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StRemorin1.3 hampers *Potato virus X* TGBp1 ability to increase plasmodesmata permeability, but does not interfere with its silencing suppressor activity

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ABSTRACT

The Triple Gene Block 1 (TGBp1) protein encoded by the *Potato virus X* is a multifunctional protein that acts as a suppressor of RNA silencing or facilitates the passage of virus from cell to cell by promoting the plasmodesmata opening. We previously showed that the membrane raft protein StRemorin1.3 is able to impair PVX infection. Here, we show that overexpressed StRemorin1.3 does not impair the silencing suppressor activity of TGBp1, but affects its ability to increase plasmodesmata permeability. A similar effect on plasmodesmata permeability was observed with other movement proteins, suggesting that REM is a general regulator of plasmodesmal size exclusion limit. These results add to our knowledge of the mechanisms underlying the StREM1.3 role in virus infection.

1. Introduction

The *Potato virus X* (PVX) is among the top 10 most important plant viruses based on its scientific and economical importance [1]. PVX is used as a model system to study plant–virus interactions and to dissect the mechanisms underlying viral propagation such as viral replication, cell-to-cell movement through plasmodesmata (PD) and suppression of post-transcriptional gene silencing (PTGS). The PVX genome contains five ORFs encoding the viral RNA-dependent RNA polymerase, the movement proteins TGBp1, 2 and 3 (Triple Gene Block), and the viral Coat Protein (CP), required for genome encapsidation and for cell-to-cell viral movement [2]. The largest TGB protein, named TGBp1 (25 kDa), is a multifunctional protein, essential for the formation of PVX viral replication complexes [3], initiation of PVX virion translation and for PVX movement [4]. TGBp1 was the first Viral Suppressor of RNA gene silencing (VSR) discovered, although its exact mechanism of action is not clearly established [5]. Recently, it was reported that it can interact with AGO proteins of *Arabidopsis* and can mediate AGO1 degradation through the proteasome pathway [6]. TGBp1 is also capable of modifying PD aperture in order to establish the transport of the PVX ribonucleoprotein complexes to adjacent cells [7]. TGBp1 "gating" activity, i.e. its propensity to expand PD Size Exclusion Limit (SEL), is presumably preceded or concomitant with its accumulation at pitfield PD. Functional characterization of TGBp1 mutants impaired in PTGS suppressor activity showed that PVX movement is dependent on the silencing suppressor activity

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of TGBp1, while the silencing suppression is not sufficient to allow virus movement between cells [8].

In 2009, we showed that *Solanum tuberosum* group 1 isoform 3 REMORIN (StREM1.3), later called REM, had an antagonist effect on PVX propagation [9]. Group 1 Remorins are proteins specific to plants, located at the plasma membrane (PM) and segregating into nanoscale sterol-dependent microdomains [10]. Membrane association is driven by a short C-terminal Anchor (REM^{CA}), which is both necessary and sufficient for PM targeting [11]. Altered expression levels of REM specifically affect PVX movement without interfering with the viral replication process [9]. In addition, restriction of the virus movement in overexpressing lines depends on REM association with the PM [11]. Of particular interest was our finding that REM can physically interact with TGBp1. Nevertheless, the mechanisms leading to the restriction of PVX infection in the presence of REM are not yet elucidated. In the present study, we investigate the effect of REM on the TGBp1 functions as VSR and as PD opening promoting factor in *Nicotiana benthamiana* plants. We also tested the role of REM, by itself or in presence of other viral proteins (Hc-Pro from *Potato virus Y* and 30 K from *Tobacco mosaic virus*), to modify PD gating.

2. Material and methods

2.1. Clones, plants and agroinfiltration

The PVX used in this work corresponds to CP2 from the International Potato Center, Peru [12]. 35S-30K:RFP clone was kindly provided by Manfred Heinlein (IBMP, Strasbourg) [33]. Constructs for REM and REM^{*} (mutated in the REM^{CA} region) are according to [9]. 35S-p19, P35S-Hc-Pro and 35S-mGFP5 (referred as 35S-GFP) were provided by Dr. Baulcombe (Cambridge University, UK). TGBp1 ORF was amplified using specific primers from pET24 vector [13], cloned into pDONR221 entry vector (Invitrogen) and then recombined into the binary vectors pK7WG2D, pK7WGF2, pK7FWG2 (Dept of Plant Systems Biology, Gent) to obtain 35S-TGBp1, 35S-TGBp1:GFP; 35S-GFP:TGBp1, vectors respectively. PVXΔTGBp1 was obtained by deletion of TGBp1 sequence from 4537 position to 5047 position of a full length infective clone pZP-PVX reported previously [14].

N. benthamiana plants were cultivated in controlled conditions (16 h photoperiod, 25 °C). *Agrobacterium tumefaciens* GV3101 strain were cultured at 28 °C until the stationary phase, washed, and resuspended in water at OD_{600nm} = 0,5 with the exception of the gating experiments, where the GFP dilution was adjusted to OD_{600nm} = 0.0002. All the above master cultures were further diluted 4 times to prepare the co-agroinfiltration mix. *Agrobacterium* cultures devoid of plasmid were used to obtain an equal number of infiltrated bacteria between the different treatments.

2.2. PTGS suppression assays

Fully expanded leaves of *N. benthamiana* wild-type or 16c line were infiltrated with *Agrobacterium* cultures carrying 35S-GFP5 alone or in combination with cultures carrying the different constructs. As a negative control of PTGS suppression, plants were agroinfiltrated with *Agrobacterium* culture without plasmid. At 5 dai, leaves were observed under UV lamp (B-100AP, UVP). Protein was extracted from ~1 cm^2 of leaf and analysed by Western Blot against GFP or against REM [9]. The band signal intensity was measured by Image J, and the densitometric data were normalized with the quantification of the rbcL subunit stained by Ponceau S or amido-black. Mann–Whitney statistical test was performed to determine whether treatment groups differed significantly from each other.

2.3. Gating assays

Fully expanded leaves of wild-type *N. benthamiana* were infiltrated with *Agrobacterium* culture carrying 35S-P19 and 35S-GFP alone (control) or in combination with the following cultures: 35S-TGBp1; 35S-REM; 35S-REM^{*}. As the PD architecture varies in different tissues or developmental stages [15], in all the experiments leaves of the same developmental stage from plants grown in the same conditions were chosen. At 4 dai, 2 leaves per condition were examined by confocal microscopy, and 50–100 clusters of GFP-expressing cells were counted. Three independent experiments were performed. The statistical significance between treatments was examined by applying a Kruskal–Wallis followed by the Dunns multiple comparison test ($P < 0,05$) on all the raw values obtained from the different experiments.

2.4. Confocal microscopy

Confocal imaging was performed as described in [9]. Aniline blue (Biosupplies) was infiltrated in leaves before analysis at 0.1 mg/ml, excited with a 405-nm laser and captured between 460 and 500 nm. FM4–64 (Invitrogen) was excited at 488 nm and captured at 650–750 nm.

3. Results

3.1. TGBp1 VSR activity is not affected by the overexpression of REM in transient PTGS suppression assays

Since REM affects viral movement, and TGBp1 is the only PVX protein that can interact with REM [9], we hypothesized that REM might affect one or more of the TGBp1 activities involved in PVX cell-to-cell movement like its PTGS-suppressor activity [8]. To evaluate the effect of REM expression on the TGBp1 VSR activity, we expressed the reporter gene GFP in wild type plants. In all the assays, we used 35S-mGFP5(GFP) [16], a variant that gives high levels of fluorescence. The co-expression of TGBp1 with GFP led to suppression of PTGS against the GFP transcripts, as shown by a stronger fluorescence at the agroinfiltrated zone at 5 dai, compared to the control condition (GFP) (Fig. 1A). The visual observations were confirmed by Western Blot as quantified against the total protein levels of large subunit of rubisco rbcL (Figs. 1B and S1). We further challenged the ability of TGBp1 to suppress the PTGS of GFP in the presence of REM, by overexpressing the three proteins together in wild type *N. benthamiana* plants. When TGBp1 and REM were coexpressed, the PTGS suppression activity of TGBp1 was not altered, compared to TGBp1 alone (Fig. 1A and B). We also tested the effect of REM on the p19 protein activity, a strong VSR from *Tomato bushy stunt virus* (TBSV), as a positive control for these PTGS assays [17]. Co-expression of p19 with GFP resulted to an average 3-fold increase of GFP expression levels, indicating a strong silencing suppression. Similarly to TGBp1, co-expression of REM with p19 and GFP, did not show any difference in GFP expression levels (Fig. 1A and B). In all cases, we confirmed by Western Blot that the REM transgene was well expressed (Fig. 1C).

In a similar experiment, we induced local silencing by infiltrating *Agrobacterium* carrying 35S-GFP (alone or in combination 35S-TGBp1 or 35S-p19, and 35S-REM) in a GFP-expressing transgenic *N. benthamiana* line, called 16c line [18]. At 5 dai, the induction of local PTGS within the agroinfiltrated zone was visualized as a decrease of GFP fluorescence in the control condition (Fig. S2A and B) while the transient co-expression of TGBp1 or P19 together with GFP resulted in a bright green fluorescence under UV light and higher GFP expression levels as shown by Western Blot

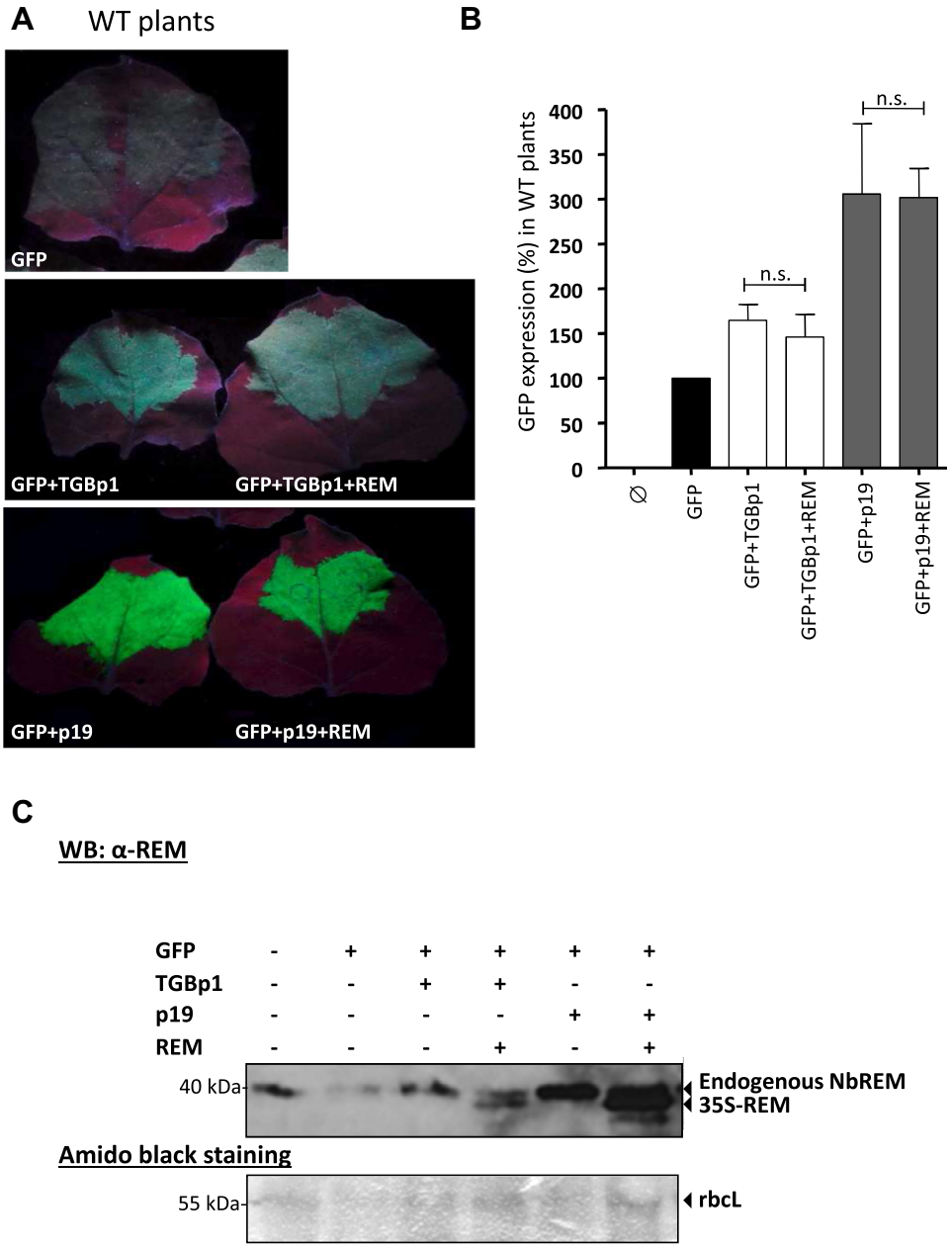

Fig. 1. The effect of REM on PTGS suppressor activity of TGBp1 and p19. Transient expression of GFP was performed in *N. benthamiana* leaves in order to estimate the PTGS activity in presence of TGBp1 or p19. (A) Similar GFP fluorescence was observed at the agroinfiltration zone under UV light at 5 dai in the presence of REM with TGBp1 or with p19; (B) The GFP protein levels from three leaf samples were analyzed by Western Blot (Fig. S1) and the normalized values were quantified as a percentage of the GFP control. ∅: *Agrobacterium* without plasmid. Error bars represent SE from independent biological repeats ($n = 3$). Statistical validations were performed between the samples expressing GFP with TGBp1/p19 alone and with REM; n.s.: not significantly different. (C) Western Blot analysis against REM from one representative assay.

(Fig. S2A–C). As expected, also in these conditions, the TGBp1 and p19 VSR activities were not significantly reduced after the overexpression of REM (Fig. S2A–C).

Taken altogether, these results obtained with wild type *N. benthamiana* or 16c lines show that REM has no significant effect on the suppression of RNA gene silencing mediated by the viral proteins TGBp1 or p19.

3.2. Sub-cellular localization of GFP fused to TGBp1 at its N- and C-terminus in the presence or absence of PVX

We next hypothesized that REM might affect the capacity of TGBp1 to modify the PD. Studies of TGBp1 subcellular localization pattern has shown PD association [7,19–23]. However, as the PVX strains used in these studies were different from ours, we decided to first examine the localization pattern of TGBp1 from the PVX strain (International Potato Center, Peru) we possessed, by transient expression assays in *N. benthamiana* leaves. We created TGBp1 variants fused to GFP at its N- (GFP:TGBp1) or C-terminus (TGBp1:GFP) and co-expressed them with the PVXΔTGBp1 construct, which includes the PVX genome deleted for the *TGBp1* ORF. In all occurrences (N- or C-terminal fusion to TGBp1), the most striking feature was the presence of cytoplasmic aggregates with particularly intense fluorescence (Fig. 2A–C and G–I). The overall shape of these cytoplasmic bodies was revealed by adjusting the confocal imaging settings (Fig. 2C and I). The GFP:TGBp1 protein fusion formed well-defined rod-like structures that were most commonly grouped in aggregates (Fig. 2A–C). Several

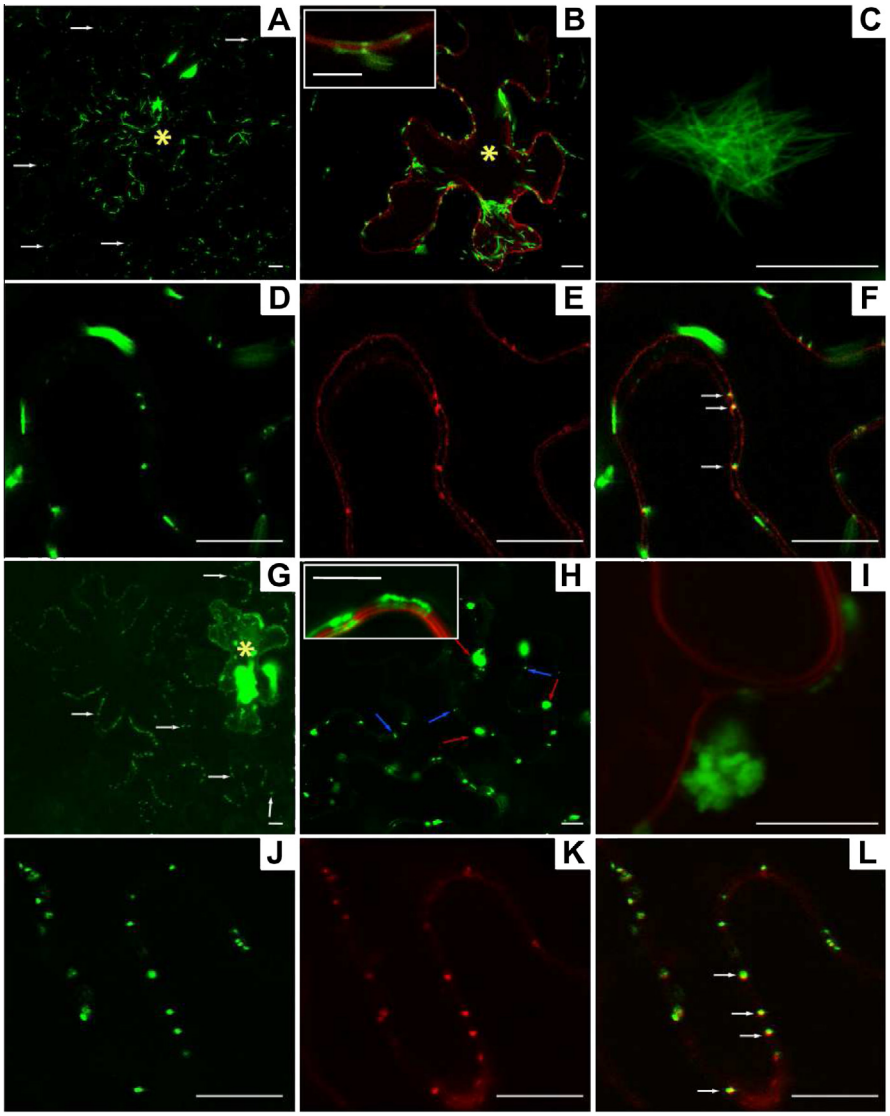

Fig. 2. Subcellular localization patterns of GFP:TGBp1 and TGBp1:GFP in presence of PVXΔTGBp1. Cellular localization pattern was observed 3 dai by confocal microscopy. (A–F), Subcellular localization pattern of GFP:TGBp1 in the presence of PVXΔTGBp1. (A) Central cell (yellow star) displaying cytoplasmic TGBp1 aggregates, whereas in neighboring cells TGBp1 associate with dots at the cell periphery (arrows). (B) TGBp1 rod shape aggregates in the cytoplasm. The PM is stained with FM4–64. Inset: TGBp1 rods aligned along the PM (C) Close up of TGBp1 rod-like structures. (D–F) Co-expression of GFP:TGBp1 with PDLP1–RFP. Cells were slightly plasmolyzed with 0.4 M Mannitol. GFP:TGBp1 co-localizes with PDLP1:RFP, a PD marker at the cell periphery, white arrows. (G–L) Subcellular localization pattern of TGBp1:GFP in the presence of PVXΔTGBp1. (G) Visualization of cytoplasmic globular aggregates in the central cell (yellow star). Punctuated dots were observed at the cell periphery (arrows white) in neighboring cells. (H) Location of cytoplasmic aggregates along the cell periphery (red arrows) and punctuated labeling in the wall (blue arrows). Inset: TGBp1 aggregates closely associated with the PM, which is stained with FM4–64. (I) Close up view of cytoplasmic aggregates. The PM was stained with FM4–64. (J–L) Co-localization of TGBp1:GFP with aniline blue in the periphery of the central cell. (J) TGBp1:GFP associate with dots at the periphery of the cell. (K) Same cell imaged to see callose staining with aniline blue. (L) Overlay of images showing the colocalization between GFP:TGBp1 and callose (white arrows). Bars = 10 μm.

individual structures could also be seen within the cytosol or aligned with the PM (Fig. 2B inset). In contrast, TGBp1:GFP formed cytoplasmic aggregates resembling globular inclusions with an amorphous shape (Fig. 2G–I). Some of these were observed closely associated with the PM and sometimes seemed to extend within the extracellular space (likely through PD) or even into the neighboring cell (Fig. 2H inset). The formation of different structures depending on the N-ter or C-ter position of the GFP tag strongly suggests a high degree of complexity in TGBp1 protein interactions. We very often observed a central cell containing numerous "aggregate" bodies, surrounded by cells displaying lesser fluorescence intensity with no or few inclusion bodies but numerous dots at the periphery, reminiscent to pitfield PD (Fig. 2A and G). To confirm the PD-association of TGBp1, we performed co-localization studies by labeling the callose deposition at the PD entry using aniline blue staining, or by co-expressing the PD marker PDLP1 (plasmodesmata-located protein 1) [34] fused to RFP (PDLP1-RFP) [24]. The results clearly showed that most TGBp1 dots detected at the cell periphery were indeed co-localized with PD (Fig. 2D–F and J–L).

The localization of TGBp1 without co-expression of PVXΔTGBp1 was also assessed using the same experimental settings (Fig. S3). In this case, the TGBp1 expression caused the formation of strongly fluorescent aggregate bodies in the cytosol, with similar morphological features as the ones we previously described. Dots were sometimes visible at the cell periphery, but clear association with PD was much less frequent than in the presence of PVXΔTGBp1. We also observed nuclear localization and diffuse fluorescence throughout the cytosol of the expressing cells.

3.3. The TGBp1 ability to gate PD and increase PD permeability is strongly restricted in presence of REM

To better understand the effect of REM overexpression on PVX cell-to-cell movement, we tested whether REM regulates TGBp1 ability to increase the PD permeability. To do so, we applied a method that indirectly measures the conductivity throughout the PD pore in presence or absence of the viral proteins. This assay monitors the expression of GFP from individual transformed cells (by infiltration of a highly diluted *Agrobacterium* culture carrying 35S-GFP) until its free diffusion to adjacent cells through the "open" PD. At 1dai individual fluorescent cells in the *N. benthamiana* leaf epidermis were identified and the spreading of the GFP fluorescence from that cell monitored for 3 days (Fig. 3A).

As expected, when TGBp1 was co-expressed in cells the GFP fluorescence diffused to 1–4 adjacent cells at 4 dai (Fig. 3A). In agreement with previous studies demonstrating that TGBp1 enabled the transfer of 10-kDa F-dextrans between cells [7,25], the diffusion of fluorescence to 2 or more adjacent cells was increased by 1.8-fold in the presence of TGBp1 compared to the control GFP alone (Fig. 3B). Interestingly, overexpression of REM together with TGBp1 strongly restricted the GFP diffusion to neighboring cells. These results suggest a role of REM in restricting TGBp1 ability to modify PD permeability.

The ability of TGBp1 to open PD was thus challenged in the presence of REM*, a REM mutated in REM^{CA}, which results in the localization of REM in the cytosol and abolishes REM function in restricting PVX movement [11]. The ectopic expression of REM* together with TGBp1 significantly restricted GFP diffusion but much less than REM. These results suggest that the PM localization of REM is a major factor for its function, as previously described in the PVX–GFP virus assays (Fig. 3B). Indeed, REM overexpression, but not REM*, led to a significant reduction of GFP diffusion in the presence of movement proteins 30 K and Hc-Pro, respectively from *Tobacco Mosaic Virus* (TMV) and *Potato Virus Y* (PVY) and in absence of viral proteins, suggesting that REM has a general role in modulating PD permeability (Fig. 3B).

4. Discussion

4.1. Does REM have an effect on the gene silencing suppressor activity of TGBp1?

PTGS is an host RNA silencing defense mechanism that specifically recognizes and degrades viral RNA [26]. TGBp1 was the first viral component described as a suppressor that blocks RNA gene-silencing defense [5]. We examined the possibility that REM might impair the ability of TGBp1 to suppress local silencing in *N. benthamiana* leaves. Our results show that there is no effect of REM on the VSR activity of TGBp1. Therefore, it is unlikely that the silencing mechanism is involved in the previously described antagonistic effect of REM on PVX propagation [9]. Silencing experiments described in this paper revealed also that REM does not modify the VSR activity of p19, an universal suppressor of PTGS process [27] that acts differently than TGBp1 [28].

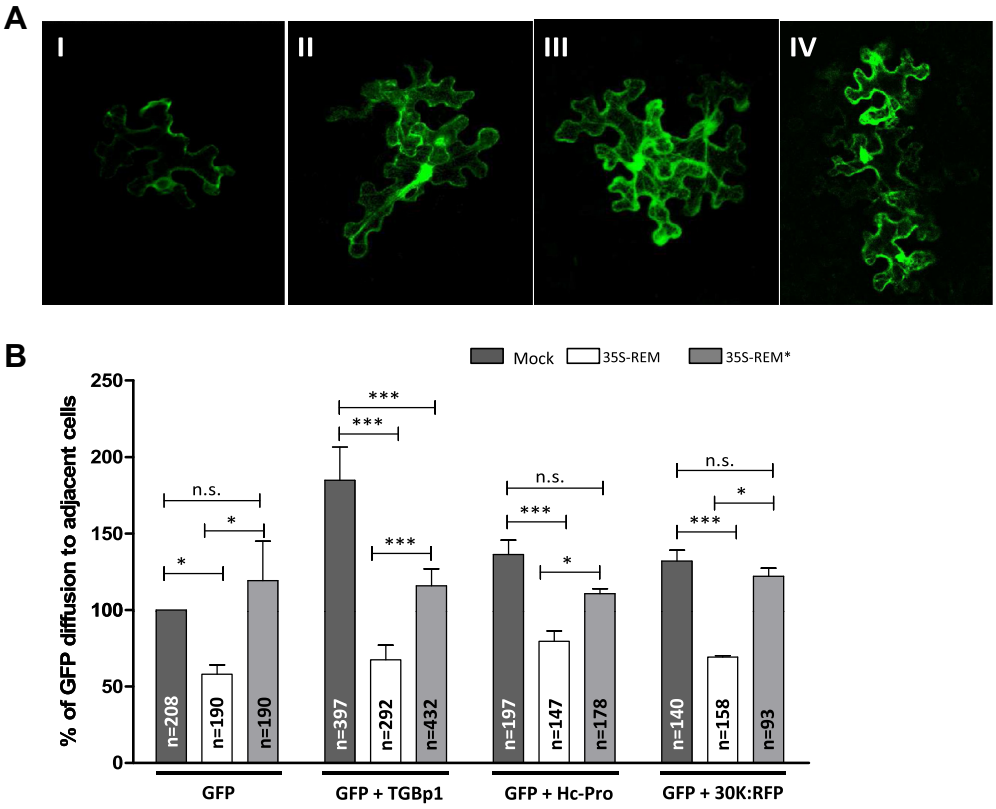

Fig. 3. Plasmodesmata gating activity of TGBp1, 30K and Hc-Pro is altered by REM overexpression. Unique GFP-expressing cells were obtained at 1 dai by infiltrating a highly diluted *Agrobacterium* culture carrying 35S-GFP in *N. benthamiana* leaves. Diffusion of GFP to surrounding cells provided a measure of molecular fluxes through PD. The gating activity of TGBp1 was challenged alone, or in presence of REM and REM* by measuring the diffusion of one unique GFP cell to neighbor cells at 4 dai by confocal microscopy; (A) Confocal images from *N. benthamiana* epidermal cells. The GFP could either be limited to unique cells (I) or diffuse to adjacent cells in clusters of two (II), three (III) or more cells (IV); (B): Graph represents the percentage of individual clusters containing two or more fluorescent cells normalized with the GFP treatment. Error bars show S.E.M. from three independent experiments (*n*: total number of GFP clusters measured). Significance was assayed by Kruskal–Wallis followed by the Dunns multiple comparison on the row values obtained from the different experiments ($^{*}P < 0{,}05$; $^{**}P < 0{,}01$; $^{***}P < 0{,}001$). The significance between GFP/GFP + TGBp1, GFP/GFP + Hc-Pro and GFP/GFP + 30 K:RFP was respectively ***, *** and **.

4.2. Does REM impair the "gating" activity of the TGBp1?

The study of protein subcellular localization is decisive to a better understanding of their possible functions and activities. REM has been extensively studied in plant cells. In Solanaceae, REM was shown to be located in the inner leaflet PM microdomains. TGBp1 has the ability to move from cell-to-cell by targeting the PD and modifying its aperture [2]. Here, we confirmed that both N-and C-terminal GFP fusions of TGBp1 are located in the cytoplasm and can target the PD (Figs. 2 and S3). In the presence of virus, we visualized a strong increase in PD targeting. The TGBp1 of our PVX strain is able to target the PD in the absence of other PVX proteins, although at very low efficiency. This result appears at variance with the one described by Tilsner et al., [23] who showed that TGB1 was recruited to PD only in the presence of the TGBp2/3 complex, but it is in accordance to the previously reported capacity of TGBp1 to move from cell to cell by itself [3].

Gating experiments revealed that, when REM is co-expressed in cells, the TGBp1-induced free GFP diffusion was strongly inhibited, indicating that previous observations showing that REM restricts PVX movement [9,11] can be explained by the ability of REM to impair TGBp1 PD opening activity. Interestingly, we found that REM overexpression can restrict GFP diffusion in the absence of viral movement protein, suggesting that REM may have a direct function on PD conductivity. Moreover, REM also hampers 30 K and Hc-Pro gating activities, suggesting that REM has a general role in the defense against viral infection in plants. We assume that the association of REM at the PM seems to be necessary to restrict PD permeability since the overexpression of a cytosolic REM (REM*) is not able to decrease GFP diffusion. In contrast, GFP diffusion promoted by TGBp1 is still slightly negatively affected by REM*. Since this cytosolic REM* can still interact with TGBp1 [11], this effect could be due to a putative sequestration of TGBp1 by direct protein–protein interaction. Interestingly, the gating activity of the other movement proteins that we tested, 30 K and Hc-Pro, was not affected by REM* overexpression, suggesting that these viral proteins might interfere with REM function by different mechanisms, perhaps without directly interacting with REM.

The effect of REM in the regulation of PD permeability should not be direct, otherwise the transgenic plants overexpressing REM should have developed an aberrant phenotype [9]. Indeed, previous reports described that overexpression of PD-associated proteins causes strong phenotypes like stunted growth, rosette-like pattern, chlorosis and cell death [29]. How could REM impair the gating activity of TGBp1? Recent studies showed that association of TGBp1 with actin is necessary for viral movement and that TGBp1 reorganizes the actin cytoskeleton at the X body replication sites [30,31]. As actin and the ER are components of PD [3], and as TGBp1 targets the PD, it is suggested that actin/ER remodeling by TGBp1 also plays a role in dilating PD and inserting the PVX ribonucleoproteic complex into PD [30,31]. Chemical inhibition of microfilaments in cells destroys the functional aggregation of GFP:TGBp1 [21]. In addition, TGBp2 and TGBp3 proteins also seem to function together with TGBp1 in PVX movement. It was demonstrated that GFP:TGBp2 ER-derived granular vesicles are necessary for virus movement. These vesicles have been also described to be alongside TGBp1-formed strands in the cytoplasm [21,22]. Finally, to enable PVX movement, TGBp1 interacts *in vivo* with TGBp2 and TGBp3, themselves recruited to GFP:TGBp1 rod-like structures [21] or to TGBp1:GFP aggregates [2,31]. TGBp2 interacts indirectly with a β1,3-glucanase, a callose-degrading enzyme [32], suggesting that one strategy used by PVX to modify PD is by inducing callose degradation.

Understanding the mechanisms underlying the function of the PM-associated REM on virus infection and specifically of its role in guarding plant intercellular communication is our future challenge. Does REM directly block PVX complexes to act as a PD-permeability modifier, or does it directly counteract the virus-mediated callose release? What are the dynamics of REM localization in the PM and PD upon virus infection? Do the REM-driven raft-microdomains play a role in the plant defense against viruses? Discovered as a phosphorylated protein, does REM function involve post-translational regulation? All these questions are currently being investigated in our laboratories.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.03.014.

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