were probed at 4 days postinfiltration with anti-myc antibodies (α-myc) to detect 6 myc-AtAGO1 or anti-flag antibodies (α-flag) to detect expression of flag tagged P0^{PL-IM} or P0^{PL-IM} mutant derivatives, as indicated on the right. Co-infiltrations were performed in the presence of P19.

Fig. 6. The $P0^{PL-IM}$ protein does not interact with *Nicotiana benthamiana* SKP1 (NbSKP1). **A**, Interactions of $P0^{PL-IM}$ and $P0^{MA}$ with NbSKP1 were investigated by yeast two-hybrid mating experiments using yeast strain Y187 transformed with BD- $P0^{PL-IM}$ or $P0^{MA}$ and strain AH109 transformed with AD-NbSKP1. The yeast strain Y187 containing $P0^{MA}$ was used as a positive control. Note the β -galactosidase expression and growth of the positive control (AD-NbSKP1 and BD- $P0^{MA}$) samples and the absence of β -galactosidase and yeast growth in the other combinations. **B**, Failure of $P0^{PL-IM}$ to interact with NbSKP1 in bimolecular fluorescence complementation tests. *N. benthamiana* leaves were co-infiltrated for expression of the following combinations of YN- and YC- fusion proteins: $P0^{PL-IM}-YN+NbSKP1-YC$, NbSKP1-YN+ $P0^{PL-IM}-YC$, $P0^{ScYLV}-YN+NbSKP1-YC$ (as a positive control), $P0^{ScYLV}-YN+YC$ (as a negative control), and $yb-YN+yb-YC$ (as a positive control). Yellow fluorescent protein fluorescence images were recorded at 2 days postinfiltration by visualization at 488 nm, and a differential interference contrast (DIC) image was photographed. Scale bars of all images represent 10 μ m.

MATERIALS AND METHODS

Plant material and growth conditions.

All *N. benthamiana* plants were grown in soil at $24 \pm 1^\circ\text{C}$ with a photoperiod of 16 h of light (approximately $7.5 \mu\text{mol}/\text{m}^2\cdot\text{s}$) and 8 h of darkness.

Plasmid constructs.

The binary expression vectors pGD (Goodin et al. 2002), pGDG, and pGDP19 were obtained from A. O. Jackson at the University of California at Berkeley, U.S.A. The $\text{wtP0}^{\text{PL-IM}}$ and flag-tagged $\text{wtP0}^{\text{PL-IM}}$ constructs used in these experiments were engineered by polymerase chain reaction (PCR) amplified from PLRV (GenBank accession number KC456052) using appropriate specific primers (Table 1). All PCR products were ligated to pMD19-T to obtain pMD19-T- $\text{P0}^{\text{PL-IM}}$ or flag-tagged $\text{P0}^{\text{PL-IM}}$. The pMD19-T- $\text{P0}^{\text{PL-IM}}$ or flag-tagged $\text{P0}^{\text{PL-IM}}$ construct was digested with *HindIII*/*BamHI* and subcloned into pGD. All mutants were produced by reverse PCR using specific primers, and the 5' and 3' primers contained *HindIII* and *BamHI* restriction sites (Table 1), respectively. The resulting fragments were inserted into pGD digested with *HindIII* and *BamHI*.

Agrobacterium-infiltration and GFP imaging.

The *Agrobacterium tumefaciens* GV3101, a gift from D. Baulcombe, was transformed with each plasmid using a freeze-

thaw method (Hofgen and Willmitzer 1988). For co-infiltrations, equal volumes of *A. tumefaciens* cultures (absorbance at 600 nm [A_{600}] = 1.0) harboring the relevant binary plasmids were mixed prior to infiltration, and the fully expanded leaves were injected with a syringe (Voinnet et al. 2000). Leaves were illuminated with a 100-W hand-held long-wave ultraviolet lamp (Black Ray model B 100AP/R; UV Products, Upland, CA U.S.A.) for photography and images were taken with a Nikon 4500 digital camera. All experiments shown in the results were repeated at least three times.

Protein extraction and Western analysis.

Infiltrated *N. benthamiana* leaves were ground in liquid nitrogen and mixed with 2× sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris [pH 6.8], 20% glycerol, 4% SDS, and 0.2% bromophenol blue) containing 10% β-mercaptoethanol (Laemmli 1970). The samples were then boiled at 100°C for 5 min, and centrifuged for 5 min at $13,000 \times g$ before loading on a gel. To detect GFP expression, proteins were separated by 12.5% SDS polyacrylamide gel electrophoresis (PAGE) and Western blot analysis was performed by first probing with a rabbit anti-GFP antibody (diluted 1:1,000) and then incubating with anti-rabbit alkaline phosphatase (Sigma, St. Louis). Finally, GFP was detected with the nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate substrates (Sigma).

For detection of the 6myc-AtAGO1, 3× flag $\text{wtP0}^{\text{PL-IM}}$, and 3× flag mutants of $\text{wtP0}^{\text{PL-IM}}$ in Western blots, proteins were

Table 1. Primer sequences used for polymerase chain reaction

Primer	Sequence (5' to 3')
PL71EH-F	CGAATTCAAGCTTATGATTGTATTGACCC
PL814B-R	AGGATCCTTATTCTTGTAATTCC
PLP03×flag-R	CGGGATCCTTATTATCGTCATCGTCTTTGTAGTCTTTATCGTCATCGTCTTTGTAGTCT TTATCGTCATCGTCTTTGTAGTACCTCCGCCTTCTTGTAATTCCTTTGGAG
PLP0V58I-F	ATTCTCCCTTTGCTCAACTGC
PLP0V58A-F	GCTCTCCCTTTGCTCAACTGC
PLP0V58-R	ATAAAGAAAAGAGCGGCATATGCGG
PLP0S102-F	ATCGTCATTAACCTTGACG
PLP0S102P-R	GGTAGGGCCCCACGATTGTATAGC
PLP0S102A-R	GGTAGCGCCACGATTGTATAG
PLP0Y174-F	CATTGGTTTCGGTCTCTATTGG
PLP0Y174H-R	CTGATGATTGCCATAAGGTC
PLP0Y174A-R	CTGAGCATTGCCATAAGGTC
PLP0T222A-F	GCACTTACCGGTTTTCCTAT
PLP0T222A-R	TCTGAAAGACTTAGCGCGCC
PLP0I228V-F	GTTTATGTCCCTCTGAAGA
PLP0I228A-F	GCTTATGTCCCTCTGAAGA
PLP0I228-R	AGGAAAACCGGTAAGTGTTT
PLP0LP59AA-F	ATGTTGCCGCTTTGCTCAACTGC
PLP0LP59AA-R	AAAGAAAAGAGCGGCATATGCGG
PLP0LP76AA-F	CATCCGGCCTTCAAGCTGCGAGGC
PLP0LP76AA-R	TTGAAATCCTGCCTCTTTTGCAGTTG
PLP0W87A-F	GCGGGATTACTCTGCGGCACC
PLP0G88A-F	TGGGCATTACTCTGCGGCACC
PLP0WG87AA-F	GCGGCATTACTCTGCGGCACC
PLP0WG87GW-F	GGATGGTTACTCTGCGGCACC
PLP0WG87AA-R	CTCAAGGCACTCATAGTGGAGG
PLP0GW139AA-F	GCTGCGGGACATGACATGGAGGC
PLP0G139A-F	GCTTGGGGACATGACATGGAGGC
PLP0W140A-F	GGTGCGGACATGACATGGAGGC
PLP0GW139WG-F	TGGGGTGGACATGACATGGAGGC
PLP0G141A-F	GGTTGGGCACATGACATGGAGGC
PLP0WG140GW-F	GGTGGATGGCATGACATGGAGGC
PLP0AWA-F	GCTTGGGCACATGACATGGAGGC
PLP0GW139AA-R	GTTTGACAATCCAGCCGATTTTG
PLP0F220R-F	AGTCTCGCAGAACACTTACCGG
PLP0F220R-R	TAGCGCGCCCTTTAGATCAAGC
PLP071E-F	CGAATTCATGATTGTATTGACCC
PLP0744S-R	GCGTCGACTTATTCTTGTAATTCC
PLP0Xba-F	GCTCTAGAATGATTGTATTGACCC
PLP0Xho-R	CCCTCGAGCAGGTCTCTCTGAGATCAGC

separated by 7% SDS-PAGE and probed with an anti-c-myc antibody (1:1,000; Sigma) or an anti-flag antibody (1:1,000; Sigma) followed by an anti-rabbit goat HRP secondary antibody (1:3,000; Bio-Rad, Hercules, CA, U.S.A.). Antibody-protein interactions were visualized using an enhanced chemiluminescence detection kit (GE Healthcare, Buckinghamshire, U.K.) according to the manufacturer's instructions.

RNA extraction and RNA gel blot analysis.

Total RNA from agroinfiltrated leaves of *N. benthamiana* was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). For detection of high-molecular-weight RNA, 5 µg of total RNA was separated on 1% agarose gels. DNA fragments were amplified by PCR and labeled with [α - ^{32} P]dCTP using the Prime-a-Gene Labeling System (Promega, Madison, WI, U.S.A.). For detection of small RNAs, 20 µg of total RNA was separated on 17% polyacrylamide-8 M urea gels. GFP-specific probes were radiolabeled with [α - ^{32} P]UTP, and hybridizations were performed as described previously (Han et al. 2010).

BiFC assays in *N. benthamiana* leaves using confocal laser-scanning microscopy.

For BiFC analysis, the full-length coding sequence of P0^{PL-IM} was amplified using the PLP0Xba-F and PLP0Xho-R primers (Table 1) and was cloned into the BiFC vectors pSPYNE-35S and pSPYCE-35S (provided by J. Kudla, Universität Münster) that had been digested with *Xba*I and *Xho*I (Walter et al. 2004). All clones generated by PCR were sequenced completely and the recombinant plasmids were confirmed by restriction analysis. *A. tumefaciens* EHA105 strains, which contained the two BiFC plasmids and the pGDP19 plasmid, were adjusted to A₆₀₀ = 1.0 and co-infiltrated into *N. benthamiana* leaves at a ratio of 0.5:0.5:0.3. Abaxial epidermal cells of infiltrated leaves were assayed for fluorescence at 2 to 3 dpi. Fluorescence analysis was performed using a Nikon ECLIPSE TE2000-E inverted fluorescence microscope equipped with a Nikon D-ECLIPSE C1 spectral confocal laser-scanning system. YFP fluorescence was detected at an excitation wavelength of 488 nm and an emission wavelength of 550 to 590 nm (Zhang et al. 2011).

Yeast two-hybrid assays.

The Clontech Matchmaker GAL4 Two-Hybrid System 3 was used to generate yeast strain Y187 containing pGBKT7-P0^{PL-IM} for yeast two-hybrid mating analyses. The P0^{PL-IM} ORF was amplified with the primers PLP071E-F/PLP074S-R (Table 1) and the PCR products were ligated to pMD19-T. The clone was then digested with *Eco*RI and *Sall*I, and the P0 *Eco*RI/*Sall*I fragments were ligated with *Eco*RI- and *Sall*I-digested pGBKT7 (Clontech, Mountain View, CA, U.S.A.) to generate pGBKT7-P0^{PL-IM} for transformation into strain Y187. Yeast transformations were carried out with a small-scale lithium acetate method. The complementary yeast AH109 mating strain harboring NbSKP1 (GenBank AF494084.1) was previously used in our lab for analysis of interactions of *Rice black-streaked dwarf virus* (Wang et al. 2013). Two-hybrid assays were performed as suggested by Clontech. Co-transformants were plated on SD minimal media minus leucine, tryptophan, adenine, and histidine (SD/-Leu/-Trp/-Ade/-His and X-Gal). Positive yeast colonies that could grow on the auxotrophic medium were tested for β -galactosidase activity and photographed.

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