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VIRUS-CELL INTERACTIONS

Geminivirus Replication Protein Impairs SUMO Conjugation of Proliferating Cellular Nuclear Antigen at Two Acceptor Sites

Manuel Arroyo-Mateos,[a,b]* Blanca Sabarit,[a] Francesca Maio,[b] Miguel A. Sánchez-Durán,[a] Tabata Rosas-Díaz,[a]* Marcel Prins,[b,c] Javier Ruiz-Albert,[a] Ana P. Luna,[a] Harrold A. van den Burg,[b] Eduardo R. Bejarano[a]

[a]Instituto de Hortofruticultura Subtropical y Mediterránea La Mayora, Universidad de Málaga-Consejo Superior de Investigaciones Científicas, Deptartmento Biología Celular, Genética y Fisiología, Universidad de Málaga, Campus Teatinos, Málaga, Spain

[b]Molecular Plant Pathology, Swammerdam Institute for Life Sciences, Faculty of Science, University of Amsterdam, Amsterdam, The Netherlands

[c]Keygene NV, Wageningen, The Netherlands

ABSTRACT Geminiviruses are DNA viruses that replicate in nuclei of infected plant cells using the plant DNA replication machinery, including PCNA (proliferating cellular nuclear antigen), a cofactor that orchestrates genome duplication and maintenance by recruiting crucial players to replication forks. These viruses encode a multifunctional protein, Rep, which is essential for viral replication, induces the accumulation of the host replication machinery, and interacts with several host proteins, including PCNA and the SUMO E2 conjugation enzyme (SCE1). Posttranslational modification of PCNA by ubiquitin or SUMO plays an essential role in the switching of PCNA between interacting partners during DNA metabolism processes (e.g., replication, recombination, and repair, etc.). In yeast, PCNA sumoylation has been associated with DNA repair involving homologous recombination (HR). Previously, we reported that ectopic Rep expression results in very specific changes in the sumoylation pattern of plant cells. In this work, we show, using a reconstituted sumoylation system in *Escherichia coli*, that tomato PCNA is sumoylated at two residues, K254 and K164, and that coexpression of the geminivirus protein Rep suppresses sumoylation at these lysines. Finally, we confirm that PCNA is sumoylated *in planta* and that Rep also interferes with PCNA sumoylation in plant cells.

IMPORTANCE SUMO adducts have a key role in regulating the activity of animal and yeast PCNA on DNA repair and replication. Our work demonstrates for the first time that sumoylation of plant PCNA occurs in plant cells and that a plant virus interferes with this modification. This work marks the importance of sumoylation in allowing viral infection and replication in plants. Moreover, it constitutes a prime example of how viral proteins interfere with posttranslational modifications of selected host factors to create a proper environment for infection.

KEYWORDS geminivirus, Rep, PCNA, begomovirus, SUMO, sumoylation, homologous recombination

Geminiviruses constitute a large family of plant viruses with circular single-stranded DNA (ssDNA) genomes packaged within geminate particles (1), which replicate in the nuclei of infected cells through double-stranded DNA (dsDNA) intermediates (2, 3). The largest geminivirus genus corresponds to begomoviruses, which can have bipartite genomes (A and B components), like *Tomato golden mosaic virus* (TGMV), or monopartite genomes, like *Tomato yellow leaf curl virus* (TYLCV). Begomoviruses encode two proteins involved in viral replication: Rep (also called AL1, AC1, and C1), a multifunctional essential protein, and C3 (also called AL3, AC3, C3, and REn), which interacts with Rep and greatly enhances begomovirus DNA accumulation in host cells (4). Rep has different functions: it mediates recognition of its cognate origin of replication in a

Received 20 April 2018 **Accepted** 22 June 2018

Accepted manuscript posted online 27 June 2018

Citation Arroyo-Mateos M, Sabarit B, Maio F, Sánchez-Durán MA, Rosas-Díaz T, Prins M, Ruiz-Albert J, Luna AP, van den Burg HA, Bejarano ER. 2018. Geminivirus replication protein impairs SUMO conjugation of proliferating cellular nuclear antigen at two acceptor sites. J Virol 92:e00611-18. https://doi.org/10.1128/JVI.00611-18.

Editor Anne E. Simon, University of Maryland, College Park

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Address correspondence to Eduardo R. Bejarano, edu_rodri@uma.es.

* Present address: Manuel Arroyo-Mateos, Molecular Plant Pathology, Swammerdam Institute for Life Sciences, Faculty of Science, University of Amsterdam, Amsterdam, The Netherlands; Tabata Rosas-Díaz, Shanghai Center for Plant Stress Biology/CAS Center for Excellence in Molecular Plant Science, Chinese Academy of Sciences, Shanghai, China.

M.A.-M. and B.S. contributed equally to this work.

geminivirus species-specific manner (5), it is required for initiation and termination of viral DNA synthesis (6–8), and it acts as a DNA helicase (9, 10).

Growing evidence strongly supports the notion that geminivirus proteins have a significant impact on a variety of host processes, including cell differentiation, cell cycle control, DNA replication, plasmodesma function, and RNA silencing (3). By these means, geminiviruses reshape their environment by co-opting cellular processes necessary for viral replication, systemic spread, and impairment of plant defenses. There are numerous mechanisms by which geminiviruses mediate their effects on the host cell, including targeting of posttranslational modification systems. Such systems play critical roles in many cellular processes because they cause rapid changes in (i) the function of preexisting proteins, (ii) the composition of multiprotein complexes, and (iii) their subcellular localization. Their versatility in regulating protein function and cellular behavior makes them a particularly attractive target for viruses. One example of a key cellular regulatory system targeted by viruses is sumoylation (11, 12), a posttranslational process mainly involved in nuclear functions that modifies protein function, activity, or localization of its targets through covalent attachment of a 10-kDa ubiquitin-like polypeptide called SUMO (small ubiquitin-like modifier) (13–15).

Briefly, posttranslational modification by SUMO involves a cascade of ATP-dependent reactions that are mechanistically similar to ubiquitination, involving sequential activation and conjugation of SUMO. SUMO activation is driven by an E1 enzyme (SUMO-activating enzyme SAE1/SAE2 heterodimer), while SUMO conjugation is mediated by a single E2 enzyme (SUMO-conjugating enzyme SCE1, also known as Ubc9 in yeast and mammals). The final transfer of SUMO from SCE1 to specific lysine residues in target proteins can occur directly or can be enhanced by SUMO ligases (14, 16). Target proteins can undergo monosumoylation of one lysine, polysumoylation (SUMO chain formation), or multisumoylation (modification of several lysines in one substrate) (17–19). SUMO can be specifically detached from modified lysines by SUMO proteases (ubiquitin-like specific proteases; ULPs), making it a reversible and dynamic process (18, 20). The consequences of sumoylation on targets are very diverse, ranging from changes in localization to altered activity and, in some cases, stabilization of the modified protein. All of these effects are frequently the result of changes in the molecular interactions of the sumoylated proteins. Sumoylation can either mask a binding site in its target, thus inhibiting its interactions with other proteins; increase the number of binding sites on its target, hence facilitating the binding of molecules, such as proteins or DNA; or produce a conformational change that modulates its activity.

In plants, the characterization of the sumoylation enzymes has largely been restricted to *Arabidopsis thaliana*, although information based on sequence analysis of other plant genomes is available (1, 21, 22). The *Arabidopsis* genome carries eight full-length SUMO genes (*AtSUMO* genes), a single gene encoding the SUMO-conjugating enzyme SCE1 (*AtSCE1a*), and a large number of ULPs. Only two SUMO E3 ligases (SIZ1 and HPY2/MMS21) have been identified and characterized in *Arabidopsis* (2, 3, 23–27). In plants, sumoylation is important for embryonic development, organ growth, flowering transition, and hormone regulation (4, 28). In addition, SUMO also plays a key role in stress-associated responses to stimuli such as extreme temperatures, drought, salinity, and nutrient assimilation (5, 29, 30). During these abiotic stresses, the profile of SUMO-modified proteins changes dramatically, greatly increasing the global SUMO conjugate levels and decreasing the pool of free SUMO (6–8, 31, 32). After exertion of stress, SUMO conjugates slowly diminish by the action of ULPs, which are fundamental players in fine-tuning SUMO conjugation/deconjugation (9, 10, 20, 33). Several observations, including pathogen manipulation of SUMO conjugation by bacterial elicitors (3, 34–36), modification of SUMO levels altering pathogen infection in plants (11, 12, 37, 38), and sumoylation influencing innate immunity (13–15, 39–41), indicate that SUMO also plays an important role in plant defense responses.

Numerous studies in recent years have shown that sumoylation also plays a role in viral infection. In animals, proteins from DNA and RNA virus families were shown to be sumoylated, and this modification seems to be important for their function. Conversely, proteins encoded by DNA viruses can modify host sumoylation, globally or only to

certain specific substrates, altering the host environment to facilitate viral replication or to overcome host defenses, either by preventing *de novo* sumoylation or by enhancing desumoylation (11, 12, 14, 16, 42, 43).

In sharp contrast with these animal pathosystems, only two examples of an interaction between viral proteins and the sumoylation machinery have been described for plants so far. The only RNA-dependent RNA polymerase of the potyvirus *Turnip mosaic virus* (TuMV), Nlb, is sumoylated and interacts with SUMO3 from *Arabidopsis* (17–19, 44). Knockout or overexpression of *SUMO3* suppresses TuMV replication and attenuates the viral symptoms (18, 20, 45). The other example is the interaction between the begomovirus protein Rep and SCE1 (37). This interaction is essential for viral infection, since Rep mutants impaired in SCE1 binding and plants with altered SUMO levels showed reduced viral replication (37, 46). Transient expression of Rep in *Nicotiana benthamiana* showed that the interaction between Rep and SCE1 does not alter the global sumoylation pattern *in planta* but rather may specifically influence SUMO conjugation of a selected subset of host proteins (46).

In this study, we identify PCNA (proliferating cell nuclear antigen) as such a plant protein whose sumoylation is altered in the presence of the begomovirus protein Rep. Using a reconstituted sumoylation assay in *Escherichia coli*, we demonstrate that tomato PCNA is readily sumoylated at two different lysines (K164 and K254). However, in the presence of Rep, SUMO attachment is compromised at both these acceptor sites. This interference is specific for PCNA, since Rep does not alter sumoylation of a control protein. It also does not depend on the physical interaction between Rep and SCE1. Finally, we are able to detect for the first time sumoylation of PCNA *in planta* and show that the reduction of PCNA sumoylation exerted by Rep also occurs in plant tissue.

(This article was submitted to an online preprint archive [47].)

RESULTS

Rep modulates the sumoylation of PCNA. Ectopic Rep expression alters the sumoylation status of specific host proteins (46). Although a full determination of the number and identity of these plant targets will require a comprehensive proteomic analysis, proteins that both interact with Rep and are known to be sumoylated are primary candidates. One such protein is PCNA, since its sumoylation has been described for PCNA homologues of yeast, *Xenopus laevis*, mammals, and *Arabidopsis* (48–54), and tomato PCNA binds to Rep from several begomoviruses (55, 56).

Sumoylation of *Arabidopsis* PCNA has been described for both homologues harbored in its genome (*AtPCNA1* and *AtPCNA2*) using a sumoylation system reconstituted *in bacteria* (53, 54). In order to assess if tomato PCNA (PCNA) is also sumoylated and if Rep then interferes with its sumoylation, we followed a similar strategy and performed sumoylation assays in *E. coli* as described previously (57). In this assay, all components of the sumoylation pathway (SAE1/2, SCE1, and SUMO1) are expressed in *E. coli* together with a potential substrate protein using an inducible system based on T7 promoters, where SAE1, SUMO1, and the target protein are expressed as His-tagged proteins to facilitate protein detection. Three compatible plasmids were comaintained in *E. coli* in this assay to simultaneously express (i) mammalian His-SAE1, SAE2, and SCE1 (Ubc9) from a polycistronic RNA; (ii) mammalian His-SUMO1; and (iii) His-PCNA from tomato or, alternatively, His-PCNA and Rep as a polycistronic mRNA. Protein expression was induced in cells cotransformed with the appropriate plasmids, and total protein extracts from these cells were analyzed with Western blots probed with anti-PCNA, anti-SUMO1/2, anti-His, or anti-Rep antibodies (Fig. 1). Cells expressing only PCNA displayed a band of the expected size (32 kDa) when the blot was incubated with anti-PCNA (Fig. 1, lane 1). An additional band (PCNA-SUMO) of approximately 55 kDa was detected when PCNA was expressed together with the complete sumoylation machinery (E1/E2 and SUMO) (Fig. 1, lane 5) but not when it was coexpressed with only the sumoylation machinery without SUMO (Fig. 1 lane 4). Since the apparent mass of this band matches the expected mass for a PCNA-SUMO dimer and a similar band was detected when the blot was incubated with anti-SUMO or anti-His antibodies, we

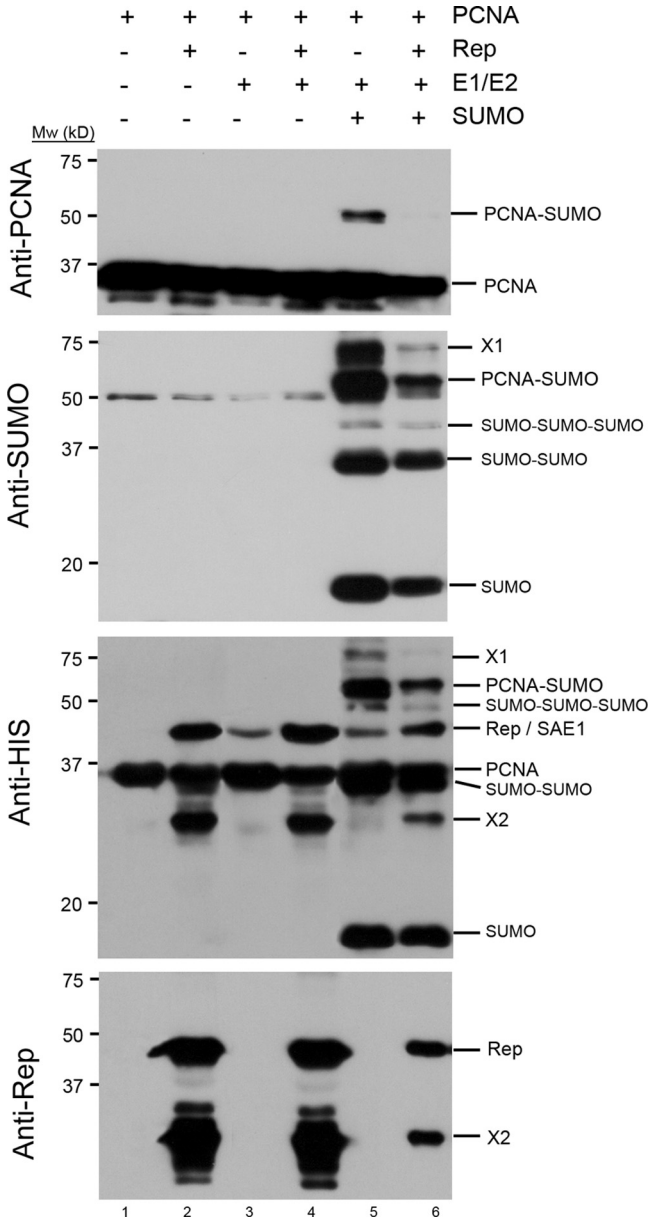

FIG 1 Sumoylation of tomato PCNA in a reconstituted SUMO conjugation system *in bacteria* is modulated by Rep. Tomato PCNA, Rep from *Tomato golden mosaic virus* (TGMV), E1/E2 (mammalian SAE1, SAE2, and Ubc9), and SUMO (*HsSUMO1*) were coexpressed in *E. coli* NCM631 cells and extracted. Protein extracts were blotted with the antibodies indicated (left side). Expression (+/−) of the individual components is indicated at the top of each lane. Relevant bands are labeled on the right side. The band labeled "X1" could correspond to a SUMO tetramer or, more likely, to a complex of PCNA-2×SUMO. Band "X2" corresponds to a truncated form of the Rep protein. Molecular weight markers (Mw) are indicated.

conclude that this 55-kDa band corresponds to the monosumoylated form of PCNA. This result indicates that tomato PCNA, like its yeast, mammalian, and *Arabidopsis* homologues, can be sumoylated. The extra protein bands observed on the membrane with the anti-SUMO antibody are similar to those reported previously (57) and likely correspond to free SUMO (SUMO) or SUMO chains (dimers and trimers, etc.). Similar bands were detected with the anti-His antibody.

When PCNA and Rep were coexpressed from the same plasmid as a polycistronic mRNA, together with the complete sumoylation machinery expressed from two accompanying plasmids, the intensity of the band that corresponds to sumoylated PCNA (PCNA-SUMO) was drastically reduced (Fig. 1, lane 6). This reduction in intensity was

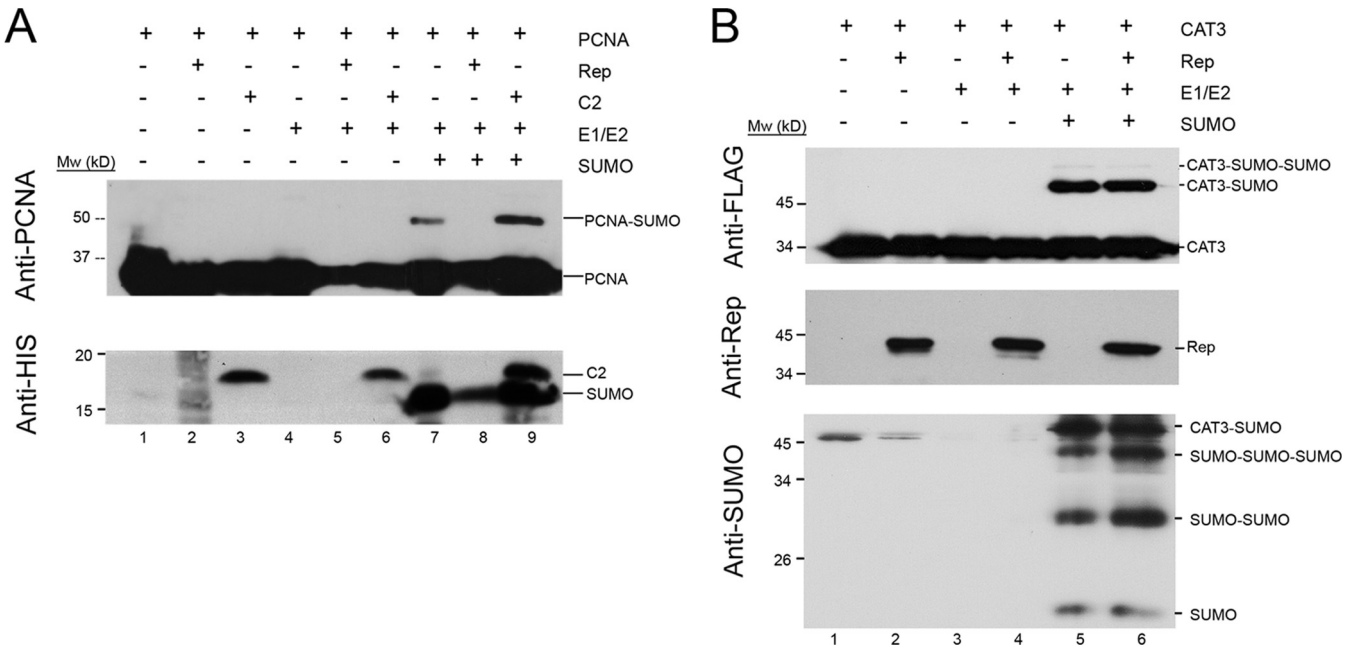

FIG 2 Rep interferes with sumoylation of only PCNA but not other proteins. (A) Tomato PCNA, Rep from TGMV, C2 from *Tomato yellow leaf curl Sardinia virus* (TYLCSV), E1/E2 (mammalian SAE1, SAE2, and Ubc9), and SUMO (*HsSUMO1*) were coexpressed in *E. coli* NCM631 cells and extracted. Protein extracts were blotted with the antibodies indicated (left side). Expression (+/−) of the individual components is indicated. Relevant bands are labeled on the right side. (B) Similar to panel A except that catalase 3 (CAT3) is coexpressed. Molecular weight markers (Mw) are indicated.

also observed for a higher-molecular-weight band (X1) detected by the anti-SUMO antibody. The size of this band is consistent with PCNA with two SUMO peptides attached. However, this extra band was not detected with anti-PCNA, arguing that it reflects a different protein (complex). Together, these results demonstrate that sumoylation of tomato PCNA is strongly reduced in the presence of Rep. When using an anti-Rep antibody, a band corresponding to the expected molecular weight (44 kDa) of the viral protein Rep was detected (Fig. 1). We did not detect any bands that could match with sumoylated forms of Rep, confirming that SUMO is not covalently attached to Rep. The additional smaller band (X2) detected with the anti-Rep antibody likely corresponds to a truncated form of the viral protein. To establish if impairment of PCNA sumoylation is strictly due to coexpression of Rep, we performed a sumoylation assay in which we replaced Rep by the C2 protein from the begomovirus *Tomato yellow leaf curl Sardinia virus* (TYLCSV). The results showed that only Rep and not C2 was capable of reducing the amount of sumoylated PCNA, thus ruling out unspecific interference with sumoylation due to the simultaneous expression of PCNA and any given protein from a polycistronic RNA (Fig. 2A, lanes 7 to 9). Expression of C2 was confirmed using an anti-His antibody.

To determine if the Rep-mediated suppression of PCNA sumoylation is due to the inhibition of SCE1 activity by the viral protein, we carried out a sumoylation assay by replacing PCNA with *Arabidopsis* catalase 3 (CAT3), a known sumoylation substrate (58). A C-terminal CAT3 fragment fused to a Flag epitope was expressed in *E. coli* cells in the presence or absence of Rep. Again, Western blots were probed with anti-Flag, anti-Rep, and anti-SUMO antibodies (Fig. 2B). In all protein extracts, a band of approximately 35 kDa corresponding to the CAT3 protein was detected with the anti-Flag antibody (Fig. 2B). When CAT3 was coexpressed with the complete sumoylation machinery (E1/E2 and SUMO), an additional band of approximately 50 kDa, consistent with monosumoylated CAT3, was detected with both the anti-Flag (Fig. 2B, lane 5) and the anti-SUMO (Fig. 2B, lane 5) antibodies. When CAT3 and Rep were expressed simultaneously from one plasmid as a polycistronic mRNA, together with the sumoylation machinery expressed from the two accompanying plasmids, the intensity of the band identified as sumoy-

lated CAT3 remained unaltered (Fig. 2B, lane 6). This indicates that Rep expression does not affect the conjugating activity of SCE1 in the *E. coli* assay in a generic way. Rep expression was confirmed by probing the Western blot with an anti-Rep antibody.

Tomato PCNA is sumoylated at residues K164 and K254. Sumoylation of yeast PCNA occurs preferentially at K164, a residue conserved in all PCNA proteins, and, to a lesser extent, at K127 (48). Sumoylation of yeast PCNA K164 requires the SUMO E3 ligase *Saccharomyces cerevisiae Sc*Siz1, whereas K127 sumoylation proceeds without an E3 ligase *in vitro* and is mediated by *Sc*Siz2 *in vivo* (48, 59). Sumoylation at K164 has been observed in other species, such as chicken cells, *X. laevis* egg extracts, and mammalian cells (49–52, 60). In the *E. coli*-reconstituted sumoylation system, *Arabidopsis* PCNA was shown to be sumoylated primarily at K254, although additional sumoylation was reported to occur at other lysine residues (K13, K14, K20, K217, and K240) but not at K164 (53).

In order to establish whether tomato PCNA also contains multiple SUMO acceptor sites and to examine if Rep interferes with sumoylation at each site, we performed sumoylation assays by expressing tomato PCNA mutants where lysines were replaced by alanines. To select PCNA lysine residues for mutagenesis, we analyzed a PCNA multisequence alignment and candidate lysines were picked according to the following criteria: (i) the residue is conserved in PCNA homologues of different organisms, (ii) the residue was previously described as a SUMO acceptor site in other PCNA homologues, (iii) the residue is located in a predicted SUMO acceptor site using GPS SUMO-gp and SUMOsp2.0 (61, 62), and/or (iv) the residue is located at the surface of the PCNA three-dimensional (3D) structure. These analyses suggested that residues K91, K164, K168, K190, and K254 have an increased probability to serve as a SUMO acceptor site, with K164 and K254 being the prime candidates (Fig. 3A). As a first step, single and double mutants were generated for residues K164 and K254 and analyzed in our sumoylation assay in *E. coli*. Coexpression of wild-type PCNA with the complete sumoylation machinery (E1/E2 and SUMO) produced a double band (PCNA-SUMO) of the expected molecular mass of sumoylated PCNA (Fig. 3B, lane 2). We did not observe this double band before, due to a lower resolution of protein separation in our previous Western blots (Fig. 1). This double band could represent PCNA monomers monosumoylated at two different positions. This phenomenon was previously described for yeast and human PCNA, which, when sumoylated at K127 and K254, respectively, migrated on SDS-PAGE gels at a different apparent molecular mass than yeast PCNA SUMO modified at K164 (48, 51, 63). When PCNA K164A was coexpressed instead of wild-type PCNA, a single band of 55 kDa (corresponding to the lower of the two bands obtained with wild-type PCNA) was observed (Fig. 3B, lane 4). On the contrary, coexpression of the PCNA single mutant K254A resulted in a decrease in the intensity of the lower band, while the intensity of the upper band remained unchanged at the levels seen for wild-type PCNA (Fig. 3B, lane 6). When the PCNA double mutant K164A/K254A was expressed, the upper band disappeared entirely, while a decrease in the intensity was detected for the lower band, similar to the situation for the single mutant K254A (Fig. 3B, lane 5). These results indicated that the double band detected with wild-type PCNA corresponds to two distinct PCNA-SUMO adducts in which SUMO is attached to two different lysines, with the upper band being the result of sumoylation at K164 and the lower band being, at least partially, the product of sumoylation at K254. To identify the alternative lysine residue(s) responsible for the residual sumoylation of the lower band in PCNA K254A, we performed sumoylation assays by introducing into the PCNA double mutant K164A/K254A additional mutations in each of the other selected lysine residues (K91, K168, or K190). However, none of these additional mutations was able to remove the weak band present in the double mutant K164A/K254A, suggesting that lysine residues other than K91, K168, or K190 are sumoylated *in bacteria* when K164 and K254 are mutated (data not shown). When coexpressing Rep, no lower band is detected, and a large reduction in the upper band is observed (Fig. 3B, lane 3), indicating that in the presence of Rep, PCNA sumoylation is impaired at all lysine residues.

Rep-mediated suppression of PCNA sumoylation is independent of the Rep-SCE1 interaction. To gain insight into the mechanism of Rep-mediated suppression of

A

Tomato PCNA

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MLELRLVQGSLLKKVLESIKDLVNDANFDCSATGFSLQAMDSSHVALVALLLRSEGFEHY      60
RCDRNISMGMNLTNMAKMLKCAGNDDIITIKADDGSDTVTFMFESPTQDKIADFEMKLMD      120
IDSEHLGIPEAEYHAIVRMPSAEFGRICKDLSSIGDTVVISVTKEGVKFSTRGDIGTANI      180
VCRQNTTVDKPEEATVIEMNEPVSLTFALRYLNSFTKASPLSNTVTISLSSELPVVVEYK      240
IAEMGYVRYYLAPKIEEDEEETKP                                          264
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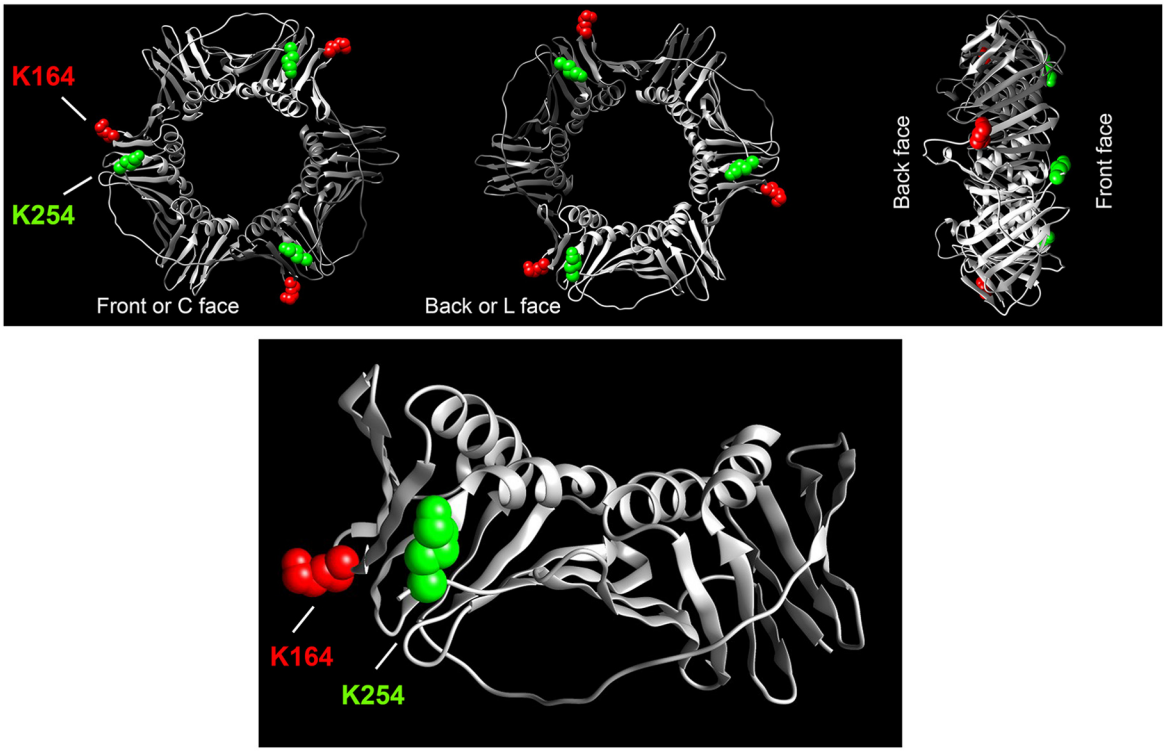

B

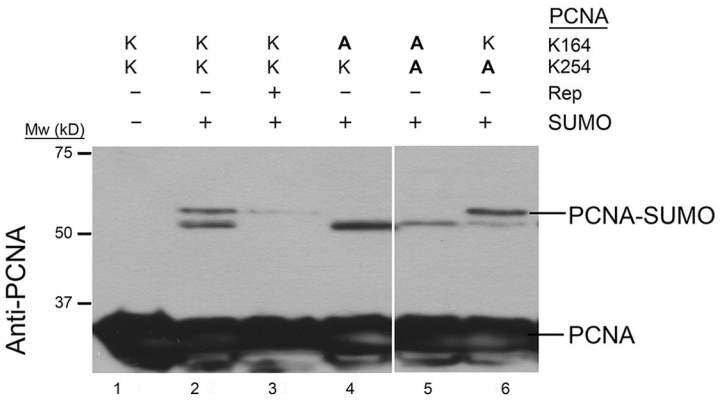

FIG 3 Identification of tomato PCNA SUMO acceptor sites. (A) Primary sequence of tomato PCNA with residues K164 and K254 highlighted (red and green, respectively) (top) and the predicted 3D model of the structure of a tomato PCNA trimer (middle) or a monomer (bottom). (B) Sumoylation assay of tomato PCNA single mutants K164A and K254A and double mutant K164A/K254A *in bacteria* while coexpressing Rep from TGMV. The assay is similar to the one described in the legend of Fig. 1. Expression (+/−) of the individual components is indicated. E1/E2 enzymes were expressed in all samples. Molecular weight markers (Mw) are indicated.

PCNA sumoylation, we analyzed whether the physical interaction of Rep with SCE1 or PCNA had a role. Previous work had mapped the SCE1-binding domain of Rep between residues 56 and 114, with the region spanning residues 56 to 85 likely forming the core of the interface with *Nicotiana benthamiana* SCE1, while the region spanning residues 86 to 114 may stabilize or enhance this interaction. Replacement of lysine residues K68

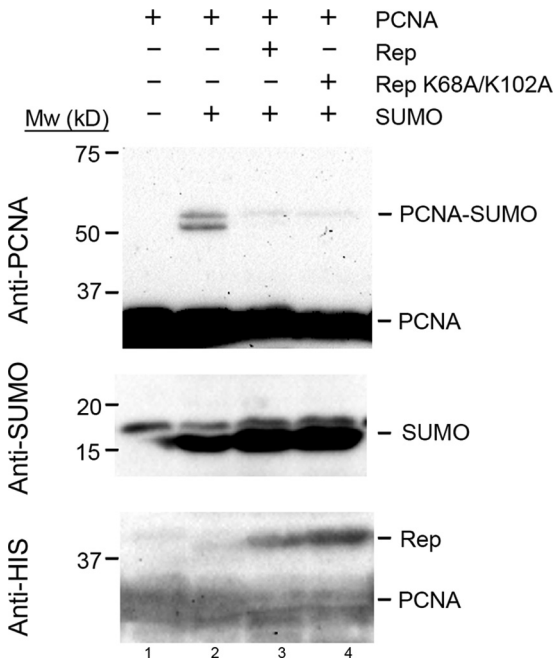

FIG 4 The Rep-SCE1 interaction is not essential to suppress PCNA sumoylation *in bacteria*. The assay is similar to the one described in the legend of Fig. 1 except that a Rep variant that fails to interact with SCE1 was coexpressed (K68A/K102A) (lane 4). Molecular weight markers (Mw) are indicated.

and K102 in the binding region of Rep impairs its interaction with SCE1 and dramatically reduces viral replication, indicating that the Rep-SCE1 interaction is required for viral DNA replication (37, 46). To determine whether the Rep-SCE1 interaction (37, 46, 55) is required for compromised PCNA sumoylation, we performed a sumoylation assay in *E. coli* coexpressing the Rep K68A/K102A double mutant, which does not interact with *N. benthamiana* SCE1 (46). As expected, the double band corresponding to sumoylated PCNA vanished when wild-type Rep was coexpressed (Fig. 4, lane 3). A similar reduction in the intensity of these two bands was seen when Rep K68A/K102A was coexpressed (Fig. 4, lane 4). This indicates that, under the conditions of our sumoylation assay, the direct SCE1-Rep interaction is not required for the Rep-mediated suppression of PCNA sumoylation.

The residues of PCNA that contribute to its interaction with Rep seem to spread across the protein, while the residues of Rep important for the interaction with PCNA appear to be concentrated in the middle part of this viral protein, spanning residues 120 to 183 (55). To further investigate the mechanism of interference, we carried out sumoylation assays by expressing truncated versions of Rep. Ten different Rep truncations were generated and expressed from the same promoter as wild-type PCNA (Fig. 5). Expression of all these Rep mutants could be successfully detected in protein extracts from *E. coli* by Western blotting using an anti-Rep antibody, except for Rep_{1-68}, Rep_{1-99}, and $Rep_{120-140}$. The latter three truncations were expressed at low or undetectable levels and therefore were excluded from our sumoylation assays (Fig. 5C). As described above, the expression of the full Rep protein interfered with PCNA sumoylation, producing a clear decrease in the intensity of the two sumoylated PCNA bands (Fig. 5B). This reduction in intensity for both SUMO-modified PCNA bands was also seen when any of the truncated Rep proteins were expressed, but only in the presence of Rep_{1-184} and $Rep_{120-352}$ was the decrease in the intensity of the lower band equivalent to the effect seen with the intact Rep protein (Fig. 5B).

Rep interferes with PCNA sumoylation in an *Arabidopsis* sumoylation system reconstituted *in bacteria*. Analyses performed *in bacteria* with *Arabidopsis* PCNA1 by Strzalka and coworkers detected sumoylation on K254 but did not detect sumoylation

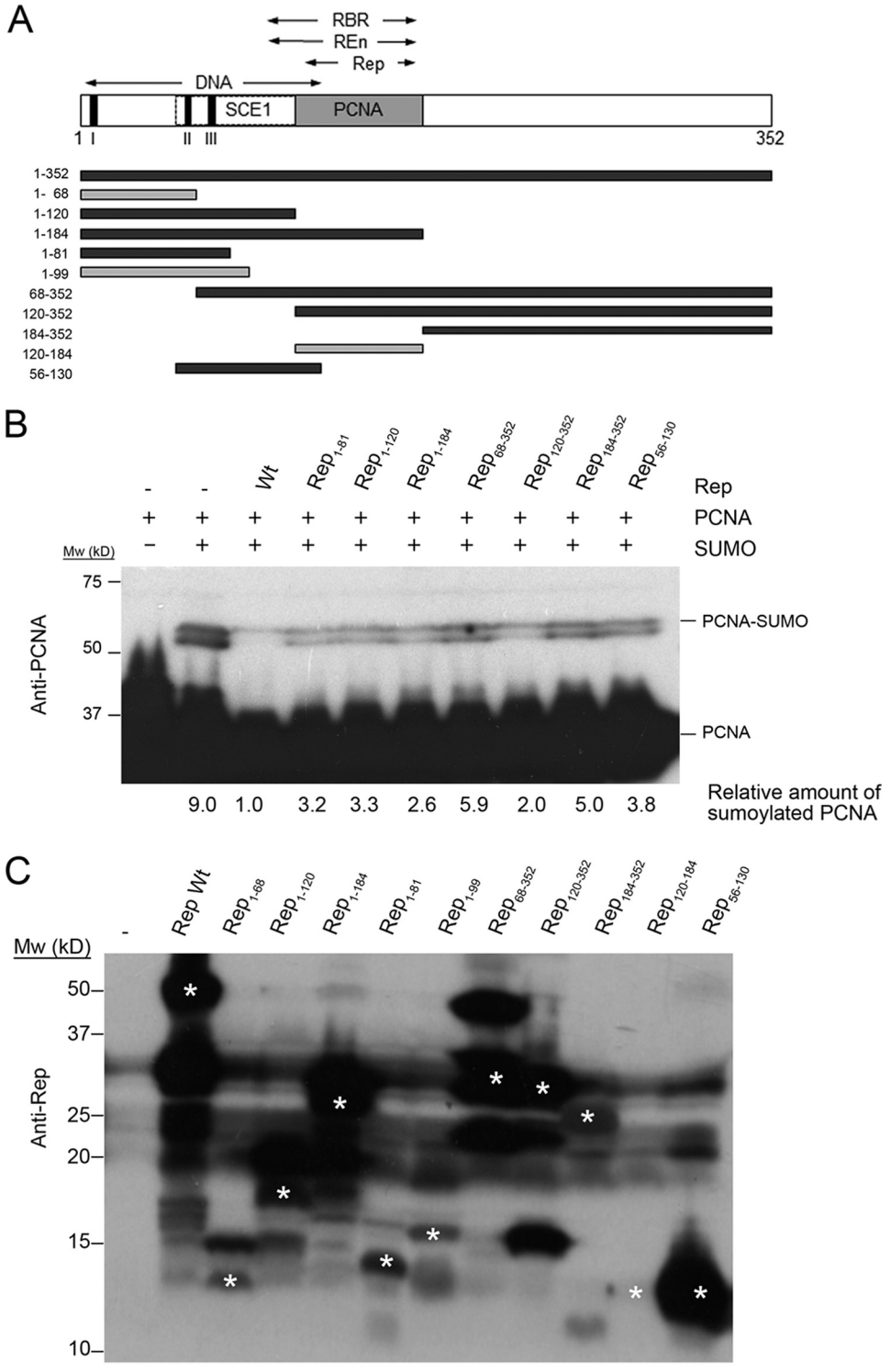

FIG 5 Identification of Rep domains involved in interference of tomato PCNA sumoylation. (A) Graphical representation of the TGMV Rep protein. The TGMV intergenic region interaction domain (DNA, residues 1 to 130), the interaction domain with itself (Rep, residues 121 to 181), the interaction domain with other viral proteins (REn, residues 101 to 180), or the interaction domain with proteins from the plant (RBR, residues 101 to 180; SCE1, residues 56 to 130; and PCNA, residues 120 to 184) are represented. Domains involved in DNA replication initiation (I, II, and III) are also indicated. Rep deletion mutants are represented by horizontal bars: dark or light gray indicates, respectively, high or low levels of accumulation of truncated Rep in the bacterial extracts (see panel C). Molecular weight markers (M_w) are indicated. (B) Sumoylation assay of tomato PCNA in the presence of wild-type or truncated versions of Rep (Rep_{xx-xxx}). Mammalian SAE1, SAE2, SCE1, and SUMO proteins and mutant and wild-type (Wt) versions of tomato PCNA proteins were overexpressed in *E. coli* NCM631 cells and extracted. Protein extracts were

(Continued on next page)

of the conserved residue K164 (53). However, our results imply that in the case of tomato PCNA, both residues can be sumoylated with similar efficiencies (Fig. 3B). The respective experimental approaches differ not only in the PCNA homologue used but also in the origin of the sumoylation enzymes used. We used an assay previously employed to identify SUMO targets in *Arabidopsis* proteins (64), originally developed by Mencía and de Lorenzo (57), that expresses mammalian E1 and E2 enzymes and human SUMO1. However, the results with *Arabidopsis* PCNA obtained by Strzalka and coworkers are based on a reconstituted sumoylation pathway that is entirely composed of *Arabidopsis* proteins. To determine if the discrepancy between these K164 sumoylation results is due to the disparate origins of the enzymes used, we redesigned the assay of Mencía and de Lorenzo by replacing the mammalian E1 and E2 enzymes with their *Arabidopsis* counterparts (AtSAE and AtSCE1). Using this system, we first determined if tomato PCNA could be SUMO modified using the *Arabidopsis* isoform AtSUMO1, AtSUMO2, or AtSUMO3. As a positive control, we used human SUMO1. Total protein extracts were analyzed by Western blotting using anti-PCNA or anti-His antibodies to confirm the expression of the SUMO isoforms (Fig. 6A). Consistent with our above-described sumoylation assay with mammalian enzymes (Fig. 3B), coexpression of PCNA and human SUMO1 produced a double band of approximately 55 kDa (Fig. 6A, lane 3). This double band was also observed in the protein extract from cells expressing either AtSUMO1 or -2 albeit at a slightly lower molecular weight due to a difference in the sequences of the *Arabidopsis* SUMOs compared to human SUMO1 (Fig. 6A, lanes 4 and 5). The same double band was also detected in the presence of AtSUMO3; however, its intensity was noticeably reduced (Fig. 6A, lane 6), even with the amount of mature free AtSUMO3 being similar to that of AtSUMO1 or AtSUMO2 (Fig. 6A, bottom, lanes 4 to 6). These results indicate that tomato PCNA can be posttranslationally modified by AtSUMO1 and AtSUMO2 but that it is a poor substrate for AtSUMO3 conjugation when using the *Arabidopsis* E1 and E2 enzymes.

To confirm that the sumoylated PCNA double band corresponds to sumoylation at K164 and K254, the tomato PCNA single mutant K164A or K254A and the double mutant K164A/K254A were coexpressed in the *Arabidopsis*-reconstituted assay with AtSUMO1. Expression of human SUMO1 was used as a positive control. The upper band observed with wild-type PCNA disappeared when the PCNA single mutant K164A was tested (Fig. 6B, lanes 4 and 8). Expression of the single mutant K254A caused a decrease of the intensity of the lower band, while the intensity of the upper band remained unaltered compared to wild-type PCNA (Fig. 6B, lane 5). In the case of the double mutant K164A/K254A, the upper band disappeared completely, and the intensity of the lower band was greatly diminished (Fig. 6B, lane 6). These results indicate that tomato PCNA is sumoylated *in vitro* on the same lysine residues, regardless of the origin of the sumoylation machinery used.

When PCNA and Rep were expressed simultaneously from the target plasmid as a polycistronic mRNA, together with the *Arabidopsis* sumoylation machinery (AtSAE/SCE1 and AtSUMO1), the intensity of the two bands identified as monosumoylated PCNA was greatly reduced compared to the intensity of those produced in the absence of Rep (Fig. 6C, lanes 3 and 4), confirming the result obtained using the reconstituted assay *in bacteria* (Fig. 1).

***In planta* sumoylation of tomato PCNA is compromised by Rep.** While sumoylation of plant PCNAs has been detected *in bacteria* using different reconstituted sumoylation systems, sumoylation of PCNA remains to be proven in plant cells, likely

FIG 5 Legend (Continued)
blotted with anti-PCNA antibody. The expression of PCNA and SUMO (+/−) is indicated. E1/E2 enzymes were expressed in all samples. Relevant bands are labeled on the right. The amount of sumoylated PCNA was quantified using ImageJ software and relativized against the amount detected in the protein extracts containing wild-type Rep. (C) Expression levels of wild-type and truncated Rep obtained by blotting bacterial extracts with an anti-Rep antibody. All protein extracts were obtained from *E. coli* NCM631 cells expressing mammalian SAE1, SAE2, SCE1, and SUMO and tomato PCNA. Bands with an asterisk are identified as the result of Rep protein expression. Molecular weight markers (Mw) are indicated.

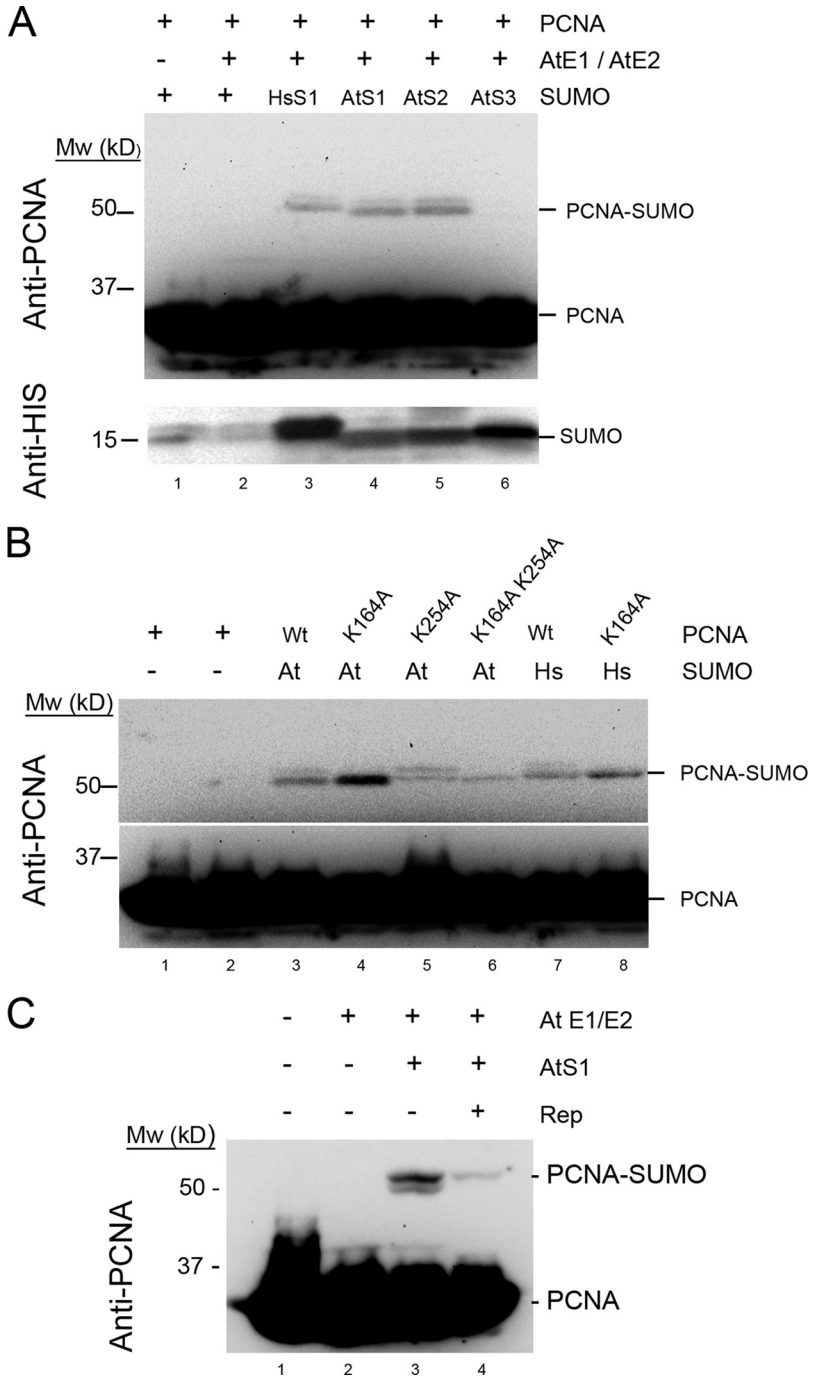

FIG 6 *Arabidopsis* SUMO conjugation enzymes modify the same lysine residues in tomato PCNA *in bacteria*. (A). Similar to the assay described in the legend of Fig. 1 except that AtE1/E2 (*Arabidopsis* SAE1, SAE2, and SCE1) was coexpressed together with the *Arabidopsis* SUMO paralogues SUMO1, -2, and -3 (AtS1/2/3). Expression (+/−) of the individual components is indicated. E1/E2 enzymes were expressed in all samples. (B) Similar to panel A except that PCNA variants in which K164, K254, or both residues were mutated to Ala were coexpressed. At, AtSUMO1; Hs, HsSUMO1 (lanes 4 to 6 and 8); Wt, wild-type tomato PCNA, as used in panel A. (C) Similar to panel A except that Rep from TGMV is coexpressed (lane 4). Molecular weight markers (Mw) are indicated.

due to the low levels of modified PCNA available, probably beyond the sensitivity threshold of the detection methods used. Increasing the amount of sumoylated PCNA might allow us to infer if Rep also modulates the posttranslational modification status of PCNA *in planta*. To this end, we transiently expressed Flag-tagged tomato PCNA

(PCNA-Flag) together with mature AtSUMO1 in young, not-fully-expanded *N. benthamiana* leaves (2-week-old plants), in which more cells are likely to be in the replicative stage of the cell cycle (previous studies showed that yeast PCNA is predominantly sumoylated during the S phase of the cell cycle [63]). In addition, we subjected the harvested leaf material to a heat shock at 37°C for 45 min to increase the levels of SUMO conjugates (31). Western blots on PCNA protein immunopurified with anti-Flag resin showed that PCNA accumulated in plants as monomers, dimers, and trimers (of approximately 35, 70, and 100 kDa, respectively) (Fig. 7A). These blots were also probed with anti-SUMO antibody, revealing that the PCNA-Flag monomers were also sumoylated *in planta*, giving rise to a band of approximately 50 kDa (Fig. 7A), equivalent to that previously detected *in bacteria* using the *Arabidopsis* system (Fig. 6). When Rep from the begomovirus *Tomato yellow leaf curl virus* (TYLCV) was coexpressed *in planta* under the same experimental conditions, the signal for the ~50-kDa band decreased, suggesting that the levels of SUMO-modified PCNA were lower when Rep was present (Fig. 7). The presence of Rep (green fluorescent protein [GFP] tagged) was confirmed by immunoblotting of total protein extracts with an anti-GFP antibody (Fig. 7). These results show for the first time that PCNA is sumoylated in plant cells and confirm that Rep also compromises PCNA sumoylation *in planta*.

DISCUSSION

PCNA is a protein highly conserved in eukaryotes that controls cell cycle regulation, DNA replication, and DNA repair. The switching between these different PCNA functions is modulated by its posttranslational modification status, mainly ubiquitination or sumoylation, which facilitates or hinders the interaction of PCNA with specific binding partners, providing a mechanism to control and switch PCNA functions (65, 66).

Sumoylation of the two PCNA homologues (PCNA1 and PCNA2) present in *Arabidopsis* has been described using a reconstituted sumoylation system in *E. coli* (53, 54). In the first report, sumoylation of PCNA1 was detected using *Arabidopsis* AtSUMO1 and AtSUMO3, while in the second, the authors showed efficient sumoylation of PCNA1 and PCNA2 using *Saccharomyces cerevisiae* SUMO (Smt3) as well as AtSUMO1, -2, -3, and -5. In this work, we show that tomato PCNA is also sumoylated *in vitro* by human SUMO1 and AtSUMO1 and -2 (Fig. 6). However, sumoylation of PCNA with AtSUMO3 was notably inefficient compared to that obtained with the other SUMO homologues, even though all SUMOs were expressed in bacteria to similar levels (Fig. 6). Taking into account that all the sumoylation enzymes used in both works were the same, this divergence must be due to the PCNA origin. The difference in sumoylation observed when using AtSUMO1/2 or AtSUMO3 is not a surprise, since *Arabidopsis* SUMO paralogues have acquired distinct expression patterns and biochemical properties (22, 41).

Sumoylation assays of tomato PCNA identified two modification products with similar molecular weights (Fig. 3B) that likely correspond to PCNA monomers monosumoylated at two alternative sites (K164 versus K254). Such a phenomenon was previously described for yeast PCNA, which migrated on SDS-PAGE gels with different apparent molecular masses when sumoylated at K127 or K124 (48, 63). In none of the experiments *in bacteria* using the *Arabidopsis* or mammalian sumoylation enzymes did we identify bands corresponding to tomato PCNA monomers simultaneously modified with two SUMO molecules (PCNA-2×SUMO), indicating that (i) the simultaneous modification of both lysine acceptor sites of one PCNA monomer or (ii) the formation of a SUMO chain (di-SUMO) on one acceptor lysine is more inefficient than previously suggested for *Arabidopsis* PCNA (53). Considering that tomato PCNA could be efficiently sumoylated at each of two lysine acceptor residues in an alternate fashion, the lack of PCNA molecules attached to two SUMO peptides simultaneously could suggest that some hierarchy is established in the sumoylation of the lysines. Whether inhibition of the consecutive modification of both lysines with SUMO was due to (i) the biochemical characteristics of the sumoylation systems when expressed *in bacteria* or (ii) the absence of additional components, such as SUMO E3 ligases, remains to be analyzed. Interestingly, previous data showed that, *in bacteria*, the expression of the *Arabidopsis*

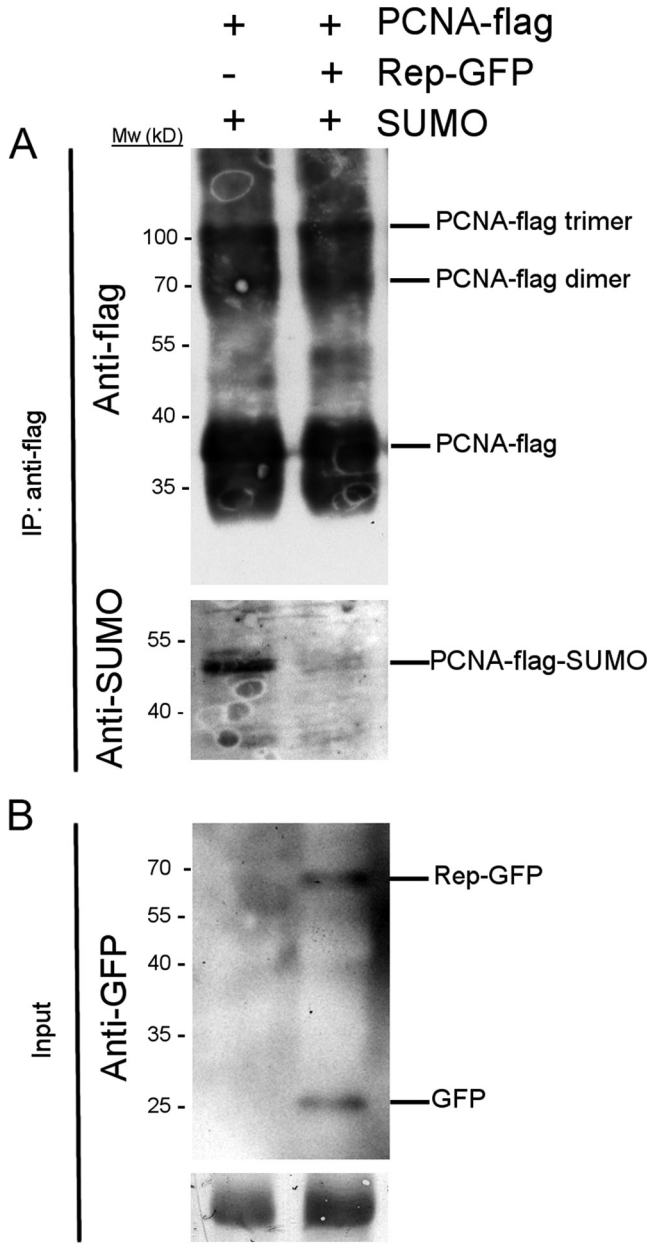

FIG 7 Rep from *Tomato yellow leaf curl virus* (TYLCV) compromises sumoylation of tomato PCNA *in planta*. (A) Flag-tagged tomato PCNA together with *Arabidopsis thaliana* SUMO1 were transiently expressed in *Nicotiana benthamiana* leaves in the presence or absence of GFP-tagged Rep from TYLCV. Total protein extracts were subjected to immunoprecipitation, and proteins that coeluted from the Flag affinity resin (IP:anti-Flag) were blotted with anti-Flag or anti-AtSUMO1 antibodies. The top panel shows the enrichment of PCNA-Flag as a monomer but also as a dimer and trimer (~70 and 100 kDa, respectively); the anti-SUMO immunoblot reveals that in the presence of Rep, the ~50-kDa band, corresponding to the sumoylated monomeric form of PCNA-Flag, is reduced when Rep is coexpressed. (B) Total protein extracts (input) were analyzed by immunoblotting using anti-GFP antibody to show the accumulation of Rep-GFP and by Coomassie staining to confirm equal protein loading. Molecular weight markers (Mw) are indicated.

SUMO E3 ligase AtSIZ1 does not impact the sumoylation of *Arabidopsis* or *S. cerevisiae* PCNA isoforms (53, 67).

PCNA sumoylation has not been detected in any of the analyses carried out *in planta* to identify plant SUMO targets (24, 32, 64, 68–71). Even the use of plant material containing a large proportion of dividing cells as a PCNA source, or transiently expressing PCNA in leaves, has failed to prove PCNA modification *in planta* (53). In this work,

we show for the first time that plant PCNA, as its animal and yeast homologues, is indeed sumoylated in plant cells. The determinant use of a heat shock to increase the accumulation of SUMO (31) allowed us to detect the sumoylation of a PCNA monomer when transiently expressing all proteins in *N. benthamiana* leaves. Interestingly, labeling of PCNA at the C terminus with a Flag epitope seemingly does not interfere with its ability to interact with itself to form homodimers or trimers.

The analysis of PCNA containing point mutations in lysine residues shows that, *in bacteria*, tomato PCNA is preferentially sumoylated at two residues that are conserved across all eukaryotic PCNAs: K164, a residue reported to be sumoylated in yeast and animals, and K254. Although both residues are at the surface of the PCNA ring, they are located at opposite sides of the PCNA ring: K164 is at the back side, while K254 is at the front side of this ring. The weak band detected in the assays with the double mutant K164A/K254A suggests that, in the absence of these two residues, another as-yet-unidentified lysine can be sumoylated. Whether or not this corresponds to a true third SUMO acceptor site or its modification is an artifact caused by the absence of the other two sites remains to be clarified.

Previous work with *Arabidopsis* PCNA identified K254 as one of the residues sumoylated *in bacteria* yet failed to detect sumoylation at K164 (53). Although that study used a reconstituted system consisting of *Arabidopsis* proteins similar to the one used here, there are some experimental differences that could explain this apparent discrepancy. Mainly, to identify SUMO acceptor sites, Strzalka and coworkers used *Arabidopsis* AtSUMO3, while we used AtSUMO1. Thus, it could be possible that K164 is sumoylated only by AtSUMO1, while K254 can be modified by AtSUMO1 and AtSUMO3, a unique SUMO paralogue that is present in only a small clade of the *Brassicaceae* (22). Moreover, it also remains unclear if maturation of pre-AtSUMO3 occurs *in planta* by SUMO proteases in order to expose the di-Gly motif needed for SUMO conjugation (67). This maturation step is skipped in the reconstituted sumoylation assays that we and Strzalka and coworkers used. In order to analyze sumoylation, Strzalka and coworkers generated *Arabidopsis* PCNA mutants by replacing all but one of the lysine residues by arginines, while in our approach, we replaced only those residues already proven to be sumoylated. Thus, we cannot rule out the possibility that with the method of Strzalka and coworkers, the overall structure of PCNA is changed, which would interfere with the access of the sumoylation enzymes to specific residues. In fact, their observation that five additional lysines (located in the inner circle of the PCNA ring) can serve as SUMO acceptor sites suggests that in the absence of the main acceptor sites, SCE1 will accept any available lysine as the substrate. Such potentially unbiased sumoylation was previously observed for FoxM1 (72). Furthermore, overexpression of the sumoylation machinery in the presence of a target with only one lysine residue could result in the generation of false positives, given that the stoichiometric conditions of such a reaction are bound to be far from physiological. However, we cannot fully exclude that *Arabidopsis* and tomato PCNA could be partially sumoylated at different residues.

Replication of the geminiviral genome fully relies on the host DNA replication machinery, including PCNA and DNA polymerases. PCNA is essential for viral replication (73), and expression of the corresponding gene is induced by the presence of Rep (74–76). Our results prove that Rep, besides binding to the PCNA protein, also interferes with its sumoylation in all modifiable lysine residues. This inhibitory effect of Rep was unique for PCNA, as it did not affect SUMO attachment to the plant protein used as a control (CAT3). The fact that PCNA sumoylation is also compromised when a Rep mutant that cannot interact with the SUMO-conjugating enzyme SCE1 is used suggests that reduced SUMO conjugation of PCNA is not due to Rep inhibiting SCE1 enzymatic activity, a mechanism previously described for the Gam1 protein of the chicken embryo lethal orphan virus (77). Our results point to the PCNA-binding domain in Rep as a determining factor for the suppression of PCNA sumoylation. The data reported by Bagewadi and colleagues (55), showing that Rep interacts with residues located all over

the PCNA molecule, could indicate that the reduction of PCNA sumoylation is a consequence of steric hindrance of SCE1 once Rep is bound to PCNA. This scenario would fit with the model described by Mayanagi and colleagues, where one PCNA interactor can block access of other interactors to the this PCNA molecule; this steric hindrance would thus fine-tune and modulate PCNA function (78–80). Specific mutants of PCNA in which the Rep-PCNA interaction is lost will be required to confirm this hypothesis.

Sumoylation of PCNA is high in particular during the S phase, where it would be involved in suppressing undesired recombination events between newly synthesized DNA molecules during normal fork progression. In yeast, PCNA sumoylation recruits the DNA helicase Srs2 that inhibits recombination by removing RAD51 from ssDNA, thereby disassembling an essential recombination intermediate structure (reviewed in reference 81). During replication, Srs2 aids in the repair of gaps by preventing ssDNA from being used to initiate recombination. Deletion of Srs2 or mutations of PCNA that impair its sumoylation cause increased levels of homologous recombination (HR) (63, 82–84). The mechanisms mediated by Srs2 seem to be conserved in eukaryotes. Sumoylation of PCNA in human cells recruits PARI, an Srs2 homologue that binds to RAD51 (52, 85). In *Arabidopsis*, an Srs2 homologue was identified and shown to act as a functional DNA helicase that can process branched DNA structures that occur in the synthesis-dependent strand annealing (SDSA) pathway of recombination (86). These properties suggest that AtSrs2 might play a role in regulating HR in plants, as predicted for its yeast homologue.

Recombination is a key evolutionary process to generate diversity among ssDNA viruses (87). Recombination among geminiviral genomes has been extensively recorded and seems to be a consequence of a general enhancement of the recombination frequency upon infection (88–93; reviewed in reference 94). Besides, several experimental results also indicate that geminiviruses alter HR in plants, as infection with the begomovirus *Euphorbia mosaic virus* induces somatic HR events for *Arabidopsis* transgenes, especially within vein-associated tissues where this virus replicates (95).

The mechanisms of HR in ssDNA viruses remain poorly defined but are most probably strongly influenced by the ways in which these viruses replicate. Geminiviruses replicate their circular ssDNA by three modes of action: complementary strand replication, rolling-circle replication, and recombination-dependent replication (RDR) (96, 97). It has been suggested that RDR is a replication system by which host recombination factors are utilized for geminiviral amplification and also lead to enhanced host DNA recombination (95). Rep is likely to have a key role in the recruitment and assembly of this viral replisome, a protein-DNA complex that includes both viral proteins and host factors, including those for HR (reviewed by in reference 3). Besides its interaction with PCNA, Rep interacts with a variety of proteins involved in replication and/or HR processes, such as RFC (98), RPA32 (99), Rad54 (100), or Rad51 (101). The relevance of this HR replication mechanism for geminiviruses has been highlighted by infecting an *Arabidopsis RAD51D* mutant with a bipartite geminivirus (102). The results obtained showed that RAD51D promotes viral replication at the early stages of infection, and its presence is required for geminiviral recombination, since in the absence of RAD51D, a significant decrease of both intra- and intermolecular recombinant molecules between the two DNA components of the bipartite geminivirus was observed. Expression of the Rep homologue from the geminivirus *Bean yellow dwarf virus* increased the frequency of gene targeting in a series of experiments to develop geminivirus-based replicons for transient expression of transcription activator-like effector nucleases (TALENs) (103). This result points to the involvement of the Rep protein in the viral control of the HR mechanism.

Combining all our data, we propose that the interaction between Rep and PCNA modulates the protein modification status of PCNA, thus switching its cellular function to create an environment suitable for viral replication. Furthermore, we suggest that the specific reduction of PCNA sumoylation, caused by the action of Rep, is a key step to induce HR in both infecting geminiviral genomes and the genomes of the infected

plant cells. We propose that during viral replication, Rep will interfere, possibly by its interaction with PCNA, with the ability of SCE1 to attach SUMO to PCNA. As a consequence, Srs2 binding to PCNA would be reduced, thus allowing maintenance of the Rad51-ssDNA nucleoprotein filaments generated from exposed ssDNA, which in turn will cause an increase in the level of HR recombination. Beside its effect on increasing geminiviral recombination, higher HR activity could also have an effect on the geminiviral replication efficiency, since HR provides a mechanism for tolerating lesions that could block the progression of replication forks.

MATERIALS AND METHODS

General methods. Manipulations of *Escherichia coli* strains and nucleic acids were performed according to standard methods (104). *E. coli* strain DH5α was used for subcloning. All the PCR-amplified fragments cloned in this work were fully sequenced. The *E. coli* NCM631 strain was used for the sumoylation assays.

Molecular graphics of tomato PCNA were made with the UCSF Chimera package (105) using human PCNA (Protein Data Bank accession no. 1AXC) as a reference. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS grant P41-GM103311).

***In bacteria* sumoylation assays.** PCNA from *Solanum lycopersicum* (obtained from pCNACT2 [56]) and Rep from TGMV (positions 2461 through 2588 to nucleotide 1416) [GenBank accession no. NC_001507], obtained from pDBRepTG [56]), each of them PCR amplified with primer pairs O01/O02 and O03/O04, respectively, were subcloned into NdeI/BamHI sites of pET28b (Novagen, EMD Millipore, Billerica, MA) to obtain the pET28-SlPCNA and pET28-Rep plasmids, respectively. An XbaI-blunt-ended/XhoI fragment from pET28-Rep containing the RBS (ribosomal binding site) and the Rep ORF (open reading frame) fused to a 6×His tag was subcloned into EcoRI-blunt-ended/XhoI sites of pET28-SlPCNA, generating pET28-SlPCNA-Rep, a vector able to produce the polycistronic mRNAs for the SlPCNA and Rep proteins.

PCNA mutants were obtained using the vector pET28-SlPCNA as the template and a QuikChange Lightning site-directed mutagenesis kit (Stratagene, Agilent, Santa Clara, CA), using primer pairs O05/O06, O07/O08, O11/O12, O13/O14, and O09/O10 for generating the mutations K164A, K254A, K91A, K190A, and K168A, respectively. For generating the double mutants K164A/K168A and K164A/K254A, pET28-SlPCNA-K164A was used as a template, and for generating the triple ones (K164A/K168A/K254A, K91A/K164A/K254A, and K164A/K190A/K254A), pET28-SlPCNA-K164A/K168A and pET28-SlPCNA-K164A/K254A were used as the templates.

To express *Arabidopsis* catalase 3 (AtCAT3) and Rep from TGMV from a polycistronic RNA, an XbaI-XhoI restriction fragment blunt ended from pET28-Rep, containing the RBS and the Rep ORF fused to a 6×His tag, was subcloned into the NotI-blunt-ended site of pGEX-AtCAT3 to yield pGEX-AtCAT3-Rep. pGEX-AtCAT3 expresses the catalase C-terminal fragment (AtCAT3Ct −419/472) fused to GST (glutathione *S*-transferase) and Flag (106). To express C2 and SlPCNA from a polycistronic mRNA, an XbaI-blunt-ended/XhoI restriction fragment of pET28-C2 containing the RBS and the C2 ORF fused to a 6×His tag was subcloned into the EcoRI-blunt-ended/XhoI site of pET28-SlPCNA, yielding pET28-SlPCNA-C2. Previously, pET28-C2 was generated by PCR amplification with primer pair O15/O16 and cloning of the C2 ORF of TYLCSV (position 1631 to nucleotide 1224 [GenBank accession no. L27708]) into EcoRI/XhoI sites of pET-28b.

To express Rep K68A/K102A, the Rep ORF was PCR amplified from pGBAL1-K68A/K102A (46) with primer pair O03/O04 and cloned into NdeI/BamHI sites of pET28b, yielding pET28-RepK68A/K102A. An XbaI-blunt-ended/XhoI restriction fragment from pET28-RepK68A/K102A, containing the RBS and the Rep ORF fused to a 6×His tag, was subcloned downstream of the PCNA ORF into EcoRI-blunt-ended/XhoI sites from pET28-SlPCNA to yield pET28-SlPCNA-RepK68A/K102A.

Truncation constructs of Rep were constructed by PCR, using pET28-Rep as a template and specific primers (O20/O27 for pET28-Rep$_{120-184}$, O18/O26 for pET28-Rep$_{56-130}$, O19/O28 for pET28-Rep$_{68-352}$, O20/O28 for pET28-Rep$_{120-352}$, and O21/O28 for pET28-Rep$_{184-352}$). PCR fragments containing the truncated Rep ORFs were cloned into NdeI/HindIII sites of pET28b to obtain pET28-Rep$_{120-184}$, pET28-Rep$_{56-130}$, pET28-Rep$_{68-352}$, pET28-Rep$_{120-352}$, and pET28-Rep$_{184-352}$. To express truncated Rep and SlPCNA from a polycistronic mRNA, PCR-amplified fragments of these plasmids (primers O17/O22 for Rep$_{1-68}$, O17/O25 for Rep$_{1-120}$, O17/O27 for Rep$_{1-184}$, O17/O23 for Rep$_{1-81}$, O17/O24 for Rep$_{1-99}$, O17/O28 for Rep$_{68-352}$, O17/O28 for Rep$_{120-352}$, O17/O28 for Rep$_{184-352}$, O17/O27 for Rep$_{120-184}$, and O17/O26 for Rep$_{56-130}$), containing the RBS and truncated Rep fused to a 6×His tag, were subcloned into SacI-HindIII sites of pET28-SlPCNA to yield pET28-SlPCNA-Rep$_{1-68}$, pET28-SlPCNA-Rep$_{1-120}$, pET28-SlPCNA-Rep$_{1-184}$, pET28-SlPCNA-Rep$_{1-81}$, pET28-SlPCNA-Rep$_{1-99}$, pET28-SlPCNA-Rep$_{68-352}$, pET28-SlPCNA-Rep$_{120-352}$, pET28-SlPCNA-Rep$_{184-352}$, pET28-SlPCNA-Rep$_{120-184}$, and pET28-SlPCNA-Rep$_{56-130}$.

The polycistronic constructs expressing *Arabidopsis* sumoylation E1 and E2 enzymes was generated as follows. AtSCE1, AtSAE1, and AtSAE2 were amplified from *Arabidopsis* Columbia-0 cDNA with primer pairs O29/O30, O31/O32, and O33/O34, respectively, and cloned into NcoI/BglII, NdeI/BamHI, or NcoI/SalI sites in pET28b in order to set them downstream of a RBS, yielding the plasmids pET28-AtSCE1, pET28-AtSAE1a, and pET28-AtSAE2. Next, an XbaI/EcoRI-blunt-ended restriction fragment of pET28-AtSCE1 was subcloned into the XbaI-blunt-ended site of pET28-AtSAE2 to obtain pETSS, and an XbaI/BamHI-blunt-ended restriction fragment of pET28-AtSAE1a was then cloned into the SalI-blunt-ended site of pETSS to obtain pETSS1a. Finally, to transfer the polycistronic construct to a vector with

P15A *ori* and chloramphenicol resistance, compatible with the other plasmids used in the sumoylation assays in *E. coli*, an SphI/EagI-blunt-ended fragment of pETSS1a was subcloned into a EcoRV site of pACYC184 (107) to yield pASS1a.

The coding DNA sequences (CDSs) corresponding to the mature proteins (GG) of *Arabidopsis* AtSUMO1, -2, and -3 were PCR amplified from *Arabidopsis* cDNA (using primer pairs O35/O36, O37/O38, and O39/O40, respectively) and cloned into the NdeI/BamHI sites of pET28b to obtain pET28-AtSUMO1, pET28-AtSUMO2, and pET28-AtSUMO3, respectively. XbaI-blunt-ended/BamHI restriction fragments of these constructs, containing ORFs of AtSUMOs fused to histidine tags, were subcloned into SphI-blunt-ended/BamHI sites of pRHSUMO (57) to substitute the human SUMO1 (*Hs*SUMO1) ORF, obtaining pRHAtSUMO1, pRHAtSUMO2, and pRHAtSUMO3.

Sumoylation assays with the mammalian enzymes were performed as previously described (57). Plasmids expressing the potential sumoylation target proteins, the sumoylation enzymes, and human SUMO1 were sequentially transformed into *E. coli*. Expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture medium in the exponential growth phase (optical density at 600 nm [OD_{600}] of 0.6). Samples were taken 4 h after induction, and proteins were extracted as described previously (57). The amount of protein loaded in all gels was normalized according to Coomassie blue staining. The mammalian and *Arabidopsis* sumoylation E1 and E2 enzymes are encoded by the plasmids pBADE12 and pASS1a, respectively. Human SUMO1 and *Arabidopsis* SUMO1, -2, and -3 are fused to His tags and expressed from plasmids pRHSUMO, pRHAtSUMO1, pRHAtSUMO2, and pRHAtSUMO3. In the sumoylation assays with pGEX-AtCAT3, human SUMO1 is expressed from pRKSUMO (57) instead of pRHSUMO.

***In planta* sumoylation assay.** For the *in planta* sumoylation assay, the ORF of *S. lycopersicum* PCNA (GenBank accession no. NM_001247915) (kindly provided by Keygene NV, Wageningen, The Netherlands) was amplified with primer pair FP5751/FP6068 to generate a fragment containing SlPCNA fused to a Flag tag at its N terminus. The fragment was subcloned into the pJL-TRBO vector (108) to generate the pTRBO-PCNA-Flag plasmid. The pK7FWG2 plasmid (109) containing Rep from TYLCV fused to enhanced GFP (EGFP) (Rep GenBank accession no. FJ956702.1) was also kindly provided by Keygene NV (referred to as pK7FWG2-Rep). The ORF of mature *Arabidopsis* AtSUMO1 (residues 1 to 91) in pDONR221 (110) was introduced into the pGWB402 destination vector (111) using a Gateway LR Clonase II reaction (Thermo Fisher) to generate the pGWB402-SUMO1 vector.

The binary constructs pTRBO-PCNA-Flag, pK7FWG2-Rep, and pGWB402-SUMO1 were introduced into *Agrobacterium tumefaciens* strain GV3101 (112) by electroporation. Single colonies were grown overnight until an OD_{600} of 0.8 of 1.5 was reached in low-salt LB medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 0.25% [wt/vol] NaCl [pH 7.0]) supplemented with 20 μM acetosyringone and 10 mM morpholineethanesulfonic acid (MES) (pH 5.6). Cells were collected by centrifugation and resuspended in infiltration medium (1× MS [Murashige and Skoog] salts [Duchefa], 10 mM MES [pH 5.6], 2% [wt/vol] sucrose, 200 μM acetosyringone). The *A. tumefaciens* cultures were mixed at a ratio of 1:1:1 and coinfiltrated (in the sample without Rep, the pK7FWG2-Rep culture was replaced with a culture harboring the empty pGWB451 vector) in leaves of a 2-week-old *N. benthamiana* plant at a final OD_{600} of 1. In addition, an *A. tumefaciens* strain carrying pBIN61 with the P19 silencing suppressor from *Tomato bushy stunt virus* was added to every infiltration mixture at an OD_{600} of 0.5 at a 2:1 ratio. Three days after infiltration, the whole infiltrated leaves of *N. benthamiana* were harvested, placed into a petri dish on wet paper, and heat shocked for 45 min while floating in a water bath set at 37°C in the dark. After this period, the leaf tissue was snap-frozen in liquid nitrogen and stored until protein extraction.

Plant proteins were extracted as described previously (113). For coimmunoprecipitations, 500 μl of the input was incubated with 30 μl anti-Flag M2 affinity gel resin (Sigma-Aldrich) (50% slurry) at 4°C for 3 h. Subsequently, the resin was collected by centrifugation (5,000 × *g*), washed 3 times with 0.5 ml washing buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10% [vol/vol] glycerol, 10 mM EDTA, 0.15% [vol/vol] NP-40, and 1 tablet of a protease inhibitor cocktail [Roche]/50 ml buffer), and incubated at 4°C for 1 h with 100 μl elution buffer (washing buffer plus 3× Flag peptide [catalogue no. F4799; Sigma-Aldrich] at 150 ng/μl). After incubation, the resin was transferred to Bio-Spin columns (Bio-Rad) and spun down at 1,000 × *g* for 1 min. The eluate (immunoprecipitated proteins [referred to as IP:anti-Flag]) was analyzed by Western blotting.

Antibodies and primers used in this work. Antibodies used in this work were anti-rat PCNA (48), anti-His tag (catalogue no. AB-3237; Biomedal), anti-NbSUMO1 and anti-Rep (AL1) (46), anti-Flag (catalogue no. F7425; Sigma-Aldrich) (114), anti-AtSUMO1/2 (catalogue no. ab5316; Abcam), anti-AtSUMO1/2 (University of Amsterdam), and anti-GFP (catalogue no. 3H9; Chromotek) (115).

The primers used are as follows (nucleotides that introduce mutations are shown in lowercase type): O01 (CCCATATGTTGGAACTACGTCTTGTTCAG), O02 (AAGGATCCTCAAGGCTTGGTTTC), O03 (CCCATATGC CATCGCATCC), O04 (AAGGATCCTTAGCTGCTCTGTGTTGA), O05 (GATACAGTTGTTATTTCGGTGACTgcgGAA GGTGTG), O06 (CACACCTTCcgcAGTCACCGAAATAACAACTGTATC), O07 (CCTGGCACCTgcaATAGAAGAGG ATG), O08 (CCTCTTCTATtgcAGGTGCCAGG), O09 (ACTgcgGAAGGTGTGgcaTTCTCAACCAGAGGT), O10 (AC CTCTGGTTGAGAAtgcCACACCTTCcgcAGT), O11 (CATCATCACCATCcgGGCTGACGATGGCAG), O12 (CTGCC ATCGTCAGCCcgGATGGTGATGATG), O13 (CAACTGTTGACgcGCCTGAAGAAGCC), O14 (GGCTTCTTCAGGCg cGTCAACAGTTG), O15 (GGGCCGAATTCATGCAATCTTCGTCACC), O16 (ACCTCGAGCCTAAAGACTCTTAAAA AATG), O17 (AAGAGCTCAGAAGAGATATACC), O18 (AACATATGCAACCTCACCTCC), O19 (AACATATGAAA TACTGCTGCC), O20 (AACATATGGACGGTCGAAGTGC), O21 (AACATATGCCGTTCCACGTCTC), O22 (AAAAG CTTTTATTTTCCCTCGAACTG), O23 (AAAAGCTTTTAGGATACCAGGTCG), O24 (AAAAGCTTTTAGGAAGACGA TTTAGC), O25 (AAAAGCTTTTAGTCGACCTGGAATTC), O26 (AAAAGCTTTTATGTTTGGCAACCTCC), O27 (AA AAGCTTTTACGGAGGAAGCCATGG), O28 (AAAAGCTTTTAGCTGCTCTGTG), O29 (CCATGGCTAGTGGAATCG

CTCG), O30 (AGAGATCTTTAGACAAGAGCAGG), O31 (CATATGGACGGAGAAGAGC), O32 (GGATCCTTAAGA GGTAAAAGAGTCGG), O33 (AACCATGGCTACGCAACAACAG), O34 (GGGTCGACCTATTCAACTCTTAT), O35 (AACATATGTCTGCAAACCAGG), O36 (AAGGATCCTCAGCCACCAGTCTGATG), O37 (AACATATGTCTGCTACT CCGG), O38 (AGGGATCCCTAACCACCAGTCTGAT G), O39 (AACATATGTCTAACCCTCAAG), O40 (AGGGATC CTTAACCACCACTCATCGC), FP5751 (AAAATTAATTAAATGGACTACAAGGACGACGATGACAAAGTCAAGCTT CTCGAGAATTCCTTGGAACTACGTCTTGTTCA), and FP6068 (AAAAGCGGCCGCCTAAAGGCTTGGTTTCCTCTT CATCC).

ACKNOWLEDGMENTS

We thank María Lois (Center for Research in Agricultural Genomics [CRAG], CSIC-IRTA-UAB, Barcelona, Spain) for providing plasmid pGEX-AtCAT3. We thank T. Nagakawa (Shimane University, Japan) for sharing plasmids.

This research was supported by a grant from the Spanish Ministerio de Ciencia y Tecnología (AGL2016-75819-C2-1-R). M.A.S.-D. was awarded a predoctoral fellowship from the Junta de Andalucía and an EMBO short-term fellowship (ASTF no. 240-05). B.S. was awarded a predoctoral fellowship from the Spanish Ministerio de Educación y Cultura. The Topsector T&U program Better Plants for Demands (grant 1409-036 to H.A.V.D.B.), including the partnering breeding companies, supported this work. F.M. is financially supported by Keygene NV (The Netherlands).

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