# Letters

## On the inhibition of RNA silencing movement by the tombusvirus P19 protein: reliance on sRNA binding and correlation with local silencing suppression

### A response to Brioudes *et al*. (2022) 'Suppression of both intra- and intercellular RNA silencing by the tombusviral P19 protein requires its small RNA binding property'

In plants, RNA silencing is considered the main antiviral defence mechanism. RNA silencing is initiated by the presence of double-stranded RNA (dsRNA) molecules, generated during the viral cycle; these molecules are processed by Dicer-like (DCL) RNase III proteins into small RNAs (viral small interfering RNAs, vsiRNAs), which are in turn bound by ARGONAUTE (AGO) proteins and incorporated onto RNA-induced silencing (RISC) complexes to direct the degradation of sequence-complementary RNA molecules (Jin *et al*., 2021). Small RNAs triggering RNA silencing can move between cells and systemically, presumably relaying the information of a viral attack ahead of the infection front. The relevance of this pathway for antiviral defence is underscored by the finding that all plant viruses studied to date encode one or several independently evolved proteins capable of interfering with this process, the so-called viral suppressors of RNA silencing (VSRs) (Jin *et al*., 2021).

Most VSRs have been described to interfere with intracellular RNA silencing, and only some of them also exert an effect on the cell-to-cell movement of the signal. One such example is the C4 protein encoded by the geminivirus tomato yellow leaf curl virus (TYLCV), which led to the identification of its interacting receptor-like kinases (RLKs) BAM1 and BAM2 as players in the inter-cellular movement of RNA silencing (Rosas-Diaz *et al*., 2018; Fan *et al*., 2021). The ability to suppress the spread of RNA silencing as well as to interact with BAM1/2 is conserved in other geminiviruses (Carluccio *et al*., 2018; Li *et al*., 2020).

The involvement of BAM1/2 in the cell-to-cell spread of RNA silencing and their targeting by geminiviral C4 led us to hypothesise that these RLKs or multiprotein complexes containing them might be convergently targeted by plant viruses. In order to test this idea, we initially selected the P19 protein encoded by the tombusvirus tomato bushy stunt virus (TBSV), one of the best characterised and most widely used VSR, which is known to interfere with the inter-cellular movement of silencing (Qu & Morris, 2002; Papp *et al*., 2003; Chapman *et al*., 2004; Brosnan *et al*., 2019). Strikingly, we found that P19 interacts with BAM1/2, and that, as in the case of C4, this interaction preferentially occurs at plasmodesmata, the channels through which the silencing signal moves from cell to cell (Rosas-Diaz *et al*., 2018; Garnelo Gómez *et al*., 2021). Of note, the movement protein of the tobamovirus tobacco mosaic virus (TMV) has also been recently shown to interact with BAM1 at plasmodesmata (Tran & Citovsky, 2021).

P19 forms dimers that bind an sRNA duplex, be it an siRNA or a microRNA (miRNA), in a size-dependent manner (Vargason *et al*., 2003; Ye *et al*., 2003): P19 sequesters 21-nt sRNA molecules, hence impairing their function and ultimately suppressing RNA silencing. As P19 could associate with BAM1/2 at plasmodesmata, we speculated that the effect of this VSR on the cell-to-cell movement of silencing may derive, at least partly, from its interaction with the RLKs, and not be solely due to its ability to titrate out sRNAs, as previously assumed. With the aim to test this idea, we used a published mutant version of P19, P19$^{W39/42R}$, which had been shown to be considerably less efficient in local silencing suppression and target cleavage assays (Vargason *et al*., 2003; Lakatos *et al*., 2006), due to replacement of the tryptophan residues in positions 39 and 42, mediating the end-capping interactions with the ends of the sRNA duplex, by arginines. This P19 mutant had also been previously used with the same purpose (to discern the contribution of sRNA binding to a P19-derived phenotype): in Sansregret *et al*. (2013), the authors used this variant to conclude that binding of sRNA is not required for P19 to induce immune responses in tobacco.

The reporter SUC-SUL transgenic *Arabidopsis thaliana* (hereafter referred to as Arabidopsis) plants express an inverted repeat of the *CHLI* gene, encoding a subunit of the magnesium chelatase required for chlorophyll biosynthesis, specifically in phloem companion cells (Himber *et al*., 2003). Expression of this inverted repeat results in the production of 21-nt and 24-nt siRNAs against *CHLI*, which leads to its silencing, ultimately reflected in a chlorotic phenotype or bleaching. Due to the ability of 21-nt siRNAs to move intercellularly through 10–15 cells beyond the source phloem companion cells, the silencing-induced chlorosis expands to the leaf lamina and is visible to the naked eye, serving as a proxy for the efficiency of the cell-to-cell spread of RNA silencing. The ability of the wild-type P19 and of its mutant version to suppress the spread of bleaching around the vasculature in SUC-SUL plants was then evaluated. The P19$^{W39/42R}$ protein did not show, in our hands, a detectable RNA silencing suppression activity in local assays in *Nicotiana benthamiana* (Garnelo Gómez *et al*., 2021), in agreement with the original description; therefore, we considered that the use of a 35S promoter was appropriate, as it would overcome potential silencing issues of double transgenic lines containing more than one expression cassette driven by the

SUC2 promoter. In order to facilitate downstream molecular analyses, P19 and P19$^{W39/42R}$ were fused to a GFP tag at their C-terminus. The resulting transgenic lines were analysed for developmental phenotypes and bleaching in the leaf lamina (Garnelo Gómez *et al.*, 2021; Fig. 1). Of 18 T1 plants expressing P19-GFP, six showed a virtual full suppression of the bleaching phenotype and had serrated leaves (a result of the interference with miRNA function; Chapman *et al.*, 2004), and did not produce seed; the other 12 lines showed suppression of bleaching and leaf serration in varying degrees. Of 15 T1 plants expressing P19$^{W39/42R}$-GFP, none had serrated leaves and one did not produce seed; nevertheless, these plants also displayed a quantitative suppression of bleaching (Fig. 1a). These effects on leaf shape and suppression of silencing could be confirmed in T2 (Garnelo Gómez *et al.*, 2021); images of T2 plants of six independent P19-GFP-expressing and five independent P19$^{W39/42R}$-GFP-expressing lines as well as of detached leaves are shown in Fig. 1b,c, respectively (for SUC-SUL/P19-GFP lines, #5 comes from a T1 plant in group 2, while the rest come from T1 plants in group 3; see Fig. 1a). Similar images of T4 plants from the lines used in Garnelo Gómez *et al.* (2021) are shown in Fig. 2.

Considering our initial assumptions and the results obtained, we concluded that sRNA binding is not required for the suppression of the inter-cellular movement of silencing mediated by P19, as opposed to the suppression of intracellular silencing. This conclusion has now been revisited by Brioudes *et al.* (2022, pp. 824–829) in this issue of *New Phytologist*, who have demonstrated that the P19$^{W39/42R}$ variant retains a certain capacity to bind sRNAs and a residual ability to suppress RNA silencing locally. The authors estimate the capacity of this mutant to bind sRNAs as *c.* 70% of that in the wild-type in transient expression assays in *N. benthamiana*, although, interestingly, its ability to suppress RNA silencing of a co-expressed GFP transgene is decreased to *c.* 25% (Brioudes *et al.*, 2022; fig. 2a). In the absence of quantification, the sRNA-binding capacity, however, seems to be lower in transgenic Arabidopsis SUC-SUL lines, at similar accumulation of the protein, for both siRNA and miRNA (Brioudes *et al.*, 2022; fig. 1c), and is dramatically diminished in the Flag/HA-tagged version (FHA-P19$^{W39/42R}$) used by Brioudes *et al.* (2022; fig. 2d).

Surprisingly, the GFP fusion protein used in Garnelo Gómez *et al.* (2021), but not the FHA fusion generated by Brioudes *et al.* (2022), displays a relaxed size specificity, binding not only the hallmark 21-nt sRNA normally selected by P19, but expanding its binding capability to 24-nt sRNAs; how a C-terminal tag, in combination with mutation of the end-capping tryptophan residues, can affect size selectivity remains to be determined. The most relevant corollary of the observations made by Brioudes *et al.* (2022), however, is that the model drawn in Garnelo Gómez *et al.* (2021) needs to be reconsidered, as the original assumptions of the properties of the P19 variant used were not correct.

Strikingly, the silencing phenotypes of the same transgenic lines show considerable quantitative differences in terms of silencing suppression in Garnelo Gómez *et al.* (2021) and Brioudes *et al.* (2022) (see, e.g. P19$^{W39/42R}$-GFP line #2). Newly grown T2 plants are shown in Fig. 1 for comparison; T4 plants from the lines originally used in Garnelo Gómez *et al.* (2021) are shown in Fig. 2. This observation argues for a strong effect of environmental conditions on this particular phenotype. This might or might not be linked to the observation by Brioudes *et al.* (2022) that the P19/BAM1 interaction is detected at plasmodesmata only occasionally, while this localisation is preponderant in our hands (Garnelo Gómez *et al.*, 2021).

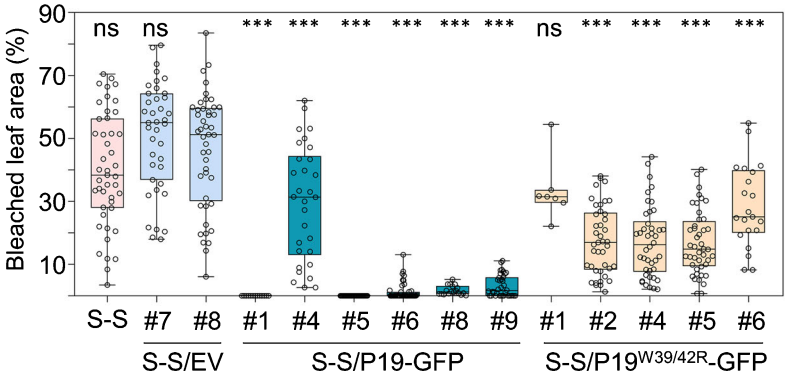
It is notable that despite the P19$^{W39/42R}$ mutant retaining only a partial capacity to bind sRNAs, which probably underlines its dramatically diminished capability to suppress local RNA silencing (Vargason *et al.*, 2003; Garnelo Gómez *et al.*, 2021; Brioudes *et al.*, 2022), its expression can result in an almost complete suppression of the vein-centred bleaching of SUC-SUL plants, in some cases comparable with that exerted by the wild-type P19. This observation raises the question of whether a differential requirement of sRNA binding exists for local silencing suppression and suppression of cell-to-cell movement of RNA silencing. In Garnelo Gómez *et al.* (2021), local silencing suppression by P19$^{W39/42R}$-HA was undetectable, while the suppression of bleaching by P19$^{W39/42R}$-GFP in SUC-SUL plants was comparable with that in lines expressing the wild-type P19-GFP (also observable in some of the plants shown in Brioudes *et al.* (2022); please refer to Fig. 1b, P19$^{W39/42R}$-GFP#5, center). In Brioudes *et al.* (2022), local

**Fig. 1** Effect of expression of P19-GFP or P19$^{W39/42R}$-GFP on plant development and bleaching phenotype in transformed SUC-SUL *Arabidopsis thaliana* reporter lines. (a) Table summarising numbers of T1 lines of transformed SUC-SUL plants expressing P19-GFP or P19$^{W39/42R}$-GFP generated in Garnelo Gómez *et al*. (2021) and their respective phenotypes. Different groups of P19-GFP-expressing T1 plants are depicted by numbers (1–3). (b) Quantification of the bleached leaf area (percentage, %) in 3-wk-old plants from different T2 lines of the three different genotypes considered in this study: P19-GFP-expressing SUC-SUL plants (S-S/P19-GFP), P19$^{W39/42R}$-GFP-expressing SUC-SUL plants (S-S/P19$^{W39/42R}$-GFP), and empty vector-transformed SUC-SUL plants (S-S/EV); original nontransformed SUC-SUL plants are also shown for comparison (S-S). In the box and whiskers graph, each dot represents bleaching value (%) per individual leaf: three leaves per plant and 15 plants per line were quantified for most groups, when possible. Error bars represents the highest/lowest values. Differences between groups were assessed by applying Kruskal–Wallis test for multiple comparisons ($P < 0.05$), followed by pairwise comparisons between each group and the reference group (S-S/EV line 8) (Mann–Whitney *U*-test, $P < 0.0038$, after Bonferroni's correction for multiple comparisons); asterisks represent statistically significant differences at: ***, $P < 0.001$; ns, not significant. (c) Images of representative bleaching phenotypes in the T2 plants (on the left) and leaves (on the right) considered for quantification in (b). Bars: (left panel) 2 cm; (right panel) 1 cm. (d) Analysis of accumulation of the different P19 variants in the T2 plants considered for quantification in (b). Top panel shows the accumulation of the different GFP-tagged P19 proteins (P19 or P19$^{W39/42R}$), after probing the membrane with an anti-GFP antibody. Middle panel shows actin (ACT) accumulation after probing the same membrane with an anti-Actin antibody. Numbers contained between top and middle panel represent densitometric data of P19 accumulation after relativising to ACT content, considering average P19-GFP accumulation as reference. Bottom panel shows Coomassie brilliant blue staining (CBB) of the membrane as total protein loading control. Two replicates per line, each of them containing material from five different plants, are shown in two separated western blot analyses (left–right). MW, molecular weight marker.

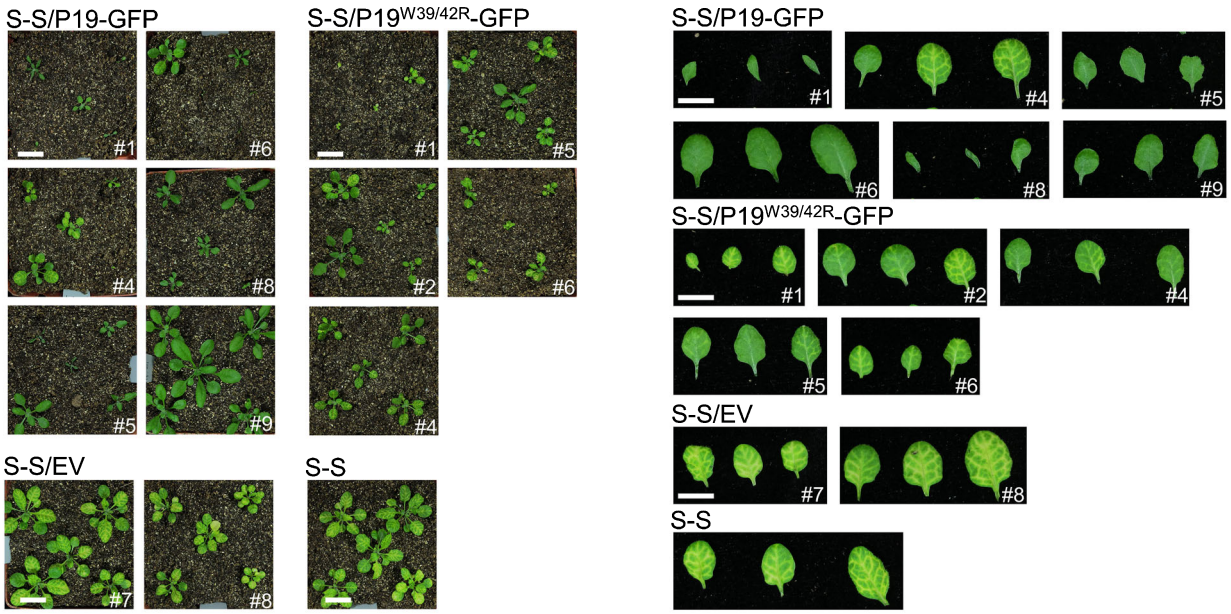
(a)

| Genotype | | | Phenotype: Leaf shape | Seed production | Bleaching |
|---|---|---|---|---|---|
| P19-GFP | | 1 | 6/18 deep serrations | Sterile | Full suppression |
| | | 2 | 5/18 mild serrations | Fertile | Variable suppression |
| | | 3 | 7/18 no serrations | Fertile | Variable suppression |
| $P19^{W39/42R}$-GFP | | | 15/15 no serrations | 1/15 Sterile | Variable suppression |

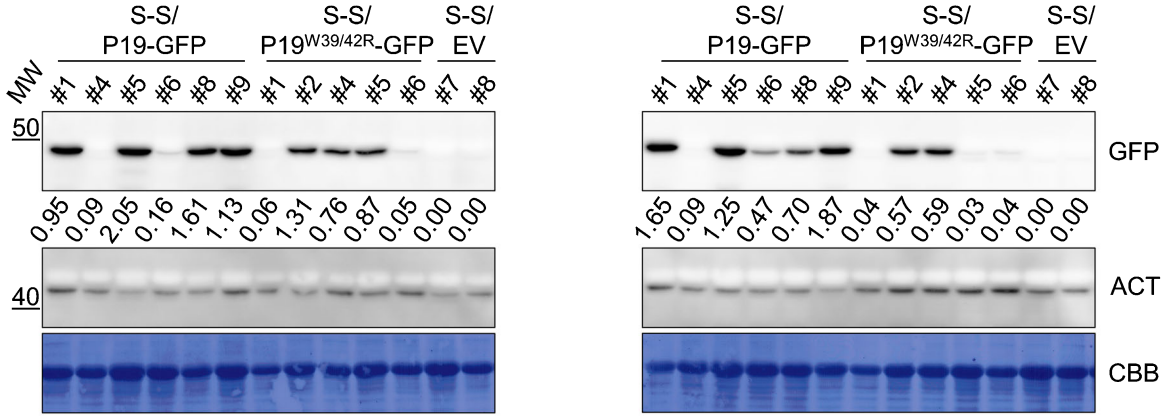
(b)




(c)

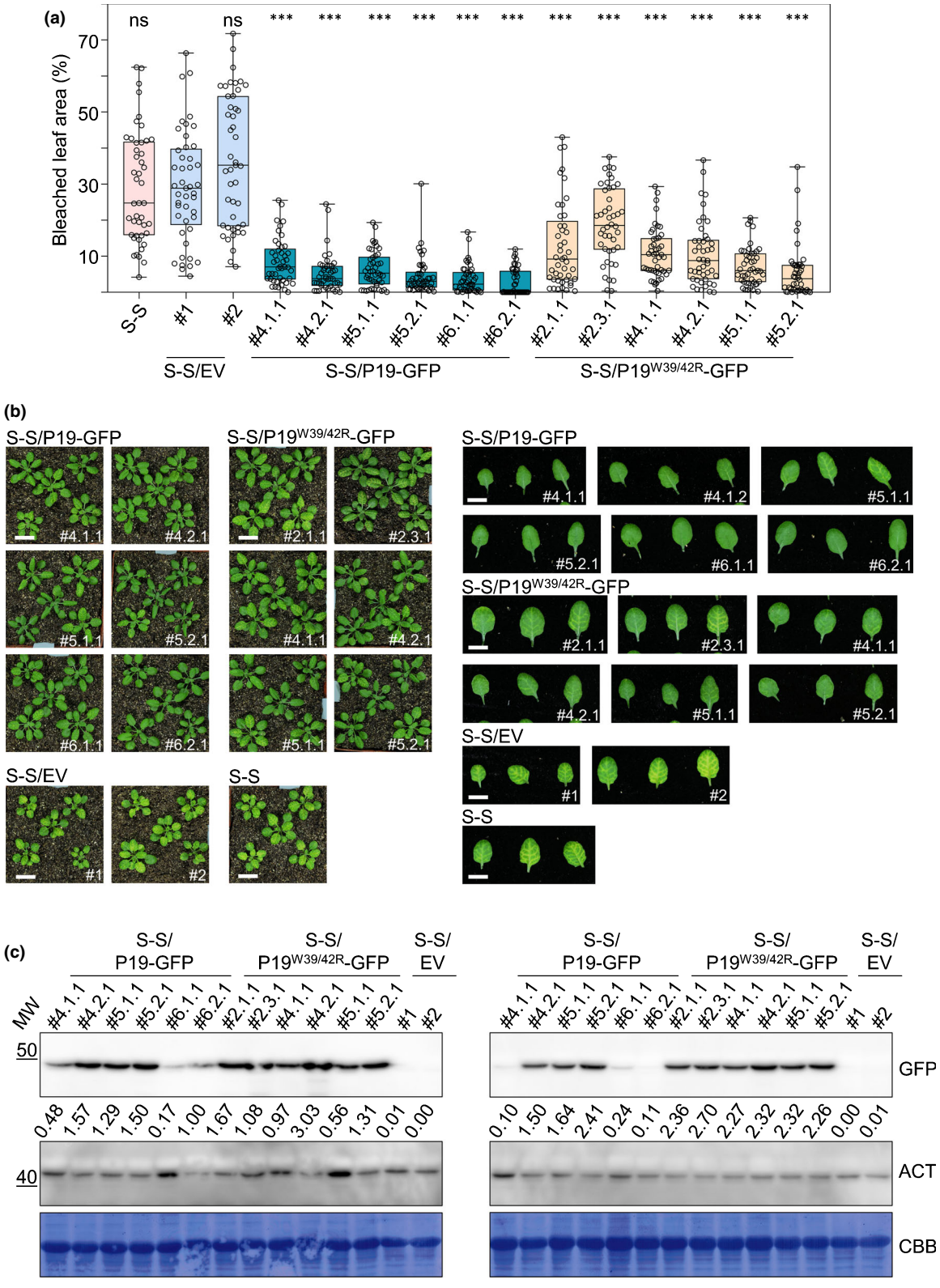



(d)




silencing suppression by FHA-$P19^{W39/42R}$ was minor, while this protein could still largely suppress the bleaching phenotype in the SUC-SUL background when accumulated to high levels (Brioudes *et al.*, 2022; fig. 2d). While the W39/42G P19 mutant version, in which sRNA binding is completely abrogated, cannot suppress local RNA silencing and does not suppress the SUL-SUL bleaching phenotype when accumulated to moderate/low levels, its effect at high doses remains to be determined.

Notably, regardless of the protein accumulation level, the W39/42R mutant does not seem to produce leaf serration (Garnelo Gómez *et al.*, 2021; Brioudes *et al.*, 2022), a side-effect of the interference with miRNA function (Chapman *et al.*, 2004); we did




not observe this phenotype in any of the 15 independent T1 lines analysed, while > 60% of 18 T1 lines expressing the wild-type P19-GFP displayed it. This is the case also in the experimental conditions used by Brioudes *et al.* (2022) despite miRNA-binding (miR160 and miR160*) occurring in both P19-GFP and P19$^{W39/42R}$-GFP lines (see lines #4 in both cases; Brioudes *et al.*, 2022; fig. 1b,c). The absence of this specific phenotype upon expression of the mutant version of P19-GFP, as opposed to that of

**Fig. 2** Effect of expression of P19-GFP or P19$^{W39/42R}$-GFP on bleaching phenotype in T4 generation of transformed SUC-SUL *Arabidopsis thaliana* reporter lines. (a) Quantification of the bleached leaf area (percentage, %) in 3-wk-old plants from selected T4 lines (from the T2 lines used in Garnelo Gómez *et al*., 2021) of the three different genotypes considered in this study: P19-GFP-expressing SUC-SUL plants (S-S/P19-GFP), P19$^{W39/42R}$-GFP-expressing SUC-SUL plants (S-S/ P19$^{W39/42R}$-GFP), and empty vector-transformed SUC-SUL plants (S-S/EV); original nontransformed SUC-SUL plants are also shown for comparison (S-S). In the box and whiskers graph, each dot represents bleaching value (%) per individual leaf: three leaves per plant and 15 plants per line were quantified per group. Error bars represents the highest/lowest values. Differences between groups were assessed by applying Kruskal–Wallis test for multiple comparisons ($P < 0.05$), followed by pairwise comparisons between each group and the reference group (S-S/EV line 1) (Mann–Whitney *U*-test, $P < 0.0035$, after Bonferroni's correction for multiple comparisons); asterisks represent statistically significant differences at: ***, $P < 0.001$; ns, not significant. (b) Images of representative bleaching phenotypes in the T4 plants (on the left) and leaves (on the right) considered for quantification in b. Bars: (left panel) 2 cm; (right panel) 1 cm. (c) Analysis of accumulation of the different P19 variants in the T4 plants considered for quantification in (b). Top panel shows the accumulation of the different GFP-tagged P19 proteins (P19 or P19$^{W39/42R}$), after probing the membrane with an anti-GFP antibody. Middle panel shows Actin (ACT) accumulation after probing the same membrane with an anti-Actin antibody. Numbers contained between top and middle panel represent densitometric data of P19 accumulation after relativising to ACT content, considering average P19-GFP accumulation as reference. Bottom panel shows Coomassie brilliant blue staining (CBB) of the membrane as total protein loading control. Two replicates per line, each of them containing material from five different plants, are shown in two separated western blot analyses (left–right). MW, molecular weight marker.

the wild-type, suggests that minor differences in sRNA binding could unequally impact different outputs of P19 activity.

These observations do not challenge any of the previous works or applications of P19, since the ability of P19 to bind and sequester sRNAs in a size-specific manner has never been called into question. Nevertheless, they raise interesting questions regarding the functioning of this silencing suppressor protein, its mode of action, and the role of BAM1/2 in the P19-mediated suppression of the intercellular spread of RNA silencing. In summary, our previous conclusion that the capacity of P19 to bind sRNA is not required for its effect on the cell-to-cell movement of RNA silencing based on the P19$^{W39/42R}$ variant is not warranted and cannot be sustained in light of the results provided by Brioudes *et al.* (2022). However, we point out that a contribution of additional molecular mechanisms has not been yet ruled out, and the relation between P19 sRNA binding, local silencing suppression, systemic silencing suppression, and the potential role of the P19/BAM1 complex in these activities remains to be fully unravelled.

Importantly, we believe this exchange and the revisiting of prior conclusions and further questions therein derived serve as an illustration of how challenging of previous results, constructive criticism, and dialogue are essential to generate the tension required to propel the advance of science, and highlight the relevance of a safe, open arena for scientific discussion and debate offered, for example, by this Letter format.

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## Author contributions

EA performed the experiments and analysed the data. EA and RL-D planned and designed the research and wrote the manuscript.

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