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Repression of turnip crinkle virus replication by its replication protein p88

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ABSTRACT

We recently reported that p28, one of the two turnip crinkle virus (TCV) replication proteins, *trans*-complemented a defective TCV lacking p28, yet repressed the replication of another TCV replicon encoding wild-type p28 (Zhang et al., 2017). Here we show that p88, the TCV-encoded RNA-dependent RNA polymerase, likewise *trans*-complemented a p88-defective TCV replicon, but repressed one encoding wild-type p88. Surprisingly, lowering p88 protein levels enhanced *trans*-complementation, but weakened repression. Repression by p88 was not simply due to protein over-expression, as deletion mutants missing 127 or 224 N-terminal amino acids accumulated to higher levels but were poor repressors. Finally, both *trans*-complementation and repression by p88 were accompanied by preferential accumulation of subgenomic RNA2, and a novel class of small TCV RNAs. Our results suggest that repression of TCV replication by p88 may manifest a viral mechanism that regulates the ratio of genomic and subgenomic RNAs based on p88 abundance.

1. Introduction

Viruses with single-stranded, positive sense (+) RNA genomes are the most common viral pathogens of plants, animals, and humans. Examples of important (+) RNA viruses include the plant-infecting tobacco mosaic virus, animal-infecting foot mouth disease virus, and human pathogens poliovirus and Zika virus. The genomes of most (+) RNA viruses are relatively small (< 15,000 nucleotides [nt]), encoding a limited number of proteins that are frequently multifunctional. A recently revealed example is the p28 protein encoded by the plant-infecting turnip crinkle virus (TCV), which exhibited opposite functions depending on protein concentration and terminal modifications (Zhang et al., 2017).

TCV is a small (+) RNA plant virus that counts model plants Arabidopsis and *Nicotiana benthamiana* as hosts (Cao et al., 2010; Zhang et al., 2012; Kuhlmann et al., 2016). Its genome of 4054 nt encodes five proteins (Fig. 1A). The 5′ proximal p28 and its read-through product (p88), both needed for genome replication, are translated directly from the genomic RNA (gRNA). The p8 and p9 movement proteins (MPs), and p38 capsid protein (CP), are translated from sub-genomic RNAs (sgRNA1 and 2) produced during viral replication (Fig. 1A). We recently reported that the auxiliary replication protein p28, upon C-terminal fusion with a double HA (2HA) tag, or a fluorescent protein (GFP or mCherry), completely shut down the replication of TCV in the same cells (Zhang et al., 2017). We further demonstrated that p28 free of any terminal modifications also potently repressed the replication of a TCV replicon, and p28 mutants with weakened repressive activity were more competent at *trans*-complementing the replication of a p28-defective TCV replicon (Zhang et al., 2017).

In the current study, we examined p88, the TCV-encoded RNA-dependent RNA polymerase (RdRp), for its ability to *trans*-complement the replication of TCV mutants in which the p88 coding region was disrupted, and to repress the replication of TCV replicons that encode functional p88 of their own. Our results showed that TCV p88 exhibited concentration-dependent repression of TCV replication, and that this repressive activity inversely correlated with the ability of the same protein to *trans*-complement the replication of a p88-defective TCV mutant. Interestingly, the repressive activity of p88 preferentially suppressed TCV gRNA amplification, but favored sgRNA accumulation. These findings suggest that p88 may play a role in regulating the relative ratio of gRNA and sgRNAs through concentration-dependent changes in its function.

2. Results

2.1. p88 expressed independently of replication trans-complements the replication of p88-defective TCV replicons

In a previous study, a defective TCV replicon was generated by changing the p28 stop codon to UAC (White et al., 1995). As a result,

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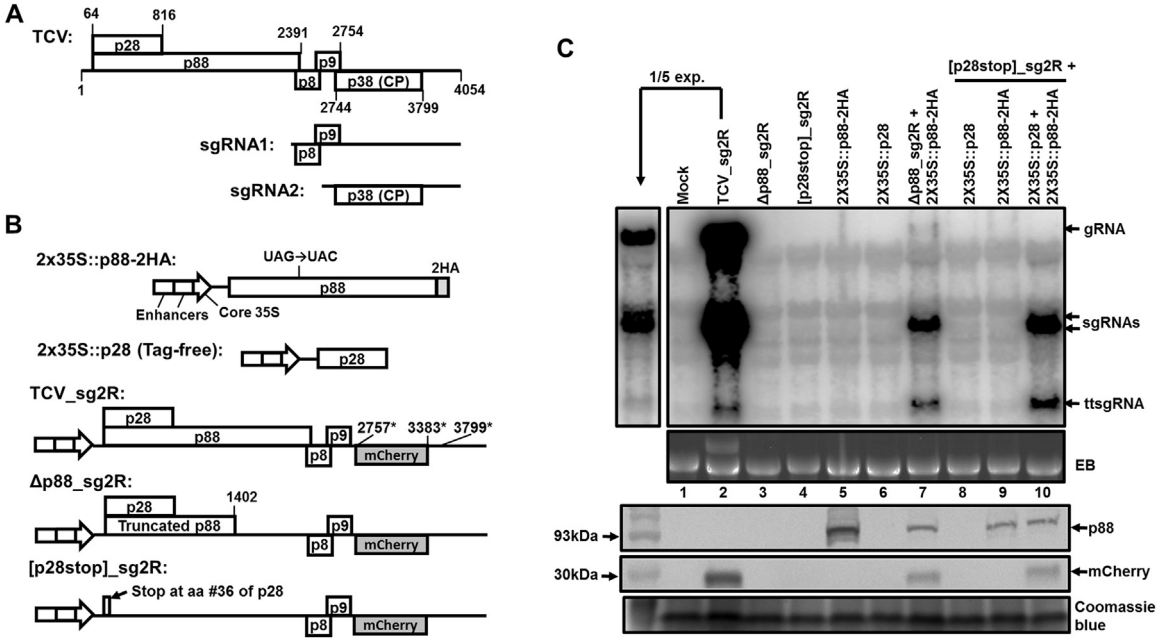

Fig. 1. TCV p88 partially *trans*-complements a p88-defective replicon. A: TCV genome organization. The nucleotide (nt) coordinates of the open reading frames encoding p28, p88, and capsid protein (CP) were highlighted. The two subgenomic RNAs, sgRNA1 and 2, serving as mRNA for movement proteins (p8 and p9) and CP respectively, were also depicted. B: Constructs used in experiments leading to C. All constructs were delivered into *N. benthamiana* cells using agro-infiltration. The first two support replication-independent expression of p88 (p88-2HA) and p28, with their transcription controlled by the strong 35S promoter with duplicated enhancers. The next three were TCV replicons with the CP ORF replaced by that of mCherry. TCV_sg2R replicates on its own as both p28 and p88 ORFs are intact. Δp88_sg2R is p88-defective as the p88 ORF was terminated prematurely by a 4-nt deletion at nt #1402. [p28stop]_sg2R translates neither p28 nor p88, as both ORFs were terminated by a stop codon introduced at aa #36. C: Partial *trans*-complementation by replication-independent expression of p88 and p28 revealed by Northern and Western blottings. EB: ethidium bromide stained agarose gel serving as the loading control for Northern blotting.

genomic RNA of that defective replicon could translate only p88 but not the smaller p28, thus was unable to launch replication on its own (White et al., 1995). Nevertheless, p88 produced by that defective replicon *trans*-complemented the replication of a reciprocal mutant encoding only p28. However, it remains to be determined whether p88 expressed from a non-replicating mRNA could complement TCV replication in *trans*. To address this question, we generated a p88-expressing binary construct, designated 2 × 35S::p88-2HA (Fig. 1B). The p88 expression from this construct is driven by the strong cauliflower mosaic virus 35S promoter with duplicated enhancers (2 × 35S). In addition, a double HA epitope tag (2HA) was added to the C-terminus of p88 to facilitate the detection of p88 protein (Fig. 1B).

To test whether p88 expressed from this non-replicating construct *trans*-complements the replication of TCV, the construct was delivered into leaf cells of *Nicotiana benthamiana* plants along with Δp88_sg2R, a mutant TCV replicon in which the p88 coding sequence was disrupted by a four nucleotide (nt) deletion (Fig. 1B). Note that Δp88_sg2R and the other two TCV replicon constructs used in the current study all harbored the 2 × 35S promoter that launches the transcription of first batch of TCV gRNA. Also note in all experiments described in the current study, a construct expressing the p19 silencing suppressor of tomato bushy stunt virus (TBSV) was always included to mitigate RNA silencing-mediated transcript degradation (not shown). As shown in Fig. 1C (top panel, lanes 3 and 7), replication of the defective Δp88_sg2R replicon was partially restored by p88-2HA. Nevertheless, the complementation was inefficient, and favored accumulation of TCV sgRNAs, especially sgRNA2, and a class of smaller TCV RNA of unknown size(s) and origin, which we call tiny TCV sgRNA (ttsgRNA) hereafter.

We next assessed whether p88-2HA, together with a tag-free p28 that was also expressed from a non-replicating construct, could complement the replication of [p28stop]_sg2R, a TCV replicon encoding neither p28 nor p88 (Fig. 1B). By comparing lanes 4, 8, 9, and 10 in Fig. 1C, we concluded that both p88-2HA and p28 were needed to achieve a partial complementation of [p28stop]_sg2R. Finally, the expression of p88-2HA protein, as well as the replication-dependent production of mCherry protein, were confirmed with Western blotting, using HA and mCherry antibodies, respectively (Fig. 1C, bottom three panels). Therefore, p88-2HA provided in *trans* was partially replication-competent.

2.2. p88-2HA represses the replication of a TCV replicon encoding intact p88

We wondered why the complementation by p88-2HA was not more efficient (Fig. 1C). Since the transiently expressed p88-2HA was expected to accumulate at much higher levels than p88 translated from TCV replicons, we reasoned that the over-expressed p88-2HA protein could partition into two states, with the first supporting replication, but the second repressing replication. This possibility was attractive to us because (i), the same p88-2HA protein partially repressed the replication of a GFP-expressing TCV replicon in a previous study (Zhang et al., 2017); and (ii), p28-2HA, the 2HA-tagged form of the smaller replication protein p28, completely repressed TCV replication (Zhang et al., 2017). Note that p28 constitutes the N-terminal 1/3 of p88 (Fig. 1A).

One prediction of this possibility is that lowering intracellular p88-2HA levels would discourage the genesis of the repressive p88-HA state, leaving more p88-2HA in the replication-competent state. To test this prediction, we tried to decrease the p88-2HA protein levels by removing both enhancers from the 2 × 35S promoter, yielding the construct Core35S::p88-2HA (Fig. 2A). As shown in Fig. 2B, the level of p88-2HA protein was substantially reduced in cells receiving this new construct (compare lanes 2 and 3), and it remained low in the presence of TCV replicons (compare lanes 6, 7, 8 and 9). Consistent with our prediction, decreased p88-2HA expression from the Core35S::p88-2HA construct led to improved *trans*-complementation of the p88-defective

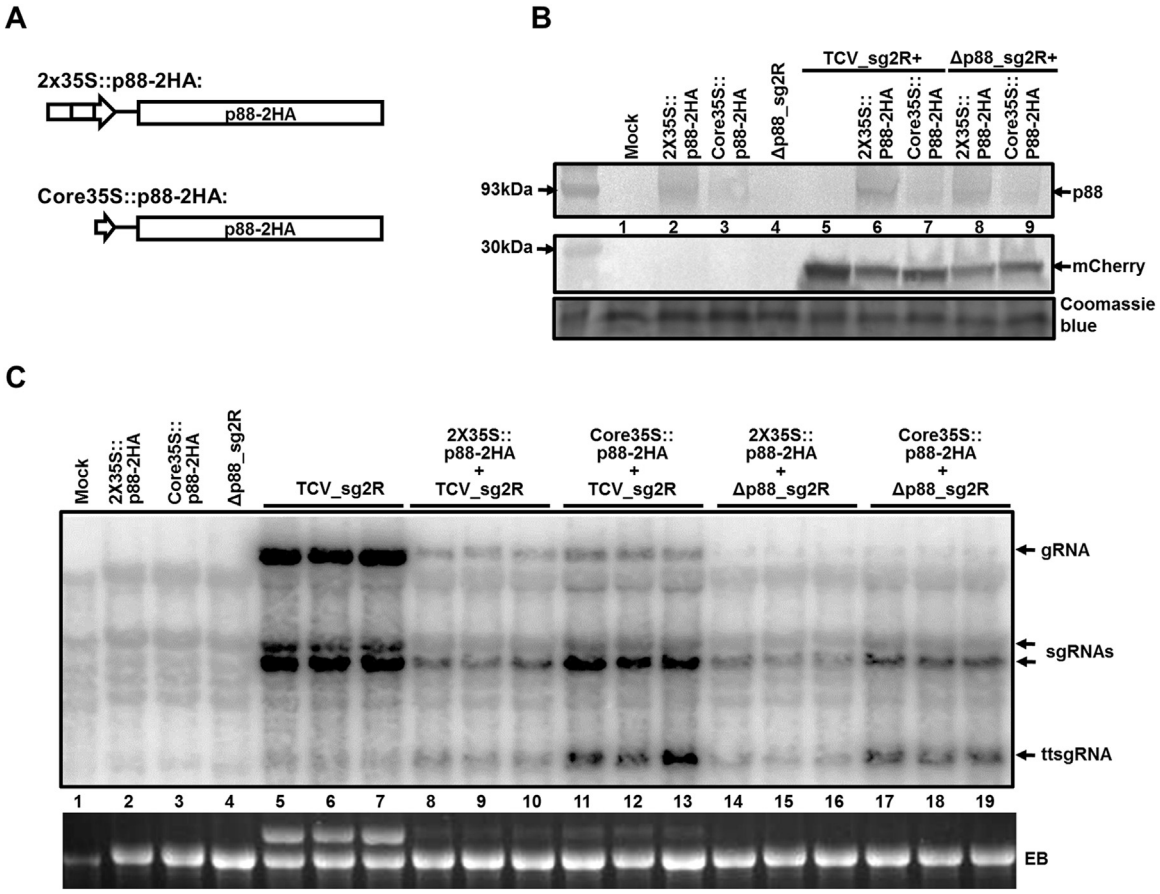

Fig. 2. The level of p88-2HA protein correlates negatively with its *trans*-complementation activity, but positively with its replicational repression activity. A: Constructs with the same p88-2HA ORF under control of promoters of different strengths. 2 × 35S: 35S promoter with duplicated enhancers. Core35S: core 35S promoter without the enhancers. B: Protein levels of the replication-independent p88-2HA and replication-dependent mCherry, in the absence or presence of two different replicons differing in their ability of producing cognate p88, as determined by Western blotting. C: The efficiency of p88-2HA-mediated *trans*-complementation (lanes 14–19) and repression (lanes 8–16), with two different p88-2HA protein levels, as determined by TCV-specific Northern blotting.

replicon (Fig. 2C, compare lanes 14–16 and 17–19. Note that the triplicate lanes 5–7, 8–10, 11–13, 14–16, and 17–19, represent three independent samples).

Both the 2 × 35S::p88-2HA and Core35S::p88-2HA constructs were then used to test whether p88-2HA repressed the replication of a TCV replicon encoding its own p88. As shown in Fig. 2C (lanes 8–10), higher p88-2HA protein level caused a dramatic reduction in both gRNA and sgRNA levels of TCV_sg2R, suggesting that this level of p88-2HA strongly repressed the replication-dependent synthesis of both gRNA and sgRNAs. Strikingly, this repression was greatly released at the lower p88-2HA protein level (Fig. 2B, lane 7, and Fig. 2C, lanes 11–13). Together the data in Fig. 2 demonstrated that (i), p88-2HA both complemented and repressed TCV replication; and (ii) higher p88-2HA protein levels correlated with lower complementation but higher repression. Therefore, the limited complementation by p88-2HA is likely caused by the intrinsic repressive activity of p88. Importantly, both *trans*-complementation and repression by p88-2HA led to a preferential accumulation of sgRNA2 and ttsgRNA (lanes 8–19). This selective amplification is particularly strong at lower p88-2HA levels (lanes 11–13, 17–19), suggesting that repression by p88-2HA preferentially targeted the synthesis of TCV gRNA.

2.3. The C-terminal 2HA tag does not appreciably affect p88 activities

In addition to being transiently overexpressed, p88-2HA also differs from the virus-encoded p88 by having a C-terminal 2HA tag. We thus needed to assess whether this tag compromised the function of p88, rendering it more potent at repression yet less competent at *trans*-complementation. This assessment is of particular importance in light of a previous report showing that activity of the RdRp of a related virus (p92 of TBSV) is strongly affected by changes within the last five amino acid (aa) residues (Wu and White, 2007). To this end, we generated a new construct to express p88 free of any terminal tags, under control of the Core35S promoter (Core35S::p88. Fig. 3). Northern blotting results in Fig. 3 showed that, compared to p88-HA under control of the same promoter (Core35S::p88-2HA), the tag-free p88 was actually slightly less competent at complementing the replication of Δp88_sg2R (lane 10 *versus* 11), but exhibited a similar repressive activity toward TCV_sg2R (lane 6 *versus* 7). Therefore, the 2HA tagged p88-2HA was functionally analogous to the tag-free p88, hence can be used as the surrogate of the latter.

2.4. Trans-complementation by p88-2HA tolerates limited N-terminal truncation

We recently reported that supplying p28 alone restored the replication of a mutant TCV replicon in which the translation of both p28 and p88 was disrupted by an early frame-shift (fs) mutation (Zhang et al., 2017. Also see Fig. 4A). We speculated that translation of p88 (and p28) could reinitiate from the second in-frame start codon 36 amino acid (aa) downstream, and that the resulting N-terminally truncated p88 (but not p28) could still be functional. Another study by Rajendran et al. (2002) found that removal of the entire p28-coding region from p88 enhanced the replication function of p88 *in vitro*. These

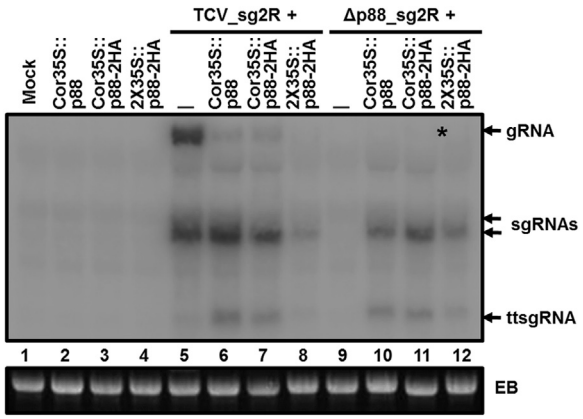

Fig. 3. The C-terminal 2HA tag does not appreciably affect p88 activities. The Core35S::p88 construct expressing a tag-free p88 was compared side-by-side with two other constructs that express varying levels of p88-2HA, a C-terminally tagged p88, for its ability to repress, as well as *trans*-complement, TCV replication.

findings promoted us to assess whether *trans*-complementation by p88-2HA *in vivo* required p88 to be full-length. To this end, we generated a series of N-terminal deletions within the p88 coding sequence (Fig. 4A). The first mutant, 2 × 35S::p88fs-2HA, contained the same frame-shift mutation described above, but in the 2 × 35S::p88-2HA backbone. All other mutants were based on the Core35S::p88-2HA backbone (Fig. 4A). ΔN36, ΔN127, and ΔN224 removed the N-terminal regions upstream of the three internal methionine residues at positions 37, 128, and 225, respectively, whereas Δ250 removed the entire p28 sequence.

As expected, both 2 × 35S::p88fs-2HA and Core35S::p88ΔN36-2HA produced a protein of the same size, which is slightly smaller than the full-length p88-2HA (Fig. 4B, top panel, lanes 2–4; also lanes 10–12). Nevertheless, the former (lane 2) accumulated to modestly higher levels, possibly due to the strong 2 × 35S promoter. Surprisingly, ΔN127 and ΔN224 deletion mutants accumulated to very high levels despite the weak Core35S promoter (lanes 5, 6, 13, 14). Finally the ΔN250 mutant lacking the entire p28 portion accumulated to a level slightly higher or comparable to the full length p88-2HA driven by Core35S promoter (compare lanes 7 and 15 with lanes 3 and 11, respectively).

Trans-complementation was observed only with the p88ΔN36-2HA mutant, expressed from the 2 × 35S::p88fs-2HA and Core35S::p88ΔN36-2HA constructs (Fig. 4B, bottom panel, lanes 10 and 12). Compared to the full length p88-2HA, the ΔN36 mutant protein was less efficient at complementing the synthesis of gRNA (lanes 10–12. * in lane 11 denotes a weak gRNA signal detectable only in the presence of full-length p88-2HA). Interestingly, this mutant protein was substantially more efficient than p88-2HA at facilitating the accumulation of sgRNA2 and ttsgRNA (lanes 10–12), suggesting that the N-terminal 36 aa region is critical for gRNA synthesis activity of TCV RdRp. None of the three mutants with larger deletions (ΔN127, ΔN224, and ΔN250) were capable of *trans*-complementation (lanes 13–15).

The inability to detect TCV gRNA in p88ΔN36-2HA-mediated complementations prompted the question of whether gRNA synthesis by p88 was completely abolished by the ΔN36 deletion. To test this, we attempted to detect the gRNA-specific, negative-sense (-) RNA using a more sensitive semi-quantitative reverse transcription (RT)-PCR procedure (Plaskon et al., 2009). As shown in Fig. 4C, p88ΔN36-2HA (lane 3), but not p88ΔN250-2HA (lane 4), facilitated the accumulation of a very low level of (-) gRNA. This experiment also confirmed that the full-length p88-2HA more efficiently complemented gRNA synthesis (compare lanes 2 and 3). Together these results indicated that deletion of N-terminal 36 aa of p88 diminished, but did not abolish, gRNA accumulation; yet strongly stimulated the synthesis of sgRNA2 and ttsgRNA.

2.5. p88 mutants capable of trans-complementation are also strong repressors of replication

We next examined the deletion mutants for their ability to repress the replication of TCV_sg2R. Interestingly, the same two mutants (2 × 35S::p88fs-2HA and Core35S::p88ΔN36-2HA) that exhibited partial *trans*-complementation (see above) also exerted a strong repression on TCV_sg2R replication (Fig. 5, top panel, lanes 10 and 12). Strikingly, both mutant proteins severely repressed the accumulation of gRNA, but actually enhanced the accumulation of sgRNA2 and ttsgRNA, leading to an RNA accumulation profile extremely similar to that of *trans*-complementation (compare Figs. 4 and 5). A similar, but not identical, RNA accumulation profile was also observed when *trans*-complementation and repression were achieved using low levels of full-length p88-2HA (Core35S::p88-2HA; Figs. 2, 3, 4, and 5). Furthermore, the repression is unlikely due to non-specific protein over-expression, as both ΔN127 and ΔN224 mutant proteins accumulated to greatly elevated levels, but exerted weak repression on TCV_sg2R replication that did not cause the preferential accumulation of sgRNA2 and ttsgRNA (lanes 13 and 14). On the other hand, the ΔN250 mutant, despite relatively low accumulation, caused a near complete blockage of TCV_sg2R replication, affecting all of the TCV RNA species (lane 15).

2.6. Replicational repression by p88 mutants partially correlates with the induction of p28 inclusions

How did p88-2HA and some of its deletion mutants repress the replication of TCV_sg2R? Since TCV p28-2HA repressed TCV replication as well (Zhang et al., 2017), we wondered if they shared the same repression mechanism. p28 with a C-terminal GFP tag (p28-GFP) displayed strong repressive activity, and formed large, intense intracellular inclusions (Zhang et al., 2017). By contrast, p28 with an N-terminal G11 (the 11th and last β-strand of GFP) tag, referred to as G11-p28, abolished the repressive activity of p28, but enhanced its *trans*-complementation activity (Zhang et al., 2017). Consistent with the role of p28 inclusions in replicational repression, G11-p28, when co-expressed with the first ten β-strands of GFP (G1–10), failed to form any inclusions, and was instead diffusely distributed throughout the cytoplasm (Zhang et al., 2017. Also see Fig. 6A). This (G11-p28 + G1–10) combination was further used as a reporter system to demonstrate *trans*-aggregation by p28, as the presence of p28 inclusions in the same cell pulled G11-p28 into the inclusions, changing the distribution pattern of the latter (Zhang et al., 2017).

We next used this reporter system to assess whether p88-2HA and its deletion mutants could *trans*-aggregate G11-p28. As shown in Fig. 6A, simultaneous expression of G11-p28 and G1–10 led to evenly distributed diffuse green fluorescence in *N. benthamiana* cells. Note that the cell nuclei also fluoresced but very faintly (white arrows). As expected, adding p28-HA or untagged p28 caused the majority of green fluorescence to congregate into very bright inclusion dots (Fig. 6B, panels 1 and 2). The remaining G11-p28 also formed tiny dots that lined the cell boundaries discontinuously. Strikingly, high level of p88-2HA expression driven by the 2 × 35S promoter led to the formation of even larger inclusions, accompanied by a near complete loss of diffuse fluorescence (Fig. 6B, panel 3), indicating that this level of p88-2HA caused strong protein aggregation, and the consequent inclusions became magnets to coalesce nearly all diffuse G11-p28. By contrast, lower p88-2HA expression driven by the Core35S promoter, though still induced the formation of G11-p28 inclusions, also left a fraction of G11-p28 in the diffuse state (Fig. 6B, panel 4). Therefore, the full-length p88-2HA likely self-associated in a concentration-dependent manner to form protein aggregates, which in turn *trans*-aggregated G11-p28.

The p88ΔN36-2HA protein still induced G11-p28 aggregation in some cells (Fig. 6B, panel 5). However, it was clearly less capable of doing so than the full-length p88-2HA, as more G11-p28 fluorescence remained diffused. This trend was even more apparent with cells

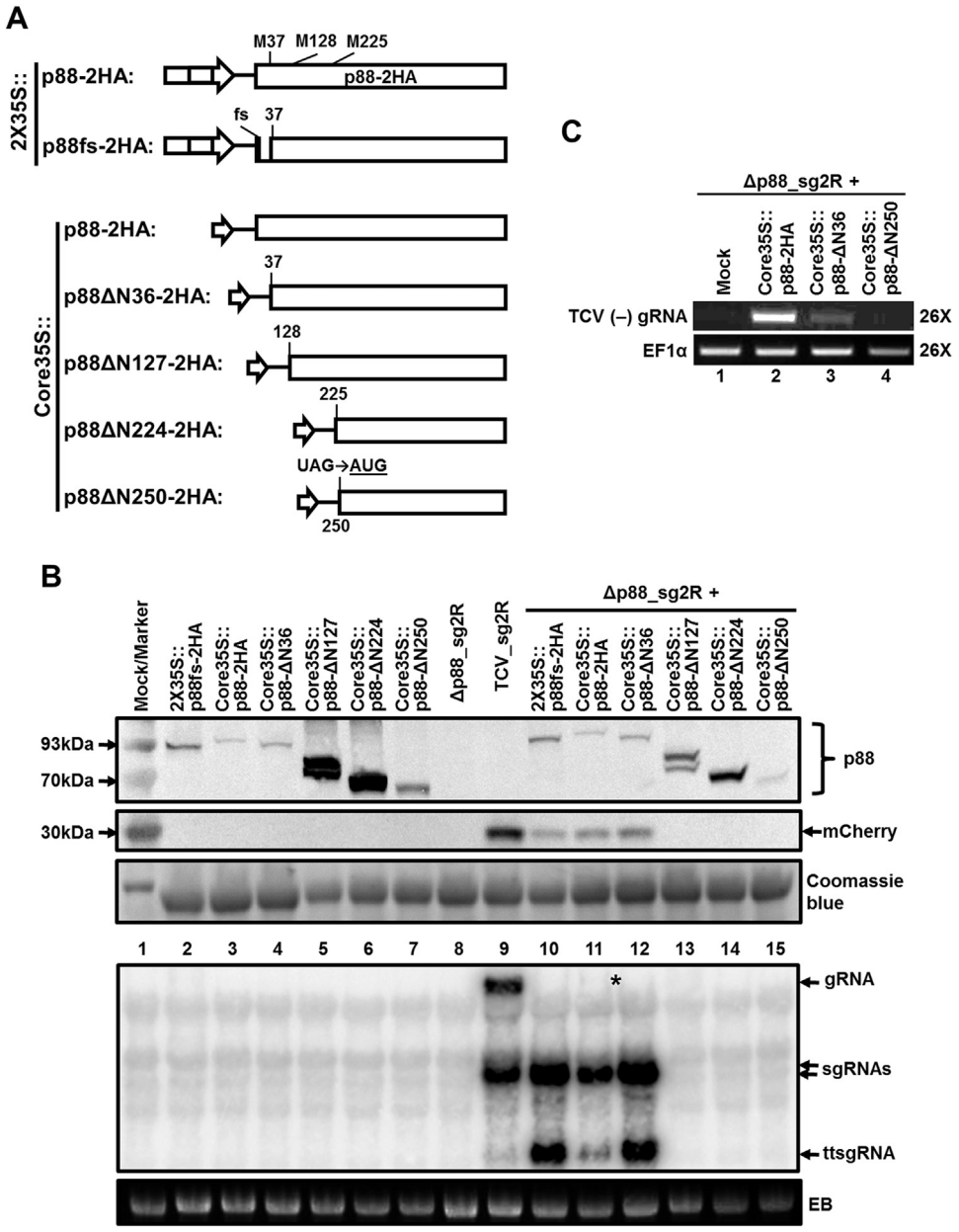

Fig. 4. *Trans*-complementation mediated by various deletion mutants of p88-2HA. A: diagrams of different deletion mutants along with the promoters used. Although the p88fs-2HA contained a one-nt deletion after fourth aa residue resulting in frame-shift (fs), it permitted translational re-initiation at aa #37 (see results in B). B: Partial *trans*-complementation by the ΔN36 mutants as revealed by Western and Northern blottings. C: Detection of (-) strand gRNA with strand-specific, semi-quantitative RT-PCR (see Materials and Methods for details).

entered by the three mutants harboring larger N-terminal deletions, where most of the G11-p28 green fluorescence remained in the diffused form. Note that the Δ127 and Δ224 mutants repressed TCV replication only marginally, whereas ΔN250 was the strongest repressor of all (Fig. 5). Therefore, while the ability to trap G11-p28 in protein aggregates could explain the repressive power of some mutants, the same explanation did not apply to the p88ΔN250-2HA mutant.

2.7. p88 with a C-terminal GFP tag forms large inclusions similar to p28-GFP

To directly address the question of whether transiently overexpressed p88 forms large protein aggregates by itself, we then tagged the full length p88, as well as the deletion mutants described earlier, with a C-terminal GFP tag. Confocal images in Fig. 6C showed that while free GFP exhibited a diffused distribution in agro-infiltrated cells (panel 1), the p88-GFP fusion protein, regardless of the strength of the promoters used, formed large, irregular inclusions that are strikingly similar to those of p28-GFP (Fig. 6C, panels 2–4). Interestingly, the GFP-tagged ΔN36, ΔN127, and ΔN224 mutants failed to form large inclusions of p88-GFP. Instead, they formed substantially smaller dots that evenly aligned the cell boundaries (Fig. 6C, panels 5–7). Finally, ΔN250-GFP protein showed a predominantly diffused distribution (panel 8). Overall these results are consistent with a role of protein aggregation in the repression of TCV replication by transiently expressed p88 and some of its mutants. However, it is important to note that protein aggregation does not explain the preferential accumulation of sgRNA2 and ttsgRNA – an observation best explained by the potential RdRp activity of overexpressed p88.

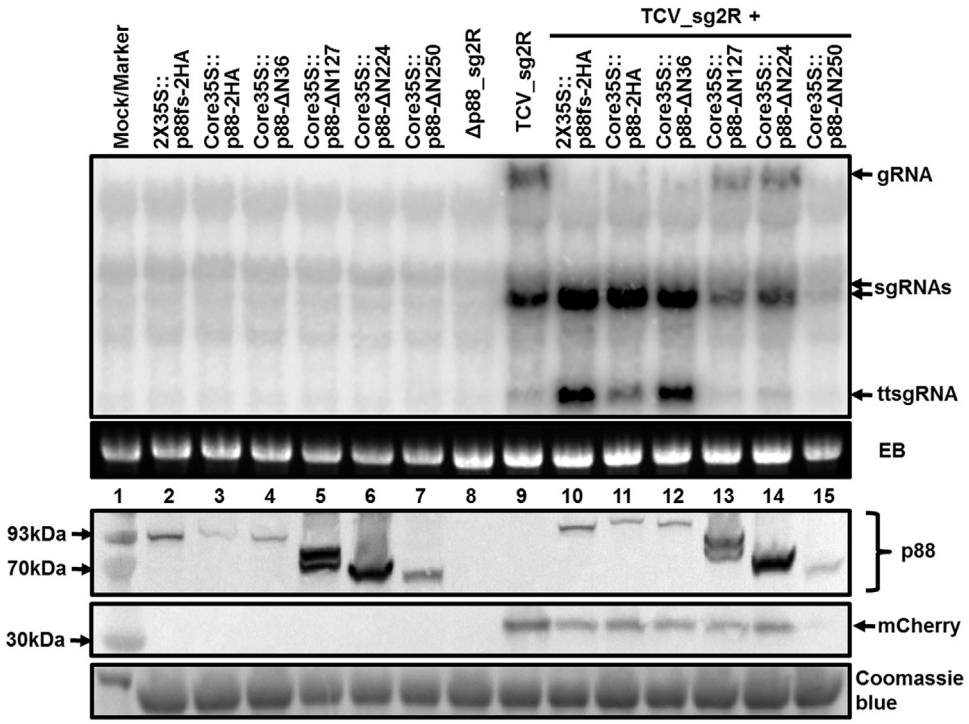

Fig. 5. Replicational repression by p88-2HA mutants depicted in Fig. 4A, as determined by Northern and Western blottings.

3. Discussion

The goals of the current study were to determine whether the TCV-encoded p88 replication protein, when expressed from a non-replicating mRNA, *trans*-complements the replication of TCV mutants lacking p88-coding capacity, and whether it interferes with the replication of TCV replicons encoding an intact p88. We recently reported that p28, the smaller TCV replication protein, *trans*-complemented the replication of a TCV replicon lacking its own p28, but repressed the replication of another replicon encoding a functional p28 (Zhang et al., 2017). We further established that p28 formed large, intense intracellular inclusions that impeded *trans*-complementation, but correlated with repression. However it is not known whether these properties are also shared by p88, the TCV-encoded RdRp.

To resolve these questions, replication-independent expression of p88 and its deletion mutants were administered in *N. benthamiana* cells, along with two different TCV replicons differing in their capacity to produce cognate p88. Based on their ability to *trans*-complement and/or repress TCV replication, these p88 variants can be sorted into four categories. First, the p88ΔN250-2HA mutant, in which the entire p28 portion of p88 was deleted, failed to *trans*-complement the replication of the p88-defective Δp88_sg2R replicon, but almost completely repressed the replication of the p88-producing TCV_sg2R replicon. The inability of this protein to substitute for p88 in *N. benthamiana* cells contrasts with the previous *in vitro* results showing a similar mutant protein being more replicationally potent than the full-length p88 (Rajendran et al., 2002), suggesting that TCV replication *in vivo* may entail additional requirements. Conversely, this protein may also use a distinct mechanism to repress TCV replication, as it did not drastically alter the diffuse distribution of G11-p28 in most cells, and its GFP-fused form likewise exhibited a diffuse distribution.

The second category of p88 mutants includes p88ΔN127-2HA and p88ΔN224-2HA, neither was capable of *trans*-complementation. Strikingly, both were only marginally repressive, despite very high levels of protein accumulation. This is particularly noteworthy, as the p88ΔN224-2HA protein was only 26-aa longer than the highly repressive p88ΔN250-2HA. Yet this short N-terminal extension seemed to have dramatically stabilized the protein while simultaneously diminished its repressive power. The simplest explanation would be that the 26-aa region (aa #225–250) shielded a repressive domain within p88ΔN250-2HA, probably through intra-molecular interactions, preventing the latter from engaging in inter-molecular interactions with full-length p88 produced from the TCV_sg2R replicon. The exact nature of these interactions will be investigated in future studies.

The third category consists of the p88ΔN36-2HA protein translated from two different constructs (2 × 35S::p88fs-2HA and Core35S::p88ΔN36-2HA), and the full-length p88-2HA protein at lower concentration (the Core35S::p88-2HA construct). Due to suboptimal construct configurations (translational re-initiation in the "fs" construct, or the weak Core35S promoter in the other two constructs), these p88 variants all accumulated relatively low levels of proteins. Nevertheless, they all partially *trans*-complemented the replication of the p88-defective replicon, leading to a unique TCV RNA profile with barely detectable levels of gRNA, but highly abundant sgRNA2 that rivaled or exceeded the control TCV_sg2R replicon, and the accumulation of the new ttsgRNA (Fig. 4). Interestingly, these three p88 forms also repressed the replication of TCV_sg2R to similar extents, and giving rise to a similar RNA profile of diminished gRNA accumulation, but elevated sgRNA accumulation (Fig. 5). This latter observation strongly suggests that these p88 proteins did not strictly repress TCV_sg2R replication, but rather preferentially enhanced the amplification of sgRNAs.

What would happen if the level of p88 protein were further reduced? This is an important question because in actual TCV infections, the p88 accumulation level is expected to be much lower still, as the read-through of the p28 stop codon is very inefficient. It seems reasonable to speculate that the very low p88 level at the early stage of TCV infection might be evolutionarily selected to ensure a sufficient amount of gRNA is synthesized in order to sustain virus infections. Conversely, a slight increase at a later stage of intracellular infection might then permit the accumulation of sgRNAs, facilitating virion assembly and intercellular spread. It could thus be inferred that during TCV replications, change in p88 concentration might exert a regulative role by adjusting the ratio of gRNA and sgRNAs according to the

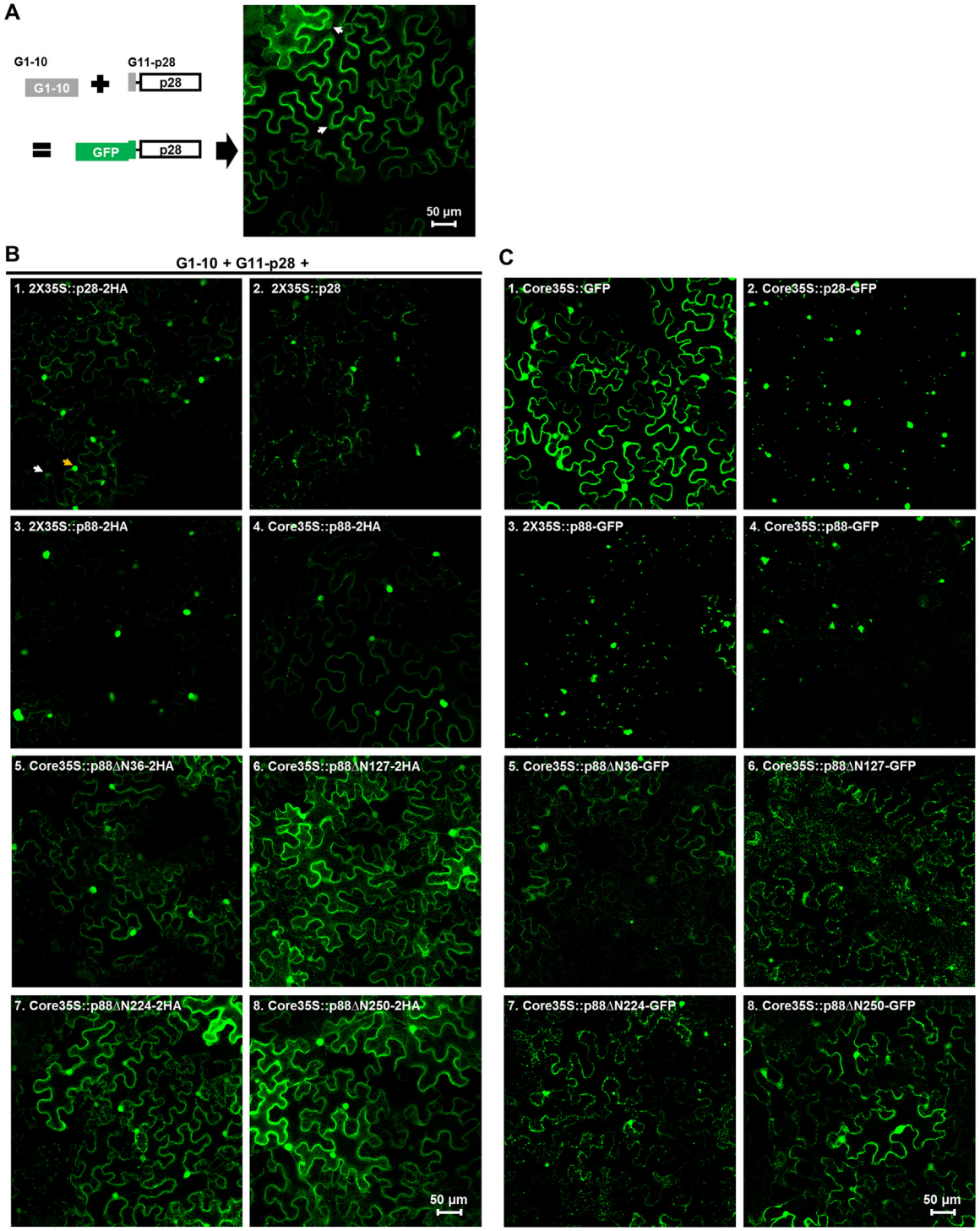

Fig. 6. Replicational repression by p88-2HA variants partially correlates with their ability to *trans*-aggregate G11-p28. A: Schematic depiction of the G11-p28 *trans*-aggregation assay. The diffuse distribution pattern of G11-p28, a p28 variant with an N-terminal G11 (the 11th β-strand of GFP) tag, could be revealed with the co-expression of G1–10 (the first 10 β-strands of GFP). The right hand image shows a confocal microscopy image. B: various p28 and p88-2HA derivatives altered the distribution of G11-p28 to varying extents (see text for details). C. p88 deletion mutants tagged at the C-termini with GFP revealing their intracellular behaviors.

progression of infection.

Finally, the highly expressed p88-2HA (from the 2 × 35S::p88-2HA) constitutes a category of its own as it exerted a very strong repression on TCV_sg2R that diminished the accumulation of both gRNA and sgRNA levels. By contrast, it was much less capable of *trans*-complementing Δp88_sg2R than its Core35S-driven counterpart, despite a substantially higher protein level. We reason that the very high level of p88-2HA could precipitate a repressive state in a manner similar to p28 (Zhang et al., 2017), by coalescing into large intracellular inclusions that could in turn *trans*-aggregate p28/p88 produced by the TCV_sg2R replicon, as well as p28 produced by Δp88_sg2R. Consistent with this idea, *trans*-aggregation of G11-p28 was much more strongly induced by 2 × 35S-driven p88-2HA than by Core35S-driven p88-2HA (Fig. 6).

In summary, this study confirmed that p88, as well as a minimally truncated form of p88 (ΔN36), was capable of *trans*-complementing the replication of a p88-defective TCV mutant in a relatively inefficient

manner that favors sgRNAs over gRNA. Lowering the expression levels of p88 caused a modest increase of complementation efficiency, revealing an inverse relationship between *trans*-complementation and p88 concentration. We further demonstrated that p88 and its various variants also repressed TCV replication by primarily targeting the accumulation of TCV gRNA, and this repressive activity positively correlated with p88 protein concentration. These results led to a hypothesis that p88 exerts a concentration-dependent control of gRNA/sgRNA ratio during the TCV replication cycle. This hypothesis will be tested with additional follow-up research.

4. Materials and methods

4.1. Constructs

Constructs 2 × 35S::p88-2HA, TCV_sg2R and Δp88_sg2R were described in previous studies (Zhang et al., 2015, 2017). Core35S::p88-2HA was generated by deleting the duplicated enhancer sequence from2 × 35S::p88-2HA, retaining only the 99-nt core promoter. The [p28stop]_sg2R mutant replicon construct was modified from TCV_sg2R by changing the methionine-coding AUG at the aa position 36 of p28 to UAG, thus introducing an in-frame stop codon in both p28 and p88 coding sequence. All of the deletion mutants of p88-2HA were generated on the Core35S::p88-2HA backbone. The identity of all new constructs was verified with Sanger sequencing.

4.2. Agro-infiltration

Upon verification, all of the constructs were introduced into *Agrobacterium tumefaciens* strain C58C1 with electroporation (Qu et al., 2003). To carry out the experiments described in the Results section, various combinations of *Agrobacterium* suspensions were mixed together and delivered into *N. benthamiana* leaves as described (Qu et al., 2003; Zhang et al., 2015, 2017). A p19-expressing *Agrobacterium* strain was included in all combinations to alleviate RNA silencing-mediated mRNA degradation.

4.3. RNA extraction and Northern blotting

Total RNA was extracted from agro-infiltrated *N. benthamiana* leaves using the Direct-zol RNA Miniprep kit (Zymo Research, Irvine, CA). To ensure consistency, four equivalent leaf sections derived from infiltrated leaves of four different plants were pooled before RNA extraction. The RNA extraction procedure included a DNase treatment step that removes DNA contamination. The RNA was then quantified with NanoDrop and subjected to Northern blotting as described (Zhang et al., 2015, 2017).

4.4. Protein extraction and Western blotting

Protein was extracted from the same *benthamiana* leaf pool using RIPA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM PMSF, and 1X ProBlockTM Gold Plant Protease Inhibitor Cocktail, the last two reagents should be added immediately before use). Western blotting was carried out as described (Zhang et al., 2015, 2017). Anti-HA and anti-mCherry antibodies were purchased from ThermoFisher Scientific.

4.5. Detection of (-) gRNA with semi-quantitative RT-PCR

The procedure for detecting (-) strand of TCV gRNA was adapted from Plaskon et al. (2009). A TCV (-) strand specific primer with a unique 5′ end tag (5′-GGAACGTCATGGTGGTGACAAAAACAGCGCTCG CAGT-GGGACT-3′) was used to prime the reverse transcription. Subsequently, a tag-specific primer (5′-GGAACGTCATGGTGGTGACA AAA-3′) and a TCV-specific reverse primer (5′-GGACAAAAGAGATCG CCTGGTC-3′) were used to amplify an (-)-strand-specific PCR product. Semi-quantification was achieved using an RT-PCR product of *N. benthamiana* EF1α mRNA as the reference.

4.6. Confocal microscopy

Confocal microscopic observations were carried out using a Leica Confocal microscope (TCS SP5) available through Molecular and Cellular Imaging Center at the Ohio Agricultural Research and Development Center, The Ohio State University.

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