A novel sweet potato potyvirus open reading frame (ORF) is expressed via polymerase slippage and suppresses RNA silencing

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SUMMARY

The single-stranded, positive-sense RNA genome of viruses in the genus *Potyvirus* encodes a large polyprotein that is cleaved to yield 10 mature proteins. The first three cleavage products are P1, HCpro and P3. An additional short open reading frame (ORF), called *pipo*, overlaps the P3 region of the polyprotein ORF. Four related potyviruses infecting sweet potato (*Ipomoea batatas*) are predicted to contain a third ORF, called *pispo*, which overlaps the 3' third of the P1 region. Recently, *pipo* has been shown to be expressed via polymerase slippage at a conserved GA4 sequence. Here, we show that *pispo* is also expressed via polymerase slippage at a GA4 sequence, with higher slippage efficiency (~5%) than at the *pipo* site (~1%). Transient expression of recombinant P1 or the ‘transframe’ product, P1N-PISPO, in *Nicotiana benthamiana* suppressed local RNA silencing (RNAi), but only P1N-PISPO inhibited short-distance movement of the silencing signal. These results reveal that polymerase slippage in potyviruses is not limited to *pipo* expression, but can be co-opted for the evolution and expression of further novel gene products.

Keywords: frameshift, gene expression, overlapping gene, RNA silencing suppression, RNA virus, transcriptional slippage.

INTRODUCTION

Several viruses of the genus *Potyvirus* (family *Potyviridae*) infect sweet potato (*Ipomoea batatas*). Currently, most fall within the monophyletic ‘*Sweet potato feathery mottle virus* (SPFMV) group’ (Untiveros et al., 2008). These include SPFMV, *Sweet potato virus C* (SPVC), *Sweet potato virus G* (SPVG) and *Sweet potato virus 2* (SPV2), but not *Sweet potato latent virus*. SPFMV is the most common virus infecting sweet potatoes worldwide. In mixed infections with *Sweet potato chlorotic stunt virus* (SPCSV; genus *Crinivirus*), it is associated with a severe sweet potato virus disease (SPVD), causing heavy yield losses (Gibson et al., 2004; Tairo et al., 2005). SPVD is particularly important in sub-Saharan Africa, where sweet potato is the second most important root crop after cassava.

The complete genome sequences of several SPFMV-group viruses have been determined (Li et al., 2012; Pardina et al., 2012; Sakai et al., 1997; Untiveros et al., 2010; Yamasaki et al., 2010). Although all members of the genus *Potyvirus* encode a P1 protein, SPFMV-group viruses encode the largest P1 among currently sequenced potyviruses (Fig. 1a) (Sakai et al., 1997; Valli et al., 2007). P1 is a serine protease that cleaves at its own C-terminus, but is not known to be involved in other polyprotein-processing events. Apart from its protease activity, the functions of P1 are not well established. It appears to play a variety of roles in different potyviruses, including enhancement of the RNA silencing suppression (RSS) activity of the helper component proteinase (HCpro), and roles in genome amplification and host specificity (reviewed in Rohožková and Navrátil, 2011; Valli et al., 2007). Although HCpro is generally considered to be the RSS protein in potyviruses (Anandakrishni et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998), in members of some other potyvirus genera, P1 has been shown to be a potent RSS. In *Sweet potato mild mottle virus* (SPMMV; genus *Ipomovirus*), *Triticum mosaic virus* (genus *Paovirus*) and *Wheat streak mosaic virus* (genus *Tritimovirus*), P1, but not HCpro, suppresses silencing (Giner et al., 2010; Tatini et al., 2012; Young et al., 2012). *Cassava brown streak virus* (genus *Ipomovirus*) lacks HCpro, and P1 suppresses silencing (Mbanzibwa et al., 2009). *Cucumber vein yellowing virus* (genus *Ipomovirus*) also lacks HCpro, but has two P1 serine protease proteins, P1a and P1b, where P1b suppresses silencing (Valli et al., 2006). In *Potato virus Y* (PVY), P1 may be needed to stabilize the RSS protein HCpro (Tena Fernández et al., 2013). However, macluraviruses lack any P1 protein (Revers and García, 2015).

The P1 protein of SPFMV-group viruses appears to contain two distinct protein domains (Fig. 1a): the C-terminal P1-pro, which is homologous to the P1 serine protease encoded by all potyviruses, and an N-terminal extension, designated P1-N, which is absent from other potyviruses, but bears homology to the N-terminal region of the P1 protein of the ipomovirus SPMMV (Untiveros...
It was long believed that the potyvirus gene expression strategy was based on a large, single, open reading frame (ORF) encoding a polyprotein, which is processed to 10 mature proteins by three viral proteinases. However, Chung et al. (2008) discovered that there is another, short ORF in a different reading frame embedded within the region encoding P3. This ORF, designated *pipo* (‘pretty interesting *Potyviridae ORF’), is conserved throughout the *Potyviridae* family. Polymerase slippage at a highly conserved GA$_6$ sequence at the 5‘ end of *pipo* results in the insertion of an extra A into 1%–2% of transcripts (Olspert et al., 2015; Rodamilans et al., 2015), leading, after translation and proteolytic cleavage, to the production of P3N-PIPO, a ‘transframe fusion’ of the N-terminal part of P3 (P3N) with the product (PIPO) of the *pipo* ORF. An additional, long alternative-frame ORF in the region encoding P1-pro was observed in the ‘SPFMV group’ of potyviruses and, in analogy with *pipo*, was named *pispo* (‘pretty interesting sweet potato potyvirus ORF’) (Fig. 1a) (Clark et al., 2012). The presence of multiple WG/GW motifs within the translation of *pispo* was noted by Clark et al. (2012), who suggested a potential role in RSS. Here, we present computational analyses of the *pispo* ORF that support its coding status, high-throughput sequencing data demonstrating that *pispo* can be expressed as part of a transframe protein P1N-PISPO via polymerase slippage at a conserved G$_2$A$_6$ slippery sequence, and experimental analyses that confirm that P1N-PISPO is a potent suppressor of RNA silencing.

**RESULTS**

**Comparative genomic analysis of the overlapping *pispo* ORF**

The polyprotein ORFs from the 28 full-length SPFMV-group sequences currently available in GenBank, plus three additional sequences determined in this study (see Experimental procedures), were extracted, translated, aligned as amino acid sequences and back-translated to a nucleotide sequence alignment. Next, the positions of stop codons in the +1 and +2 reading frames relative to the polyprotein ORF, in all 31 sequences, were plotted. This confirmed the conserved presence of a long +2-frame ORF overlapping the P1 region of the polyprotein ORF (Fig. 1b). The ORF has 230 codons in SPFMV and SPVC, and 228 codons in SPVG and SPV2 (measured from a ubiquitous 5‘ G$_2$A$_6$ sequence; see below). The statistical significance of such a long ORF being conserved by chance over such a degree of phylogenetic divergence (estimated >900 phylogenetically independent substitutions within *pispo* in the SPFMV-group sequence alignment) was assessed as described previously (Fang et al., 2012). Using this method, the probability of such a long +2 frame ORF occurring by chance at this location is $P < 1 \times 10^{-16}$.

Next, the alignment was analysed for enhanced conservation at polyprotein-frame synonymous sites (an indicator of
overlapping functional elements), as described previously (Firth, 2014). The analysis revealed regions of enhanced synonymous site conservation where the polyprotein ORF is overlapped by the pispo ORF (Chung et al., 2008), and at the 3' end of the polyprotein ORF, where overlapping replicational RNA elements have been mapped in other potyvirus species (Fig. 1c) (Haldeman-Cahill et al., 1998). Enhancement of polyprotein-frame synonymous site conservation within the region overlapped by pispo was modest, suggesting that the PISPO amino acid sequence is not subject to strong purifying selection. Nonetheless, the mean rate of synonymous substitutions in the region of the polyprotein ORF overlapped by pispo was 10% below the genome average, with a corresponding total P value of 0.01 (Fig. 1c).

At the 5' end of the pispo ORF, there is a conserved G2A6 sequence (Figs 2a and S1, see Supporting Information). In SPFMV and SPVC isolates, this takes the form GG_AAA_AAA, where underscores separate polyprotein codons. In SPVG and SPV2 isolates, it takes the form G_GAA_AAA_A, displaced a few codons upstream from the sequence in SPFMV and SPVC (Fig. 2a). A similar highly conserved sequence, G1–2A6–7 (G2A6 in SPFMV-group viruses), is present at the 5' end of the pispo ORF in nearly all potyvirids (Chung et al., 2008). The presence of the same motifs at the 5' end of pispo suggests that pispo is expressed by the same slip-page mechanism as pipo. This would result in the production of a P1N-PISPO fusion protein (Fig. 2b). In the Ruk73 strain of SPFMV, P1 has a predicted mass of 77.1 kDa, whereas P1N-PISPO has a predicted mass of 75.7 kDa. Analysis of additional sequences with only partial coverage of the polyprotein ORF, but with coverage of the 5' end of pispo and/or the entire pispo ORF, confirmed the conserved presence of the potential slippage site (14 of 14 sequences) and the conserved presence of the pispo ORF (nine of nine sequences).

High-throughput sequencing reveals polymerase slippage at the pispo and pipo G2A6 sites

The occurrence of the G2A6 sequence at the 5' end of the pispo and pipo ORFs in different phases with respect to the polyprotein reading frame suggests that pispo and pipo are unlikely to be expressed via translational frameshifting, although, in principle, both are compatible with -1 ribosomal frameshifting (reviewed in Firth and Brierley, 2012). Further, using RNA-folding software, such as alidot (Hofacker et al., 2002) and pknotsRG (Reeder et al., 2007), we failed to predict a convincing 3' RNA secondary structure at a canonical spacing (i.e. 5–9 nucleotides) downstream of the slippery sites for the stimulation of -1 ribosomal frameshifting. To evaluate whether frameshifting could occur instead during
RNA synthesis, we first utilized high-throughput small RNA sequencing data obtained from SPFMV-infected sweet potato plants and SPV2- and SPVG-infected Ipomoea setosa plants. At both the pispo and pipo G2A6 sequences, a fraction of small RNA reads contained an additional ‘A’ insertion and, for most samples, insertions at these sites occurred well above background frequencies (Fig. 3; Table 1). In these data, ‘A’ insertions were found to occur at a frequency ranging from 0.9% to 11.4% at the pispo G2A6 site and 0.0% to 1.0% at the pipo G2A6 site, depending on the sample. The 0% values are probably a result of low coverage and the lower rate of insertions at the pipo site relative to the pispo site (see below). In comparison, average single nucleotide insertions at other sites across the genome were estimated to occur at a frequency ranging from 0.001% to 0.01% per nucleotide, depending on the sample (Table 1).

Although these results support the interpretation that polymerase slippage occurs at the pispo and pipo G2A6 sites, low coverage, variability between samples and low accuracy of insertion calling as a result of short read lengths mean that the slippage frequency is likely to be poorly estimated by this approach. Therefore, a separate sequencing experiment was conducted in which both the pispo and pipo sites in viral RNA were specifically targeted for amplification and sequencing. This experiment was conducted with RNA extracted from sweet potato or I. nil infected with SPFMV-Ruk73, and led to sequencing depths ranging from 6.1 to 32.5 million reads for each slippage site region (Fig. 4; Table 2). Single ‘A’ insertions were observed within the pispo G2A6 sequence for 5.0% and 5.5% of sweet potato and I. nil reads, respectively. Within the pipo G2A6 sequence, single ‘A’ insertions were observed for 0.9% and 1.0% of reads, respectively. Control DNAs for the same regions showed no insertions at all in more than eight million reads, indicating that insertions did not occur during amplification, library preparation or sequencing. Parallel work with the Turnip mosaic virus G2A6 pipo slippage site indicated that any insertional slippage occurring during reverse transcription appears to be rare.

| Sample              | WT | FS | FS % | Background % insertion rate |
|---------------------|----|----|------|----------------------------|
| SPFMV-10*           | 31 | 4  | 11.4 | 0.0106                     |
| SPFMV-12*           | 576| 43 | 7.0  | 0.0013                     |
| SPV2†               | 226| 2  | 0.9  | 0.0035                     |
| SPVG†               | 219| 12 | 5.2  | 0.0023                     |

*10, sweet potato only infected with SPFMV; 12, sweet potato infected with SPFMV + Sweet potato chlorotic stunt virus (SPCSV).
†Ipomoea setosa infected with SPV2 or SPVG.
transcription is less than \(\sim 0.05\%\) (Olspert et al., 2015). At a lower level, deletions were also observed to occur (Fig. 4), although these could be an artefact of deletional slippage during reverse transcription (Olspert et al., 2015).

PISPO contains GW/WG motifs

The amino acid sequence of PISPO is less conserved than the amino acid sequence of the part of P1 that it overlaps (Table S1, see Supporting Information), e.g. there is 29%–40% amino acid identity in PISPO, but 48%–67% identity in P1-pro between potyviruses that encode PISPO. Application of BLASTP, CS-BLAST, InterProScan, hhpred and phyre-2 to PISPO revealed no significant homology to previously annotated or characterized proteins, as expected, as PISPO clearly evolved by ‘overprinting’ the ancestral P1-pro domain (Altschul et al., 1990; Angermüller et al., 2012; Kelley and Sternberg, 2009; Quevillon et al., 2005; Söding et al., 2005). In addition, \(\sim 60\%\) of PISPO was predicted to be disordered according to PONDR-FIT (Xue et al., 2010) (Fig. S2, see Supporting Information), again typical of many overlapping genes (Rancurel et al., 2009).

Further inspection of the amino acid sequence revealed WG/GW (‘AGO-hook’) motifs. Such motifs play a key role within the P1-N domain of SPMMV P1 (Giner et al., 2010) and some other plant viral silencing suppressors (Azevedo et al., 2010; de Ronde et al., 2014). Among 31 sequenced isolates, the PISPO sequences contain between one and three GW/GW motifs, whereas full-length P1 and full-length P1N-PISPO contain between two and four GW/GW motifs (Fig. 5). It should be noted, however, that WG/GW di-amino acids are not particularly uncommon in generic protein sequences.

P1 and P1N-PISPO are RNA silencing suppressors with distinguishable phenotypes

To test whether P1 and/or P1N-PISPO have RSS activity, we used a standard silencing assay, whereby transgenic Nicotiana benthamiana line 16c plants that constitutively express green fluorescent protein (GFP) were agroinfiltrated with recombinant Agrobacterium tumefaciens strains harbouring binary expression constructs for GFP (pBIN:GFP) and a test protein (e.g. pBIN:P1). Overexpression of GFP mRNA from pBIN:GFP induces sense-mediated silencing of GFP and results eventually in a red leaf patch under UV light, unless the test protein suppresses silencing, in which case the leaf patch will show enhanced green fluorescence under UV light. We tested various potyvirus proteins in this system (Fig. 6). At 4 days post-infiltration (dpi), tissues co-infiltrated for expression of GFP and P1 or P1N-PISPO showed slightly brighter GFP fluorescence than those infiltrated for expression of SPFMV HCpro, Sweet potato latent virus (SPLV) P1

Table 2 Targeted high-throughput sequencing of the pispo and pipo G2A6 slippage sites.

| Sample                  | # reads | %      | # reads | %      | # reads | %      | # reads | %      |
|-------------------------|---------|--------|---------|--------|---------|--------|---------|--------|
| PIPO Ipomoea batatas    | 9149358 | 0.90   | 82637   | 0.02   | 1717    | 0.44   | 40314   |        |
| PIPO Ipomoea nil        | 37396166| 1.03   | 385513  | 0.02   | 8711    | 0.52   | 193787  |        |
| PIPO DNA                | 8449879 | 0.00   | 0       | 0.00   | 0       | 0.07   | 5808    |        |
| PISPO I. batatas        | 7298433 | 4.98   | 363120  | 0.27   | 19936*  | 0.08   | 5771    |        |
| PISPO I. nil            | 30255818| 5.45   | 1650021 | 0.31   | 95093†  | 0.10   | 28964   |        |
| PISPO DNA               | 8142625 | 0.00   | 0       | 0.00   | 0       | 0.03   | 2366    |        |

*74% G2A8, 26% G2A6.
†70% G2A8, 27% G2A6, 4% G2A10.
or a β-glucuronidase (GUS) control (Fig. 7a), indicating that P1 and P1N-PISPO have RSS activity. Leaf tissues expressing *Potato virus A* (PVA) HCpro exhibited the strongest GFP signals (Fig. 7a). These phenotypic differences correlated positively with the accumulation of GFP (Fig. 7b) and GFP mRNA (Fig. 7c), and were negatively correlated with the accumulation of GFP mRNA-derived small interfering RNA (siRNA) in the leaf tissue (Fig. 7c). The expression of P1 and P1N-PISPO was verified by western analysis using peptide antibodies (Abs) generated against an epitope (Fig. 2c) common to P1 and P1N-PISPO (blot ‘P1’ in Fig. 7b) and peptide Abs specific to P1N-PISPO (Fig. 7b). The observed bands were of slightly different sizes than expected, possibly as a result of post-translational modifications.

The suppression of silencing by P1 and P1N-PISPO was quite apparent at 6 dpi (Fig. 7d) and at 8 dpi (Fig. 7e), as indicated by GFP fluorescence in the co-infiltrated leaf tissue, whereas the leaf spots infiltrated for the expression of SPFMV HCpro, SPLV P1 and GUS had lost GFP fluorescence (Fig. 7e). However, P1 and P1N-PISPO differed in that a red halo developed around the leaf tissue infiltrated for the expression of P1. The red halo was most apparent at 6 dpi (Fig. 7d,e, leaf spot 1), indicating short-distance movement of the silencing signal. A red halo also developed around the leaf spots infiltrated for the expression of SPFMV HCpro, SPLV P1 and GUS (Fig. 7e). In contrast, only a faint red halo, if any, developed around the leaf tissue infiltrated for the expression of P1N-PISPO (Fig. 7d,e, leaf spot 2), indicating interference with the short-distance signalling for silencing.

As no Abs were available for detection of SPFMV HCpro and SPLV P1, these proteins were also expressed tagged with the N-proximal part (YN) of yellow fluorescent protein (YFP) (Fig. 6) and tested by western blot using anti-YN Abs. YN-P1 (SPLV) was detected at 2 dpi (Fig. S3, see Supporting Information), but only occasionally at later time points (3–5 dpi), whereas YN-HCpro could not be detected at any time point (2–10 dpi) in eight independent experiments.

As it is possible that the P1 construct could also express P1N-PISPO via translational or transcriptional frameshifting, we generated another P1 expression construct, P1\(_{DPISPO}\), in which expression of the pispo ORF was prevented by the insertion of a stop codon (Fig. 6). As with P1 (leaf spot 1; Fig. 8a), P1\(_{APISPO}\) (leaf spot 3; Fig. 8a) suppressed GFP silencing and was associated with the formation of a red halo.

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**Fig. 5** Locations of GW/WG sequences in P1 and P1N-PISPO proteins of *Sweet potato feathery mottle virus* (SPFMV)-group viruses. Schematic diagram of the P1 protein of *Sweet potato mild mottle virus* (SPMMV) and the P1 and P1N-PISPO proteins of SPFMV-group viruses, showing the positions of GW/WG sequences. The P1 sequence is indicated in light blue; the PISPO sequence is indicated in pink. GW/WG sequences are indicated by vertical coloured lines, with homologous instances (by BLASTP alignment) indicated with the same colour. SPV2, *Sweet potato virus* 2; SPVC, *Sweet potato virus* C; SPVG, *Sweet potato virus* G.

**Fig. 6** Binary vectors for the expression of viral proteins in leaves by agroinfiltration. *Cauliflower mosaic virus* 35S promoter-driven binary vectors were designed to express the following proteins: P1, P1 of *Sweet potato feathery mottle virus* (SPFMV) [the shaded bar indicates the position of the +2 frameshift pispo open reading frame (ORF) encoding 230 residues]; P1N-PISPO, engineered transframe sequence for the expression of the N-proximal part of P1 together with PISPO; P1\(_{APISPO}\), P1 including a stop codon inserted in the +2 frame pispo ORF to ensure that P1N-PISPO is not produced; P1N-PISPO\(_{AWG2,3,4}\), P1N-PISPO mutated at three positions (W\(_{514}\)G\(_{515}\), W\(_{624}\)G\(_{625}\) and W\(_{665}\)G\(_{666}\); designated as WG motifs 2, 3 and 4, respectively) to substitute the tryptophan residues for alanines; P1\(_{AWG1}\), P1 mutated to substitute residue W\(_{25}\) for alanine (WG motif 1); P1N-PISPO\(_{AWG1}\), P1N-PISPO with W\(_{25}\) mutated to alanine; P1N-PISPO\(_{AWG1,2,3,4}\), P1N-PISPO with all four WG motif tryptophans mutated to alanine; HCpro, HCpro of SPFMV; YN-HCpro, the N-proximal part of the yellow fluorescent protein (YN) fused to the N-terminus of SPFMV HCpro; P1(SPLV), P1 of *Sweet potato latent virus*; YN-P1(SPLV), the N-proximal part of the yellow fluorescent protein (YN) fused to the N-terminus of SPLV P1.
The WG motif at the N-terminus of P1 is pivotal for suppression of RNA silencing

The completely conserved WG motif near the N-terminus of SPFMV-group P1 (residues 25–26) aligns with the first WG motif in SPMMV P1 (Fig. 5), which has been shown to be required for SPMMV P1 RSS activity (Giner et al., 2010). To test whether this WG motif is also required for the RSS activity of SPFMV P1 and/or P1N-PISPO, we mutated the tryptophan residue (W25) to alanine and tested the mutated P1 and P1N-PISPO for RSS activity, as above. In contrast with P1 and P1N-PISPO (Fig. 8b, leaf spots 1 and 3, respectively), the mutated constructs for P1(DWG1) and P1N-PISPO(DWG1) (Fig. 6) were unable to suppress silencing (Fig. 8b, leaf spots 2 and 4, respectively). Similarly, when all four WG motifs were mutated [P1N-PISPO(DWG1,2,3,4)] (Fig. 6), no suppression of silencing was observed (Fig. 8b, leaf spot 6). In contrast, similar mutations introduced in all three WG motifs in the PISPO domain [P1N-PISPO(DWG2,3,4)] (Fig. 6) did not prevent the suppression of silencing (Fig. 8b, leaf spot 5). Taken together, these results indicate that the WG motif near the N-terminus of P1 is essential for suppression of RNAi by both P1 and P1N-PISPO.

DISCUSSION

Potyviruses were believed to use a single polyprotein gene expression strategy until the recent discovery of the overlapping pipo ORF (Fig. 1a) (Chung et al., 2008). The pipo ORF has a highly conserved G1–2A6–7 sequence at its 5’ end. Previously, it was proposed that the expression of P3N-PiPO might depend on translational or transcriptional frameshifting occurring at this site (Chung et al., 2008). However, the true nature of the expression mechanism was not confirmed until recently, when, during the preparation of this article, both we and others observed the
production of virus transcripts with single nucleotide insertions at the **pipo** \(G_{1,2}A_{6,7}\) sequence in various potyviruses, thus allowing the expression of P3N-PIPO (this study; Olspert et al., 2015; Rodamilans et al., 2015). The ubiquitous presence of a \(G_{2}A_{6}\) sequence at the 5′ end of the **pispo** ORF, conserved in all SPFMV-group sequences, suggested that **pispo** might be expressed via the same polymerase slippage mechanism, leading to a P1N-PISPO trans-frame fusion protein. The potential for P1N-PISPO to be expressed in this way was confirmed by the observation of edited transcripts in our small RNA sequencing data for SPFMV, SPV2 and SPVG, and our targeted high-throughput sequencing of SPFMV RNA. The latter data also indicate that slippage at the SPFMV **pispo** slip site (5.0%–5.4%) is significantly more efficient than at the SPFMV **pipo** slip site (0.9%–1.0%). Whilst this article was in preparation, a similar difference was also observed by others (Rodamilans et al., 2015). The reasons for the differences between the two sites are unknown, but may be a result of the flanking nucleotide context which may affect the stability of the template:nascent RNA duplex adjacent to the slippage site (Hausmann et al., 1999).

Although we have currently been unable to detect P1N-PISPO in infected plants by western analysis (perhaps because of low expression, masking of the Ab epitope and/or the kinetics of virus infection and protein turnover), the presence of edited transcripts implies that P1N-PISPO can indeed be expressed during virus infection.

P1 is the most variable potyvirus protein in terms of length and sequence (Adams et al., 2005; Valli et al., 2007). The discovery of PISPO adds a new dimension to the functional variability occurring in the P1 region. Unlike PIPO, which is found universally in the **Potyviridae** family, PISPO is restricted to potyviruses in the SPFMV group. It does not occur in the related SPLV, nor in the ipomovirus SPMMV with which SPFMV-group viruses share a related P1-N domain. The PISPO sequence is highly variable (Table S1) (Li et al., 2012) and less conserved than the overlapping region of P1-pro, in clear contrast with PIPO and the region of P3 that it overlaps (Chung et al., 2008). Differences in the evolutionary plasticity of **pipo** and **pispo** may reflect the functional importance of the corresponding transframe proteins. P3N-PIPO is an essential protein, and appears to be of ancient origin, as attested by its conservation throughout the **Potyviridae** family, whereas P1N-PISPO seems to be a more recent evolutionary development and may therefore not yet have acquired a critical role, allowing more sequence plasticity, as has been observed for other recently evolved overlapping viral genes (Sabath et al., 2012). PISPO is
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predicted to be largely disordered (Fig. S2). The function of such an intrinsically unstructured protein region may be largely independent of most of the primary sequence, allowing for rapid evolution and exploration of sequence space (Gitlin et al., 2014).

Although it is clear that PISPO is biologically relevant, as an ORF of this length would not be conserved by chance ($P < 1 \times 10^{-16}$) over the given phylogenetic divergence, at present there is no infectious clone of SPFMV, and so it is not possible to verify the functional relevance of P1N-PISPO during infection. However, given the importance of WG/GW domains in other silencing suppressors (Giner et al., 2010; de Ronde et al., 2014), the evaluation of P1N-PISPO for RSS activity was an obvious course. Surprisingly, we found that both P1N-PISPO and P1 are RNA silencing suppressors in SPFMV. Intrinsic silencing suppression has not been described previously for P1 proteins of potyviruses (Rajamäki et al., 2005; Tena Fernández et al., 2013), nor was it found herein for P1 of SPLV (which clusters as an immediate outgroup to the SPFMV group). Most recent reports assign potyvirus P1 a role for increasing the accumulation of HCpro and consequently enhancing its silencing activity (Tena Fernández et al., 2013). We also showed that P1 and P1N-PISPO seem to have different silencing activities in SPFMV, a strategy described for different RSS proteins of other viruses as well (Lu et al., 2004; Senshu et al., 2011). P1 operates only at a single-cell or local level, as evidenced by the formation of the red halo around the patch (Fig. 7), as also reported for SPMMV P1 and a few other virus silencing suppressors (Baumberger et al., 2007; Giner et al., 2010). The effect of P1 on silencing was a result of the sole action of this protein, and participation of traces of P1N-PISPO can be discarded as the phenotype was identical to P1\textsc{\textalpha}PISPO (Fig. 8), in which PISPO is made untranslatable by introducing a stop codon. However, P1N-PISPO transiently inhibited RNA silencing at both local and short-range levels, as reported for suppressors that sequester siRNA, including PVA HCpro and tombusvirus P19 (Lakatos et al., 2004; Shiboleth et al., 2007). The elimination of the three PISPO WG/GW motifs did not remove the suppressor capacity of P1N-PISPO (Fig. 8). However, the elimination of the single WG near the N-terminus of P1-N completely eliminated RSS activity of both P1 and P1N-PISPO. Thus, the differing C-termini of P1 and P1N-PISPO modulate the RSS activity resident within P1-N.

The results of our experiments differ from those of a previous study in which P1 of SPFMV was reported not to suppress RNA silencing (Szabó et al., 2012), probably because a different experimental system and SPFMV isolate were used.

The functional characterization of P1 and P1N-PISPO requires further experimental work, in particular during SPCSV synergisms. SPCSV RNase3 protein mediates these synergisms through the suppression of antiviral defence in sweet potato plants, allowing potyviruses to enhance titres (Cueilar et al., 2009). Nevertheless, symptom severity, even among SPFMV-group viruses, varies greatly (Gutierrez et al., 2003; Kokkinos and Clark, 2006; Untiveros et al., 2007), and this is not necessarily related to the enhancement of viral replication (Kokkinos and Clark, 2006). This suggests that additional viral determinants may be required for the development of unusually severe disease in sweet potato plants co-infected with SPFMV and SPCSV. Such determinants might include P1 and P1N-PISPO. Therefore, the results of this study may have important implications for understanding the devastating synergistic diseases caused by these viruses in sweet potato.

**EXPERIMENTAL PROCEDURES**

**Viruses**

SPFMV isolate Ruk73 from Uganda (Tugume et al., 2010) was used to generate all the SPFMV-derived expression vectors. In addition, a Taiwanese isolate of SPLV (Wang et al., 2013), available from the International Potato Center (CIP) reference virus collection, was used to generate the corresponding P1 expression construct. SPFMV-Piu3 (Kreuze et al., 2009), and SPVG-LSU1 and SPV2-LSU2 (kindly provided by Dr Christopher A. Clark, Louisiana State University, Baton Rouge, LA, USA), available from the CIP reference isolate collection, were used for small RNA sequencing.

**Sequencing of SPVG and SPV2 by assembly of siRNAs**

siRNA sequencing (provider: Fasteris Life Sciences SA, Plan-les-Ouates, Switzerland) and assembly were performed on leaf samples from *I. setosa* plants infected with SPVG-LSU1 or SPV2-LSU2, as described by Kreuze et al. (2009). Original siRNA reads can be found at: https://research.cip.cgiar.org/confluence/display/cpx/CIP.sweetpotato.2015. The full genome sequences of SPVG (10 771 nucleotides) and SPV2 (10 732 nucleotides) were assembled at an average sequencing depth of 342- and 557-fold, respectively, and deposited in GenBank (Table S2, see Supporting Information).

**Computational analysis**

The complete genomes of SPFMV-Ruk73, SPVG-LSU1 and SPV2-LSU2 were determined in this study (see above). Additional SPFMV-group nucleotide sequences in GenBank were identified using *BLASTN* (Altschul et al., 1990) (see Table S2 for accession numbers). Coding sequences were extracted, translated, aligned and back-translated to produce nucleotide sequence alignments using EMBOS and *CLUSTALW* (Larkin et al., 2007; Rice et al., 2000). Synonymous site conservation was calculated as described previously (Firth, 2014). The probability of the conserved absence of stop codons in the *pispo* ORF occurring by chance (i.e. if *pispo* was actually non-coding) was assessed via P1-frame alignment codon column shuffling, as described previously (Fang et al., 2012).

**Plasmids**

A schematic diagram of the vectors used in this study is displayed in Fig. 6. Briefly, DNA fragments containing the complete coding regions of SPFMV P1 and HCpro (isolate Ruk73), and SPLV P1 (KC443039.1), were
amplified by polymerase chain reaction (PCR) using Phusion high-fidelity DNA polymerase (Thermo Scientific, Waltham, MA, USA) and cloned into the pBIN61 binary vector using XbaI/Xmal restriction sites. The sequence encoding the YFP N-proximal fragment (YN) was added to the 3’ end of SPFVMV HCpro and SPLV P1 using a similar approach to that described previously (Halkonen et al., 2013). Appropriate primers were used to introduce an AUG start or a UAA stop codon for the cloned regions where required. Thus, binary vectors containing wild-type viral proteins included pBIN61:SPFMV-P1, pBIN61:YN-SPFMV-HCpro and pBIN61:YN-SPLV-P1.

In addition, SPFVMV P1 (hereafter called P1) was cloned into the expression vector pQE 30 (Qiagen, Venlo, the Netherlands) using HindIII/BamHI restriction sites, generating plasmid pQE30:P1. From this plasmid, other constructs were developed, including P1-PISPO, P1ΔPISPO, P1-PISPO(ΔWG2,3,4), P1(ΔWG1), P1-PISPO(ΔWG1) and P1-PISPO(ΔWG1,2,3,4), using appropriate primers and the Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The transframe P1N-PISPO construct contains a 1-nucleotide ‘A’ insertion at site 1464 (i.e. G2A to G2A mutation), resulting in a frame change and the production of a P1N-PISPO protein identical to that expected from polymerase slippage. In addition, two ‘A’ to ‘G’ mutations were introduced in P1N-PISPO at genomic sites 1460 and 1463 within the G2A, sequence (Lys 448 and 449; GG_AA_AAA_A to GG_AAG_A) in order to prevent translational or transcriptional slippage occurring on the run of seven ‘A’s. P1ΔPISPO is a modified version of P1 including a synonymous mutation (GCA to GCT) at serine-456 in the polyprotein frame, resulting in a +2 frame stop codon that prevents any accidental expression of P1N-PISPO from this construct. P1-PN-PISPO(ΔWG3,4) has the tryptophan residue in each of the three WG motifs in P1N-PISPO from this construct. P1N-PISPO(ΔWG3,4) was amplified by polymerase chain reaction (PCR) using Phusion high-fidelity DNA polymerase (Thermo Scientific, Waltham, MA, USA) and cloned into the relevant proteins.

Agroinfiltration assays

Agroinfiltration was carried out as described in Johansen and Carrington (2001). Agrobacterium tumefaciens C58C1 strains carrying the indicated plasmids were combined before infiltration. The different Agrobacterium cultures were diluted with infiltration medium so that equal final optical densities were reached before combining cultures in a 1:1 ratio for infiltration. Transgenic N. benthamiana line 16c, which expresses GFP, was used for infiltration (seeds kindly provided by Professor D. Baulcombe, Department of Plant Sciences, University of Cambridge, UK). Co-infiltration was carried out with two A. tumefaciens strains expressing GFP and P1, P1N-PISPO, SPFMV HCpro, SPLV P1, PVA HCpro, GUS, P1ΔPISPO, P1N-PISPO(ΔWG2,3,4), P1(ΔWG1), P1N-PISPO(ΔWG1) or P1N-PISPO(ΔWG1,2,3,4). GFP fluorescence was monitored daily by epi-illumination with a hand-held UV source (Blackray UVP 100 AP, Thermo Scientific). Photographs were taken with a digital camera, and images were prepared with Photoshop software (Adobe, San Jose, CA, USA).

Antibodies and western blotting

Polyclonal Abs to predicted antigens within PISPO and P1N (present in both P1 and P1N-PISPO) were prepared by GenScript (Piscataway, NJ, USA). Anti-P1N was raised against peptide sequence KTQTPGEVHHKMVR. Anti-PISPO was raised against peptide sequence RAHASSPENSLQKG. Rabbits were injected with one of the two peptides, and Abs were affinity purified from immune sera. The detection of GFP YN-tagged proteins and GFP was achieved using GFP-specific polyclonal rabbit antiserum (Invitrogen, Carlsbad, CA, USA). Total proteins were extracted from leaves as follows: 20 mg of leaf tissue were homogenized in liquid nitrogen and resuspended in 80 mL of 2 × extraction buffer [0.1 M Tris-HCl, 20% glycerol, 10% (v/v) β-mercaptoethanol, 4% (w/v) sodium dodecyl sulfate (SDS), 0.2% bromophenol blue], boiled for 5 min and clarified by centrifugation. When needed, proteins were concentrated from larger amounts of plant material using acetone precipitation. Proteins were analysed on a 12% SDS-polyacrylamide gel by electrophoresis and electroblotted onto a Hybond-P polyvinylidene fluoride membrane (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK). Membranes were blocked in 50 mL of Tris-HCl (TBS) with 0.1% skimmed milk powder, and then probed with the corresponding Abs (1: 5000 P1, PISPO) at 4°C overnight. Following washing with 1 × TBS containing 0.1% Tween 20, the membrane was incubated with polyclonal donkey anti-rabbit immunoglobulins (IgGs) conjugated with horseradish peroxidase (HRP) (GE Healthcare). Proteins were detected with the enhanced chemiluminescence method using Super Signal West Femto chemiluminescent substrate (Thermo Scientific), and visualized by exposure to photographic film (Kodak, Rochester, NY, USA).

RNA analysis

The accumulation of GFP mRNA and GFP mRNA-derived siRNA was evaluated by northern blot analysis in leaf tissues agroinfiltrated for co-expression of GFP and P1, P1N-PISPO, SPFMV HCpro, SPLV P1, PVA HCpro or GUS. Total RNA was extracted with TRIzol LS Reagent (Invitrogen) according to the manufacturer’s recommendations and low-molecular-weight (LMW) and high-molecular-weight (HMW) RNA fractions were separated as described in Cuellar et al. (2009). LMW RNA (15 µg/lane) and HMW RNA (10 µg/lane) were separated by electrophoresis in a denaturing 15% polyacrylamide gel and 1.2% (w/v) agarose gel, respectively, both containing formaldehyde. The two gels were transferred to a nylon membrane (GE Healthcare) and cross-linked chemically with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (Pall and Hamilton, 2008) or by UV light (Sambrook and Russell, 2001), respectively. A GFP mRNA-specific radioactive RNA probe was synthesized, fractionated with carbonate hydrolysis and used to probe the membranes for GFP mRNA and GFP mRNA-derived siRNA, as described previously (Cuellar et al., 2009). Signals were detected by autoradiography on an X-ray film (Kodak).

High-throughput sequencing

Targeted high-throughput sequencing was performed on total RNA extracted from SPFVMV-RuK73-infected sweet potato or I. nil plants, as
described in Olspert et al. (2015), except that reverse transcription-polymerase chain reaction (RT-PCR) was performed with primers to amplify the regions surrounding the putative slippage sites for pispo (target site, excluding primer and adaptor sequences: ACTCGTTTGG AAAAACGAC, corresponding to SPMV-Ruk73 nucleotides 1448–1469) and pipolo (target site, excluding primer and adaptor sequences: TCAATGTGAAAAAATTGGGAT, corresponding to SPMV-Ruk73 nucleotides 4017–4037).

High-throughput sequencing of small RNA was performed on RNA extracted from infected sweet potato (SPFMV-Piu3) or I. setosa (SPVG-LSU1 and SPV2-LSU2) plants as described above. Short-RNA reads, both forward and reverse sense, were mapped to the respective reference genomes using BWA (Li and Durbin, 2009), and positions of insertions were determined from the BWA CIGAR and TAG report fields. Initial back-ground single nucleotide insertion rates were calculated by dividing the observed number of insertions (excluding those at the pispo and pipolo G2A6 sites) by the product of the mean read depth and genome length. As a result of the short length of reads (21–24 nucleotides) and BWA not reporting insertions or deletions (indels) that occur within 5 nucleotides of the termini of reads, these initial values were adjusted for the potentially unreported indels by multiplying by the correction factor \( \sum_{i} p_i \hat{i}(i-10) \), where \( i \) ranges over the read lengths (i.e. 21–24 nucleotides), \( p_i \) is the fraction of mapped reads that have length \( i \), and \( \hat{i}(i-10) \) is the reciprocal of the proportion of insertions occurring within a read of length \( i \) that would be detected by BWA (i.e. are not within 5 nucleotides of the read termini).

**Accession numbers**

GenBank sequence accession numbers are provided in Table S2.

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**NOTE ADDED IN PROOF**

After this manuscript was accepted a complementary study by Mingot et al. was accepted in Journal of Virology (doi:10.1128/JVI.02360-15; posted online). The study by Mingot et al. largely corroborates our findings and confirms the existence of PISPO expression in infected plants by mass spectrometry.

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sequences. SPV2, Sweet potato virus 2; SPVC, Sweet potato virus C; SPVG, Sweet potato virus G.

**Fig. S2** PONDR® disorder predictions for P1 and P1N-PISPO. Common regions predicted to be unstructured or disordered include a small region of ~25 residues within the N-terminus of P1N, and the hypervariable region (residues 200–300). In P1, a third region of predicted disorder occurs around residues 459–522, whereas, in P1N-PISPO, a third region of predicted disorder encompasses almost the entire PISPO domain starting around residue 444. Predicted non-disordered domains have scores no higher than 0.5.

**Fig. S3** Expression and detection of YN-P1(SPLV) in agroinfiltrated leaf tissue of green fluorescent protein (GFP)-transgenic *Nicotiana benthamiana* line 16c. (a) Neither (1) YN-P1(SPLV) (left half of the leaf) nor (2) YN-HCpro (right half of the leaf) of *Sweet potato feathery mottle virus* (SPFMV) suppressed *gfp* silencing and no enhancement of GFP fluorescence was observed at 4 days post-infiltration (dpi). The infiltrated tissue was surrounded by a red edge indicating short-distance movement of the silencing signal. Scale bar indicates 2 cm. (b) Expressed YN-P1(SPLV) protein was detected at 2 dpi using anti-YN antibodies, but not at 3, 4 or 5 dpi. The upper band indicated with an arrow (2d) corresponds to the expected size (72 kDa) of YN-P1(SPLV). The small arrow on the left indicates the position of the 70-kDa protein marker. Staining of total proteins by Coomassie blue was used as a loading control.

**Table S1** Mean amino acid inter- and intra-species identities. Identities (%) calculated for the PISPO (*italics*; upper left) and P1-pro (roman; lower right) domains.

**Table S2** Virus species and accession number for sequences used for the computational analysis of the *pispo* open reading frame (ORF). Sequences of *Sweet potato virus G* (SPVG)-LSU1 and *Sweet potato virus 2* (SPV2)-LSU2 were determined in this study, and partial sequences with coverage of *pispo* also included are indicated in a separate column.

**Table S3** Primer sequences used in this study.