Turnip mosaic virus is a second example of a virus using transmission activation for plant-to-plant propagation by aphids

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Abstract

Cauliflower mosaic virus (CaMV, family *Caulimoviridae*) responds to the presence of aphid vectors on infected plants by forming specific transmission morphs. This phenomenon, coined transmission activation (TA), controls plant-to-plant propagation of CaMV. A fundamental question is whether other viruses rely on TA. Here, we demonstrate that transmission of the unrelated Turnip mosaic virus (TuMV, family *Potyviridae*) is activated by the reactive oxygen species H_2O_2 and inhibited by the calcium channel blocker $LaCl_3$. H_2O_2-triggered TA manifested itself by the induction of intermolecular cysteine bonds between viral HC-Pro molecules and by formation of viral transmission complexes, composed of TuMV particles and HC-Pro that mediates vector-binding. Consistently, $LaCl_3$ inhibited intermolecular HC-Pro cysteine bonds and HC-Pro interaction with viral particles. These results show that TuMV is a second virus using TA for transmission, but using an entirely different mechanism than CaMV. We propose that TuMV TA requires ROS and calcium signaling and that it is operated by a redox switch.

Importance

Transmission activation, i.e. a viral response to the presence of vectors on infected hosts that regulates virus acquisition and thus transmission, is an only recently described phenomenon. It implies that viruses contribute actively to their transmission, something that has been shown before for many other pathogens but not for viruses. However, transmission activation has been described so far for only one virus, and it was unknown whether other viruses rely also on transmission activation. Here we present evidence that a second virus uses transmission activation, suggesting that it is a general transmission strategy.

Key words

Plant virus; aphid vector; host plant; virus transmission; virus vector host interactions; reactive oxygen species; calcium; signaling

Abbreviations

CaMV, cauliflower mosaic virus; TuMV, turnip mosaic virus; TA, transmission activation; ROS, reactive oxygen species; TB, transmission body; HC, helper component; HC-Pro, helper component protease; CP, capsid protein

Introduction

Transmission is an obligatory step in the life cycle of parasites but it is also an Achilles's heel, because parasites must leave the comparably comfortable environment of the host they are installed in, and face a potentially adverse environment during the passage to a new host. Some pathogens rely on resistant dormant states like spores to persist in the "wild" until they reach a new host passively, e.g. carried by the wind. Most pathogens, however, actively use vectors for transmission and they can manipulate both hosts and vectors in an impressive number of ways, all potentially increasing transmission (1–4). In the most sophisticated cases, pathogens "*use exquisitely controlled mechanisms of environmental sensing and developmental regulation to ensure their transmission*" (5). This concept, implying active contribution of the pathogen, is widely accepted for eukaryotic parasites (for example plasmodium, shistosoma, wucheraria, dicrocoelium), which developed fascinating transmission cycles to control and adapt vector-host or primary-secondary host interactions for their propagation (4). We have recently discovered a remarkable phenomenon for a virus. Cauliflower mosaic virus (CaMV, family *Caulimoviridae*) responds to the presence of aphid vectors on infected host plants by forming transmission morphs at the exact time and location of the plant-aphid contact (6). This process, coined Transmission Activation or TA (7), is characterized by the formation of transmission complexes between CaMV virus particles and the transmission helper component (HC), the CaMV protein P2, which mediates vector-binding (1). P2 and virus particles are spatially separated in infected cells since the cell's pool of P2 is retained in specific cytoplasmic inclusions called transmission bodies (TBs) while most virus particles are contained in another type of viral inclusion, the virus factories (8, 9). In such cells, aphid punctures trigger instant disruption of TBs and the liberated P2 relocalizes onto microtubules. Simultaneously, the virus factories release virus particles that associate with P2 on the microtubules (10) to form P2/virus particle complexes, which is the virus form that aphid vectors can acquire and transmit. TA is transient; P2 reforms a new TB (6) and the virus particles return to virus factories (10) after aphid departure. TA implies that CaMV passes, induced by yet unknown mechanisms, from a non-transmissible to a transmissible state. It has been suggested that this phenomenon exists to economize host resources and to invest energy in transmission only when relevant, i.e. in the presence of vectors (7). Whether this hypothesis is true or not, inhibiting TA inhibits transmission, pointing to the importance of TA for CaMV. A fundamental question that arises is whether TA, which is reminiscent of the active transmission strategies employed by eukaryotic parasites, is exclusive to CaMV or whether it could be a general phenomenon in the virus world. Therefore, we studied transmission of the turnip mosaic virus (TuMV, family *Potyviridae*), which is entirely unrelated to CaMV, but uses also an HC for aphid

transmission. The HC of TuMV and of other potyviruses is the viral protein helper component protease (HC-Pro). It is a multifunctional protein that other its HC function bears no structural, functional or other similarity with P2. Our results show that TuMV is a second virus relying on TA for transmission, but using a totally different mechanism.

Results and discussion

Signaling molecules modify TuMV transmission by aphids

TA requires a signaling cascade that connects the initial recognition of the presence of aphids, most likely via a yet unknown elicitor, with a cellular response that is hijacked by the virus. Since TA is fast for CaMV and likely also for TuMV, which uses the same transmission mode, reactive oxygen species (ROS) or calcium are good signaling candidates. We therefore tested the effect of the ROS signaling compound hydrogen peroxide (H_2O_2), and of a general inhibitor of calcium signaling, lanthanum(III)chloride ($LaCl_3$), on TuMV transmission, using infected protoplasts as virus source (6, 11). Aphid transmission tests performed with H_2O_2-treated protoplasts showed a drastic increase of TuMV transmission (Figure 1A) whereas treatment of protoplasts with $LaCl_3$ caused a strong reduction of transmission (Figure 1B). This effect was not due to modified cell viability (Figure 1C,D). Furthermore, transmission increase by H_2O_2 and inhibition by $LaCl_3$ was clearly a biological effect requiring living cells, since no effect was observed when the experiments were repeated using cell extracts, i.e. dead cells (Figure 1E,F). The same control experiments indicate also that H_2O_2 and $LaCl_3$ did not modify aphid feeding behavior, which might have been an alternative explanation for the observed differences in transmission rates. Taken together, our data show that TuMV transmission can be artificially enhanced or inhibited.

The protoplast system is a useful but simplified biological system because the cells are individualized and not in their natural symplasmic context in a tissue. Hence, we sought to validate the protoplast results by using leaves on intact infected plants as virus source. We applied H_2O_2 or $LaCl_3$ to leaves by spraying treatment (12) and used these plants for aphid transmission assays. To rule out any interference, only one leaf of the same developmental stage was sprayed on each plant and different plants were used for each condition. H_2O_2 treatment increased significantly and $LaCl_3$ treatment decreased significantly plant-to-plant transmission rates of TuMV (Figure 1G,H). This confirmed the results obtained with protoplasts and showed that TuMV TA is observed similarly in intact plants. Compared to the protoplast experiments, higher $H_2O_2$and $LaCl_3$ concentrations were required to observe significant effects. This was probably due to dilution of these substances during leaf penetration. Combined, these results suggest that

the TA phenomenon exists for TuMV, like for CaMV, and that calcium and ROS signaling might be important for TA of TuMV.

The increase in virus transmission correlates with the formation of HC-Pro/TuMV transmissible complexes

Next, we wanted to know how TuMV TA manifests itself in infected cells. TA of CaMV is characterized by relocalization of CaMV particles and CaMV helper protein P2 from viral inclusions to microtubules (6, 10). Therefore, we performed immunofluorescence experiments on TuMV-infected protoplasts with antibodies directed against HC-Pro and the viral capsid protein CP to determine whether H_2O_2 and $LaCl_3$ induced relocalization of TuMV virus particles and/or HC-Pro. In untreated cells, HC-Pro and CP localized in the cytoplasm as reported for other potyviruses (13, 14). Treatment with H_2O_2 and $LaCl_3$ did not induce any visible rearrangement of HC-Pro or of CP (Figure 2). Thus, TA of TuMV is not characterized by the redistribution of HC-Pro and/or virus particles within infected cells.

We thus hypothesized that HC-Pro and virus particles, both evenly distributed in the cytoplasm, could pass from a non-associated state to an associated state, i.e. to transmissible HC-Pro-virion complexes, upon TA. To visualize such complexes *in situ*, we resorted to the Duolink® technique (15), an antibody-based version of the proximity ligation assay allowing detection of intermolecular interactions. Duolink® performed with HC-Pro and CP antibodies showed that H_2O_2 treatment indeed increased the number and intensity of HC-Pro/CP interaction spots (Figure 3A,B), indicative of binding of HC-Pro to virus particles. Interestingly, incubation of protoplasts with $LaCl_3$ decreased the number of transmissible complexes (Figure 3C). Thus, the increase and decrease of HC-Pro/CP interactions, triggered by application of ROS or of a calcium channel blocker, respectively, correlated with an increase and decrease of transmission (compare Figures 1 and 3).

Transmission activation of TuMV is characterized by formation of cysteine bridges between HC-Pro molecules

We wanted to understand how HC-Pro and virus particles could rapidly transit from "free" to virus-associated forms. Since ROS like H_2O_2 change directly or indirectly the cellular redox potential, the formation of HC-Pro/TuMV transmissible complexes might be controlled by the redox state of HC-Pro and CP, both of which contain cysteine residues that can form disulfide bridges under oxidizing conditions. Therefore, we performed non-reducing SDS-PAGE/Western blots to detect HC-Pro and CP migration profiles altered by intramolecular or intermolecular cysteine disulfide bridges. H_2O_2 and $LaCl_3$ did not modify the migration profile of CP (Figure 4A). However, H_2O_2 treatment increased the amount of oligomeric HC-Pro and especially of its

dimeric form (Figure 4B) that was previously reported to be active in transmission (16–18). $LaCl_3$ treatment had the inverse effect and decreased the amount of HC-Pro oligomers (Figure 4B). The effect of H_2O_2 was concentration-dependent and clearly visible using physiologic H_2O_2 concentrations (0.25 mM, Figure 4C). Thus, the increase in transmission induced by H_2O_2 correlated not solely with formation of HC-Pro/TuMV complexes, but also with the appearance of HC-Pro oligomers hold together by intermolecular cysteine bridges.

To have a biological significance, HC-Pro oligomerization should be completed within the duration of an aphid puncture, i.e. within seconds. Kinetics of formation and breakup of HC-Pro oligomers showed that both occurred within 5 seconds of incubation with H_2O_2 and $LaCl_3$, respectively (Figure 4D,E). The effect of both treatments was transient because HC-Pro oligomers disappeared (H_2O_2) or reappeared ($LaCl_3$) after ~30 min incubation. Furthermore, removal of H_2O_2 by washing protoplasts showed reversibility of HC-Pro oligomerization (Figure 4D). Induction of HC-Pro oligomers by H_2O_2 was not restricted to TuMV or to turnip hosts, because experiments with lettuce protoplasts infected with another potyvirus, *Lettuce mosaic virus*, yielded similar results (not shown).

To better establish that formation of disulfide bridges between HC-Pro monomers contributes to oligomerization, infected protoplasts were treated with the disulfide bonds-reducing agent dithiotreitol (DTT) or with N-ethylmaleimide (NEM) that does not break existing disulfide bridges but prevents formation of new ones by blocking free thiols. Figure 4F shows that DTT treatment abolished appearance of H_2O_2-induced HC-Pro oligomers in SDS-PAGE/Western blot. This confirmed that oligomerization of HC-Pro requires establishment of intermolecular disulfide bridges. NEM treatment blocked the appearance of HC-Pro oligomers in SDS-PAGE/Western blots when applied before the H_2O_2 treatment, but NEM did not prevent their appearance when applied after H_2O_2 treatment (Figure 4G). This is a further confirmation of the involvement of disulfide bridges in HC-Pro oligomerization. Note that NEM treatment caused a mobility shift of HC-Pro. This might have been due to disulfide shuffling during denaturation of the samples as reported for papilloma virus (19). To establish a direct role of intermolecular HC-Pro disulfide bonds in TuMV transmission, we performed transmission assays. Because of the toxicity of NEM, we did not use plants as virus source but resorted to the protoplast system where exposure of aphids (and the experimenter) to the substance is minimized by confining it in the protoplast medium. The NEM treatment reduced virus transmission drastically (Figure 4H) but did not affect protoplast viability (Figure 4I), suggesting that *de novo* formation of

intermolecular HC-Pro disulfide bonds is required for formation of transmissible complexes and thus for aphid acquisition of TuMV.

Model of TuMV transmission activation

In this study, we demonstrate that TA exists for a second virus, TuMV. TuMV TA was induced by the ROS H_2O_2 and inhibited by the calcium channel blocker $LaCl_3$. ROS and calcium signaling are both important in early perception of parasites including insects (20) and recently aphid punctures were described to induce rapid calcium elevations around feeding sites (21). Since ROS and calcium signaling are often interconnected (22, 23), TuMV TA likely hijacks an early step of at least one of these pathways. The initial eliciting event remains unknown. It might be a direct effect of aphid saliva-contained ROS or ROS-producing peroxidases (24) that are injected into cells during feeding activity. Alternatively, an aphid or aphid-induced plant factor might interact in a classic pathogen-associated molecular pattern (PAMP)-triggered immunity reaction with a pattern recognition receptor (PRR) (25) that prompts calcium and ROS mediated downstream events. Interestingly, a recent study has demonstrated that the red clover necrotic mosaic virus (RCNMV) requires ROS for replication (26). The authors proposed that plant viruses may have evolved a complex mechanism to manipulate the ROS-generating machinery of plants to improve their infectivity, or, transferred to this case, transmission.

TA of TuMV manifests itself by creation of HC-Pro intermolecular disulfide bridges, driven by oxidation of the cellular redox potential. We propose that oxidation of HC-Pro induces a functional switch rendering HC-Pro able to interact with virus particles and form transmissible complexes (Figure 5). Functional switching (moonlighting) by redox-driven modification of disulfide bridges has been reported for other proteins and is operated by conformation changes affecting the secondary, tertiary or quaternary structure of proteins (27–29). Why would there be such a switch? HC-Pro is a multifunctional protein involved not only in aphid transmission (30) but also in pathogenicity (31), viral movement (32) and suppression of plant RNA silencing (33–35). One (or more) functional switches could assist to coordinate these multiple functions by allowing interaction with virions and formation of transmissible complexes only when transmission is possible, i.e. when the aphids puncture cells This would help to economize finite plant resources as proposed earlier (7).

Unfortunately, we cannot provide an empirical proof that the aphid punctures directly trigger TuMV TA. In contrast to CaMV, where TA was directly visible using qualitative immunofluorescence observation of P2 and virus particle networks (the characteristic

manifestation of CaMV TA) in cells in contact with aphid saliva sheaths, TuMV TA cannot be revealed by a qualitative analysis. The quantitative Duolink® approach we used to demonstrate TuMV HC-Pro/CP interactions in protoplasts required an enormous number of cells for analysis and statistical validation. Identifying a comparable number of cells in tissue and in contact with aphid stylets is barely feasible. The same restrictions apply to electron microscopy techniques to localize HC-Pro on virus particles by immunogold labeling. Thus, proof of aphid implication in TA of TuMV remains indirect, for the time being.

Nonetheless, we here demonstrate TA for a second virus, TuMV, different from CaMV, suggesting that transmission activation might be a more general phenomenon. The great phylogenetic distance between TuMV and CaMV makes it likely that the phenomenon of TA arose independently for the two viruses during evolution. An obvious question is whether yet other viruses use TA for their transmission.

Materials and methods

Plants, viruses and inoculation

Turnip plants (*Brassica rapa* cv. Just Right) and lettuce (*Lactuca sativa* cv. Mantilla and Trocadero) were grown in a greenhouse at 24/15 °C day/night with a 14/10 h day/night photoperiod. Two-weeks-old turnip plants were mechanically inoculated with wild-type TuMV strain C42J (36), and two-weeks-old lettuce plants with *Lettuce mosaic virus* (LMV) strain E (37). Plants were used for experiments at 14 days post inoculation (dpi).

Isolation of protoplasts

Protoplasts from turnip leaves were obtained by enzymatic digestion as described (6).

Preparation of infected cell extracts

TuMV-infected turnip protoplasts were sedimented and resuspended in SAKO buffer (500 mM KPO_4 and 10 mM $MgCl_2$ pH 8.5) (38). Then sucrose was added to a final concentration of 15 % and the suspension was vortexed to homogenize protoplasts.

Drug treatments and cell viability assay

For drug treatments of protoplasts, the following substances were added from stock solutions for the indicated times to 500 µl of protoplast suspension: 1 mM $LaCl_3$ (5 min), 2 mM H_2O_2 (5 min),

232 3 mM NEM (20 min), 5 mM DTT (30 min). Protoplasts were incubated at room temperature
233 with gentle stirring (5 rpm). 15 min after treatments, protoplast viability was determined with the
234 FDA test (39). For drug treatments of plants, one leaf per plant was sprayed with 10 mM $LaCl_3$,
235 20 mM H_2O_2 or with water, and the leaf, still attached to the plant, used for transmission
236 experiments after the applied solutions had evaporated.

237 *Aphid transmission tests*

238 A nonviruliferous clonal *Myzus persicae* colony was reared under controlled conditions (22/18 °C
239 day/night with a photoperiod of 14/10 h day/night) on eggplant. The transmission tests using
240 protoplasts were performed as described (6), with an acquisition access period of 15 min and
241 transferring 10 aphids to each test plant. For plant-to-plant transmission tests, an acquisition time
242 of 2 min was used and only one aphid was transferred on each turnip plant for inoculation.
243 Infected plants were identified by visual inspection for symptoms 3 weeks after inoculation.

244 *Antisera*

245 The following primary antibodies were used: commercial rabbit anti-TuMV (sediag.com) and
246 mouse and rabbit anti-HC-Pro (recognizing HC-Pro from different potyviruses, produced against
247 the conserved peptide SEIKMPTKHHLVIGNSGDPKYIDLP by proteogenix.fr and
248 eurogentec.com, respectively). The following secondary antibodies were used: Alexa Fluor 488
249 and 594 anti-rabbit and anti-mouse conjugates (thermofisher.com) for immunofluorescence, anti-
250 rabbit IgG conjugated to alkaline phosphatase (www.sigmaaldrich.com) for western blotting and
251 corresponding Minus and Plus probes (www.sigmaaldrich.com) for Duolink®.

252 *Immunofluorescence*

253 Protoplasts were fixed with 1 % glutaraldehyde and processed as described (6). The primary and
254 secondary antibodies were used at 1:100 and 1:200 dilutions, respectively.

255 *Western blotting*

256 Drug treatments of protoplasts were stopped by lysing protoplasts in non-reducing 2x Laemmli
257 buffer (v/v) (40) except where indicated otherwise. Optionally, oligomer formation was stabilized
258 by incubating protoplasts with 3 mM NEM for 20 min before lysis. This step yielded sharper
259 oligomer bands. Samples were then resolved by 10 % SDS-PAGE. Proteins were transferred to
260 nitrocellulose membranes and incubated with primary and secondary antibodies as described (6)

except that TuMV-specific primary antibodies (1:1000 dilution) were used. Antigens were then revealed by the NBT/BCIP reaction. Equal protein charge on the membranes was verified by coloring the RuBisCO with Ponceau S Red.

Duolink® proximity ligation assay

In situ protein/protein interactions were detected by proximity ligation assay using the Duolink® kit (www.sigmaaldrich.com). Protoplasts were isolated from healthy or infected (14 dpi) turnip leaves and fixed with 3 % paraformaldehyde in 100 mM cacodylate buffer (pH 7.2) or 100 mM phosphate buffer (pH 7.4). The fixed protoplasts were immobilized on L-polylysine-coated slides. Antibody incubation with rabbit anti-TuMV and mouse anti-HC-Pro, ligation and probe amplification were performed according to the manufacturer's instructions. The slides were mounted with Duolink® *in situ* mounting medium with DAPI (www.sigmaaldrich.com).

Microscopy

Immunolabeled protoplasts were observed with an Olympus BX60 epifluorescence microscope (olympus-lifescience.com) equipped with GFP and Texas Red narrow band filters and images acquired with a color camera. Duolink® images were acquired with a Zeiss LSM700 confocal microscope (zeiss.com) operated in sequential mode. DAPI was exited with the 405 nm laser and fluorescence collected from 405-500 nm, Duolink® probes and chlorophyll were excited with the 488 nm laser and fluorescence collected from 490-540 nm (Duolink® signal)or from 560-735 nm (chlorophyll autofluorescence). Raw images were processed using ZEN or ImageJ software. Quantification of Duolink® interactions was performed on maximum intensity projections with the Analyse_Spots_Per_Protoplast macro for ImageJ, developed for this experiment (41).

Statistical analysis

Statistics and box plots were calculated with R software version 3.4.0 (r-project.org). Transmission rates and cell viability were analyzed with generalized linear models (GLM). Quasi-binomial distributions were used in order to take overdispersion into account, and p-values were corrected with the Holm method (42) to account for multiple comparisons.

Analyzing the Duolink® experiments required the calculation of the total fluorescence intensity (F_{tot}) of labeled foci as:

$$F_{tot} = \frac{n \times \bar{s} \times \bar{I}}{A}$$

where n is the number of labeled foci, $\bar{s}$ the average size of a focus, $\bar{I}$ the average fluorescence intensity of a focus and A the size of the protoplast. F_{tot} was log-transformed (to normalize the distribution) and analyzed with linear models using "treatment" and "replicate" as categorical explanatory variables.

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Legends to Figures

Figure 1. Effect of H_2O_2 and $LaCl_3$ on TuMV transmission by aphids. (A-B) Turnip protoplasts were incubated for 5 min with 2 mM H_2O_2 (A) or 1 mM $LaCl_3$ (B) and then employed in transmission assays. (C-D) Cell viability of protplasts was measured to determine if the altered transmission rates were due to modified viability. (E-F) Cell extracts from protoplasts were treated identically with H_2O_2 (C) or $LaCl_3$ (D) and used in transmission assays. (G-H) Leaves on intact plants were sprayed with 20 mM H_2O_2 (E) or 10 mM $LaCl_3$ (F) and then employed in transmission assays. Means of infected test plants (horizontal black bars in the box plots) are calculated from a pool of three independent experiments in which a total of 360 tests plants were used per condition. Each experiment had 6 repetitions for each condition and 20 tests plants per repetition (see Supplementary Data Set S1 for raw data). *p* designates p-values obtained by generalized linear models (see materials and methods). The box plots here and in the other figures present medians, upper and lower quartiles, the ends of the whiskers present lowest and highest datum still within 1.5 IQR of the lower and higher quartile, respectively, and the circles show outliers.

Figure 2. Immunofluorescence of turnip protoplasts infected with TuMV. TuMV-infected protoplasts were treated as indicated and double-labeled against HC-Pro (green, first column) and viral capsid protein CP (red, middle column). The right column (Merge) represents superposition of HC-Pro and CP labels, with co-labeling appearing in yellow. Control, untreated protoplasts; H_2O_2, incubation with 2 mM H_2O_2 for 15 min, $LaCl_3$, incubation with 1 mM $LaCl_3$ for 15 min. Scale bars 50 µm.

Figure 3. *In situ* Duolink® proximity ligation assay on turnip protoplasts infected with TuMV. (A) Untreated control protoplasts or protoplasts incubated with either H_2O_2 or $LaCl_3$ were processed by Duolink® for detection of HC-Pro/TuMV particle interactions using HC-Pro and CP antibodies and corresponding Duolink® probes. Interactions are visible as green fluorescing spots. Nuclei were counterstained with DAPI (blue) and chloroplast autofluorescence is presented in grey to reveal the cell lumen. Scale bars: 20 µm. (B-C) Quantitative analysis of the Duolink® signal shows that (B) H_2O_2 increased and (C) $LaCl_3$ decreased HC-Pro/CP interactions. The box plots presents data from three independent experiments using between 56-115 protoplasts for each condition. The y-axes show HC-Pro/CP interactions, presented as total fluorescent intensity (F_{tot}). *p* designates p -values obtained by generalized linear models (see material and methods).

Figure 4. Non-reducing SDS-PAGE/Western blotting analysis of HC-Pro and CP from TuMV-infected turnip protoplasts. The samples were lysed in a buffer without reducing agents to conserve the disulfide bridges. (A) H_2O_2 and $LaCl_3$ treatments did not modify the migration profile of the capsid protein (CP), whereas they (B) induced (H_2O_2) or inhibited ($LaCl_3$) formation of HC-Pro oligomers. (C-D) The concentration range and the kinetics of H_2O_2 incubation shows that HC-Pro oligomerization (C) was induced by a minimum concentration of 0.25 mM and (D) that it was rapid and reversible, either by extended H_2O_2 treatment (left panel) or by washing protoplasts (right panel). (E) Also inhibition of HC-Pro oligomerization by $LaCl_3$ was rapid and reversible. (F-G) HC-Pro oligomers are formed by intermolecular disulfide bridges because (F) incubation of protoplasts with DTT, either alone or after H_2O_2 treatment, abolished HC-Pro oligomers, and (G) treatment with NEM before but not after previous incubation with H_2O_2 prevented their formation. (H) Transmission tests using NEM-treated protoplasts show a drastic diminution of TuMV transmission. Transmission tests were performed three times using 320 plants per condition and analyzed by generalized linear models as described in Figure 1. (I) Protoplast viability assays show that NEM treatment did not change cell viability under the conditions used. TuMV, samples of TuMV-infected protoplasts; Non inf., samples of non infected protoplasts; $LaCl_3$, treatment with 1 mM $LaCl_3$ for 5 min; H_2O_2, treatment with 2 mM H_2O_2 for 5 min; wash, H_2O_2 was removed by centrifugation and resuspension of protoplasts in fresh medium; DTT, treatment with 5 mM DTT for 30 min; NEM, treatment with 3 mM NEM for 20 min. Equal loading of lanes is shown by Ponceau Red staining of the large RuBisCO subunit (Rub). A precolored ladder and the molecular masses in kDa are indicated at one side of each blot. *p* in (H) designates p-value obtained by generalized linear models from three independent experiments.

Figure 5. Model of TuMV acquisition by aphids. For simplicity, aphids, viral components and the plant cell are not drawn to scale. (1) Before the arrival of aphid vectors, the redox potential of the cytosol of TuMV-infected cells has 'normal' values, i.e. it is reduced. Consequently, the cytosolic HC-Pro protein (blue circles) is in a reduced form (the red points in HC-Pro present reduced cysteines) and contains no intermolecular disulfide bridges. This form of HC-Pro is presumably not associated with virus particles (purple lines). It is likely but remains to be confirmed whether reduced HC-Pro is dimeric as presented here. (2) When an aphid feeds on a leaf infected with TuMV, an unknown elicitor is recognized by the plant cell and induces the opening of calcium channels (pink cylinder) and triggers directly or indirectly ROS production in the cell. During this activation stage, the ROS in the cytoplasm increases (red lightning) the redox potential of the cell cytoplasm and oxidizes one or more HC-Pro cysteines. This oxidation

generates disulfide bridges (red lines) between different HC-Pro molecules. The intermolecular disulfide bridges either induce oligomerization of a portion of HC-Pro or change the conformation of a part of existing oligomers, presented by the transition of the circles to squares. For simplicity, higher HC-Pro forms are not shown. (3) Whatever the case, oxidation of a fraction of HC-Pro results in a functional switch of the protein and the oxidized tertiary or quaternary conformation allows interaction between HC-Pro and TuMV particles and the formation of TuMV transmissible complexes, symbolized by square HC-Pro aligned with a virion. Now the infected cell is switched into transmission mode and this stage allows efficient acquisition of TuMV. (4) The aphid acquires transmissible complexes and transmits the TuMV during the next punctures on another plant. After vector departure, the redox potential of cell cytoplasm lowers again and HC-Pro is reduced. This changes its conformation and induces dissociation of the transmissible complexes, leaving HC-Pro free to fulfill its other functions during infection. The aphid drawing is modified from (43), published under open CC3.0 license.