The Rysto immune receptor recognises a broadly conserved feature of potyviral coat proteins

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Summary

- Knowledge of the immune mechanisms responsible for viral recognition is critical for understanding durable disease resistance and successful crop protection. We determined how potato virus Y (PVY) coat protein (CP) is recognised by Rysto, a TNL immune receptor.
- We applied structural modelling, site-directed mutagenesis, transient overexpression, co-immunoprecipitation, infection assays and physiological cell death marker measurements to investigate the mechanism of Rysto–CP interaction.
- Rysto associates directly with PVY CP in planta that is conditioned by the presence of a CP central 149 amino acids domain. Each deletion that affects the CP core region impairs the ability of Rysto to trigger defence. Point mutations in the amino acid residues Ser25, Arg157, and Asp201 of the conserved RNA-binding pocket of potyviral CP reduce or abolish Rysto binding and Rysto-dependent responses, demonstrating that appropriate folding of the CP core is crucial for Rysto-mediated recognition.
- Rysto recognises the CPs of at least 10 crop-damaging viruses that share a similar core region. It confers immunity to plum pox virus and turnip mosaic virus in both Solanaceae and Brassicaceae systems, demonstrating potential utility in engineering virus resistance in various crops. Our findings shed new light on how R proteins detect different viruses by sensing conserved structural patterns.

Introduction

The plant immune system is a multilayered detection and signalling network. To successfully restrict and thwart viruses, plants use RNA silencing, translation repression, and Resistance (R) gene-mediated mechanisms (Leisner & Schoelz, 2018; Wersch et al., 2020). Most plant R genes encode intracellular nucleotide-binding leucine-rich repeat (NLR) receptors. Some of these receptors carry an N-terminal TIR domain and are termed TIR-NLRs (TNLs), whereas CC-NLRs (CNLs) carry an N-terminal coiled-coil domain (Jones et al., 2016).

A growing body of evidence suggests that in plants, as in mammals, NLR activation results from induced oligomerisation of NLRs to form signalling-active scaffolds (Saur et al., 2020), imposing induced proximity on an N-terminal signalling domain. Recent structural elucidation of the CNL: HOPZ-activated RESISTANCE 1 (ZAR1) and TNL-type: ROQ1 (recognition of XopQ 1) and RPP1 (Resistance to Peronospora parasitica 1) immune receptors has provided new insights into plant immune receptor activation (Wang et al., 2019; Martin et al., 2020). In its resting state, ZAR1 is an ADP-bound monomer that, upon activation, forms a pentameric funnel-shaped structure that functions as a calcium-permeable channel in the plasma membrane (Wang et al., 2019; Bi et al., 2021). ROQ1 and RPP1, when activated, form tetrameric complexes that activate an intrinsic NAD+ cleavage activity exerted by the TIR domains (Ma et al., 2020; Martin et al., 2020).

The activation of NLRs leads to a suite of downstream defence responses, frequently culminating in a hypersensitive cell death response (HR) at the infection site (Lolle et al., 2020). This mode of resistance is often referred to as effector-triggered immunity (ETI) and involves direct or indirect NLR-dependent pathogen recognition of effector proteins, historically termed avirulence (Avr) factors (Zipfel, 2014). Most viral effectors function as movement proteins, replicases or coat proteins (CPs), playing diverse roles during infection and replication (Leisner et al., 2018).

Some NLR proteins may recognise more than one effector from various pathogens. For instance, Rx, a resistance protein against potato virus X (PVX), was shown to recognise conserved CP motifs from various potexviruses (Baurès et al., 2008). Similarly, the N protein that confers resistance against the tobacco mosaic virus (TMV) via recognition of the helicase domain of TMV replicase (p50), can perceive several tobamoviruses (Whitham et al., 1994). Conversely, a single NLR can recognise independently evolved effectors. Sequence-unrelated
**Materials and Methods**

**Plant material**

*Nicotiana tabacum* cv Xanthi-nc. *Ry*<sub>so</sub>–transgenic *N. tabacum*, *N. tabacum* *NahG*, *N. benthamiana* wild-type (WT), *NahG*, as well as all *Nicotiana* knockouts used in this study were grown for 6 wk in soil under controlled environmental conditions (22°C, 16 h : 8 h, light : dark) as described previously (Hoser *et al.*, 2013). Transgenic tobacco plants were regenerated following *Agrobacterium tumefaciens* leaf disc transformation as described by Grech-Baran *et al.* (2020). Transgenic *Arabidopsis* plants (ecotype Columbia) were obtained using the floral dipping method as described (Clough & Bent, 1998). Plants were then cultivated in Jiffy7 pots in controlled-environment chambers (Percival Scientific, Perry, IA, USA) at 22°C, 40% humidity, under 8 h of light.

**Molecular modelling**

Each of selected CP proteins of Potyviridae were modelled by homology using the algorithm implemented in YASARA structure package (Krieger & Vriend, 2014). In all cases the same three template structures from watermelon mosaic virus (5ODV), TuMV and (6T34) and virus-like particles based on potato virus Y (6HXZ) were selected. For each template, up to five alternative sequence alignments were allowed, and up to 50 different conformations were tested for each modified loop. Each of the obtained models was assessed for structural quality (dihedral distribution, backbone, and side-chain packing), and the ones with the highest scores were then used to create a hybrid model that was built using the best fragments (e.g. loops) identified in the particular models. The above procedure was then repeated for the folded parts of the proteins identified in the initial models. The structures of protein complexes with RNA were modelled using as the template two uridine pentanucleotides bound to watermelon mosaic virus protein (5ODV, molecules a b and A, respectively). In the latter case the side-chain conformation was tuned by the 10 succeeding rounds of FOLDX 5.0 (https://doi.org/10.1093/bioinformatics/btz184) ‘repairPDB’ procedure. Finally, the effect of all single residue replacement of RNA on the stability of the protein–RNA complex was assessed using the ‘RNAscan’ procedure implemented in the FOLDX 5.0.

The phylogenetic tree was generated from protein sequences of the known potyviruses obtained from National Center for Biotechnology Information (NCBI). Sequences were aligned using CLUSTALW 1.7416 and the alignments were imported to the Mega717 to build a maximum-likelihood phylogenetic tree using the Jones–Taylor–Thornton (JTT) substitution model and 100 bootstraps.

**Plasmids generation**

The sequences for all the primers used for all CPs cloning, site-directed mutagenesis, and truncated PVY CP versions, as well as Ry and N cloning, are listed in Supporting Information Tables S1–S4.

The PVY CP, encoding sequence was amplified by reverse transcription-PCR from total RNA extracted from PVY-infected tobacco leaf tissue as described previously (Grech-Baran *et al.*, 2020) and inserted into the pENTR/D-TOPO vector (Thermo Fisher Scientific, Waltham, MA, USA). The sequences of: PRSV (X67673–35K), potato virus M (M96425.1), SMV (GT015011), pepper severe mosaic virus (NC_008393), bean common mosaic necrosis virus (AB734777.1), maize dwarf mosaic virus (AF395135.1), sunflower chlorotic mottle virus (GU181199.1), pea-seed born mosaic virus (HQL85577.1), SPFMV (AF015540.1), TuMV (NC_002509.2), cassava brown

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**References**

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streak virus (NC_014791.1) and PPV (AB576045.1) CPs were synthesised in vitro (Twist Bioscience, San Francisco, CA, USA) and inserted into the pENTR/D-TOPO vector. The resulting entry clones were LR recombined with the Gateway pGWB: 411, 441, 454 or 502-C-SF-TAP destination vectors (Nakagawa et al., 2007; Golisz et al., 2013).

Side-directed mutagenesis was used for PVY CP single (CP S125A, CP R157D and CP D201R) as well as double (CP S125A/R157D, CP S125A/D201R) or triple (CP S125A/R157D/D201R) mutant generation. The presence of the mutation was confirmed by sequence analysis.

The Rsfo and N cDNA sequences were amplified from Rsfo and nontransgenic tobacco, respectively. PCR products were then inserted into the pDONR201 entry vector (Thermo Fisher Scientific) followed by an LR Gateway reaction into the pBAV150 destination vectors (Nakagawa et al., 2007).

Semiquantitative RT-PCR
Total RNA was treated with DNase I (Thermo Fisher Scientific) and subjected to reverse transcription using a mix of random hexamers and oligo-dT primers and a Revert Aid first Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Semiquantitative RT-PCR was performed using DreamTaq (Thermo Fisher Scientific) with 30 amplification cycles followed by electrophoresis on 2% agarose gel stained with ethidium bromide. Primer pairs used in the PCR reaction are listed in Table S5.

Gene expression analysis- RT-qPCR
Gene expression analysis via RT-qPCR was performed using a LightCycler480 instrument and LightCycler480 SYBR Green Master Kit reagents (Roche). Relative gene expression levels were determined using a standard curve method, and the value for each gene was normalised against the mean expression values of two reference genes: EF1 (AAD28440.1) and L23 (TC19271-A2g39460), as described previously (Liu et al., 2012). Each sample was tested with four technical replicates and two dilutions. Primers for qPCR are provided in Table S5.

Ion conductivity
At the indicated time points, eight leaf discs (1 cm diameter) were cut from infiltrated zones and floated abaxial side up on 5 ml MilliQ water for 10 min at 18°C with gyratory agitation (50 rpm). The conductivity of the water was measured with a WTW InoLab Multi 9310 IDSCDM83 benchtop meter and expressed in μS cm⁻¹.

Cell death scoring
Agrobacterium tumefaciens strain GV3101 carrying the appropriate constructs was suspended in infiltration buffer (10 mM MgCl₂, 10 mM MES, pH 5.6, 150 mM acetosyringone) and mixed before infiltration at final OD₆₀₀ adjusted to 0.6. Bacteria were infiltrated into leaves of c. 4-wk-old N. benthamiana plants using a 1-ml needleless syringe. At 5 d post infiltration (dpi), detached leaves were photographed under UV light with a yellow coloured filter (Wratten K2 Yellow Filter no. 8; Kodak) attached to the camera lens and images were taken with a long exposure of 2 s, F8.0 and ISO 400 and scored using IMAGEJ software according to the scale presented in Fig. S4.

Western blotting
Agroinfiltrated leaves were collected, frozen and ground in liquid nitrogen. Total proteins were extracted as previously described (Grech-Baran et al., 2020). Homogenates were centrifuged at 10,000 g for 10 min, and the supernatants were collected. All tested samples were run on 4–20% precast SDS-PAGE gels (Bio-Rad) and subjected to immunoblot analysis using primary monoclonal anti-red fluorescent protein (anti-RFP), anti-green fluorescent protein (anti-GFP) or anti-Strep-tag II antibodies (Abcam, Cambridge, UK) and alkaline phosphatase-conjugated anti-rabbit secondary antibodies (Sigma Aldrich).

To detect PPV or TuMV, alkaline phosphatase-conjugated anti-PPV or anti-TuMV antibodies (Bioreba, Reinach, Switzerland) were used. For cassava brown streak virus (CBSV) detection primary anti-CBSV antibodies (Bioreba) and alkaline phosphatase-conjugated anti-rabbit secondary antibodies (Sigma Aldrich) were used. Immunoblots were developed using a NBT/ BCIP colourimetric detection kit (BioShop Inc., Burlington, ON, Canada).

Co-immunoprecipitation (IP)
The desired proteins were co-expressed in Nicotiana leaf tissue using Agrobacterium-mediated transient expression. The tissue was frozen in liquid nitrogen and ground using a mortar and pestle. The samples were suspended in immunoprecipitation buffer (150 mM NaCl, 150 mM Tris–HCl pH 7.5, 10 mM dithiothreitol, 10% (v/v) glycerol, protease inhibitor cocktail and 1% Triton X-100) and centrifuged (10 min, 10,000 g, 4°C). Proteins were immunoprecipitated for 1 h using GFP Trap Beads (ChromoTek, Planegg-Martinsried, Germany). The samples were washed five times with immunoprecipitation buffer, before elution by boiling with Laemmli buffer. The anti-GFP western blots were performed using anti-GFP antibody (Abcam) as the primary antibody and anti-rabbit alkaline phosphatase-conjugated secondary antibodies (Sigma Aldrich). Anti-RFP primary antibody (Abcam) was used for the anti-RFP western blots followed by secondary anti-rabbit alkaline phosphatase-conjugated antibody (Sigma Aldrich).

SA measurement
Free SA was extracted and quantified essentially as described by Malamy et al. (1990) and HPLC assayed as described (Krymowska et al., 2007). For each extraction 1 g of fresh tissue was used, four biological repeats and two technical replicates were used for each variant set tested. The experiment was repeated three times.
Transient expression assay

*Agrobacterium GV3101* strains carrying plasmids expressing Rysto, N, PVY CP, its variants or coat proteins of other Potyvirus strains were used to infiltrate *R. tomentosspinosus* or control *Nicotiana* plants, as described previously (García-Ruiz et al., 2020). HR-related phenotypes were assessed 3 d after infiltration. Each experiment was performed at least twice and included at least three independent biological replicates.

Viral infections

PVY-NTN isolate (AJ585342.1) infection was performed as described previously (Garcia-Ruiz et al., 2020). CCSV isolate (DSMZ no. PV-0957) or PPV isolates: 0001 (DSMZ nos. PPV-0001; MT613316) and 2233 (DSMZ nos. PPV-0233; MW854267) were inoculated, as recommended by German Collection of Microorganisms and Cell Cultures GmbH. (www.dsmz.de). For PPV-GFP and TuMV-GFP infection, plants were infected using PPV-GFP or TuMV-GFP infectious clones (Fernández-Fernández et al., 2001; Garcia-Ruiz et al., 2015), as described previously (Salvador et al., 2008; Garcia-Ruiz et al., 2015).

Confocal laser scanning microscopy

Subcellular localisation of the fusion proteins was evaluated using a Nikon C1 confocal system built on TE2000E and equipped with a ×60 Plan-Apochromat oil immersion objective (Nikon Instruments BV Europe, Amsterdam, the Netherlands). GFP was excited by the Sapphire 488 nm laser (Coherent, Santa Clara, CA, USA) and observed using the 515/530 nm emission filter, whereas RFP was excited by the 543 nm helium–neon laser and detected using the 605/75 nm barrier filter. GFP and RFP scanning was performed in sequential mode to prevent bleed through. Fluorescence microscopy was performed in the Fluorescence Microscopy Facility in IBB PAS, Poland. Confocal images were analysed using free viewer EZ-C1 and IMAGEJ software.

Statistical analysis

Statistical analyses were conducted in R 3.2.2 within R Studio 0.99.483. Technical replicates consisted of replicate readings from the same plant in the same experiment, whereas biological replicates consisted of measurements obtained from independent plants. Data were analysed using the following pipeline: data were assessed for their suitability for parametric analysis by testing for the normal distribution of the residuals using the Shapiro–Wilk test. If the data were suitable for conducting parametric tests, repeated-measures analysis of variance (ANOVA) followed by Tukey’s honestly significant difference (HSD) (**, \(P<0.001\)) test were performed.

Results

PVY CP associates with Rysto during PVY infection

Nucleotide-binding leucine-rich repeat immune receptors either interact directly with recognised pathogen effectors, or indirectly recognise them based on their activity on cellular targets (Cesari, 2018). We recently showed that Rysto perceives PVY CP (García-Ruiz et al., 2020) and set out to further investigate if Rysto and PVY interacted directly during the infection process. Rysto or tobacco N gene as a negative control (Whitham et al., 1994), were tagged at their C-termini and transiently expressed in PVY-NTN-infected *Nicotiana* plants. Total proteins were then extracted and immunoprecipitated (IPd) with anti-GFP antibody, followed by PVY detection with specific antibodies. The results indicated that PVY CP associates physically with Rysto in planta but not with N (Fig. 1a).

The core region of CP is both necessary and sufficient for eliciting Rysto-mediated cell death

To investigate the molecular mechanisms underlying PVY CP perception by the Rysto protein, a PVY CP structural model was analysed using the YASARA structure package (Krieger & Vriend, 2014). A single PVY CP unit consists of 267 amino acids with a globular core subdomain defined from Q75 to K226, that forms seven α-helical folds and a β-hairpin. This central domain is flanked by putative disordered regions, that cannot be modelled, comprising 76 and 41 amino acid residues at the N- and C-termini, respectively (Figs 1b, S1). To determine which CP region is recognised by Rysto, we generated a full-length CP construct and truncated CP variants: CP77–226, CP1–226, and CP77–226, that were RFP epitope tagged, and transiently expressed in *R. tomentosspinosus* or control *N. tabacum* plants (Fig. 1c,d). Neither the N- nor C-terminus of PVY CP was required to elicit the resistance response in Rysto *N. tabacum*, as the CP77–226 mutant still induced cell death (Fig. 1c). Based on an in silico model of the globular part of PVY CP, we removed two amino acids from each end of the CP core (CP79–226 or CP77–224) because this was predicted to disrupt the distal α-helices and was likely to hamper recognition by distorting the CP structure. Neither of these truncated constructs could elicit cell death in Rysto *N. tabacum* (Fig. 1c), indicating that the CP core region and its intact structure was required for Rysto-dependent HR development.

In a parallel set of co-immunoprecipitations (Co-IPs), we tested whether the CP core interacted directly with the Rysto receptor after transient expression in nontransgenic *Nicotiana* plants. For this purpose, Rysto-GFP was co-expressed together with full-length CP-RFP, CP core (CP77–226)-RFP, CP79–226-RFP, or Myc-RFP as a negative control. Rysto was found to interact with all tested CP variants, including the CP79–226-RFP that was unable to elicit HR, but not with Myc-RFP (Fig. 1d). These data support the view that an in planta receptor–ligand interaction is necessary for the HR but might not be sufficient if overall ligand structure is impaired.

Conserved CP residues at the protein–RNA interface are crucial for eliciting cell death in an Rysto-dependent manner

The cryo-EM model of PVY (Kezar et al., 2019) suggests that the structure of PVY virions is stabilised by protein–ssRNA interactions involving the conserved residues S125, R157 and D201 at...
Fig. 1 Direct interaction of potato virus Y (PVY) coat protein (CP) with Rysto, and minimal fragment of CP required for the Rysto-mediated hypersensitive cell death response (HR). (a) Detection of interaction of Rysto with CP during PVY infection in planta by immunoprecipitation (IP) followed by western blot analysis. Total proteins were extracted from Nicotiana plants infected with the PVY-NTN strain followed by transient expression of Rysto-GFP or N-GFP as a negative control. IP was performed with an anti-GFP antibody and immunoblots were probed with anti-GFP or anti-PVY CP monoclonal antibodies. (b) Schematic representation of PVY CP and corresponding truncations. Blue, unstructured N- and C-terminal regions; pink, the core subdomain; white and crossed out, the deleted fragments. (c) Development of cell death in leaves of Rysto or control tobacco plants transiently expressing intact PVY CP and its truncations. Photographs were taken at 3 d post infiltration (dpi). (d) In planta accumulation of RFP-tagged CP variants. For anti-RFP immunoblots, total protein extracts were prepared at 3 dpi. Staining of Rubisco with Ponceau S was used as a loading control. (e) Detection of Rysto interaction with truncated variants of PVY CP in transient expression assay in planta. Total proteins were extracted from Nicotiana plants expressing Rysto-GFP together with full-length CP-RFP, CP(77–226)-RFP or CP(79–226)-RFP, whereas Myc-RFP was used as a negative control. IP was performed with an anti-GFP antibody and immunoblots were probed with anti-GFP or anti-RFP antibodies.
the ssRNA binding site of the CP core (Zamora et al., 2017) (Fig. 2a). We investigated the impact of PVY CP structure-guided mutations on the activation of Ry<sub>sto</sub>-dependent immune responses using HR assays in transgenic Ry<sub>sto</sub> tobacco plants.

Constructs carrying single (S<sub>125</sub>A, R<sub>157</sub>D and D<sub>201</sub>R), double (S<sub>125</sub>A_R<sub>157</sub>D and S<sub>125</sub>A_D<sub>201</sub>R), or triple (S<sub>125</sub>A_R<sub>157</sub>D_D<sub>201</sub>R) mutations were generated and tagged with C-terminal RFP epitope. Following the transient expression of these CP
variants in $R_{yst}$ plants, three phenotypes were observed (Fig. 2b). A typical cell death phenotype was observed for the single substitutions $S_{125}A$ and $R_{157}D$ and double $S_{125}A_R_{157}D$, whereas the $D_{201}R$ mutant and double $S_{125}A_D_{201}R$ variant partially retained CP recognition and triggered a weaker response. By contrast, no cell death was observed when the triple mutant was tested (Fig. 2b). To prevent the synthesis of protein variants in which steric hindrances would disable proper folding, the free energy change was calculated for each variant of the CP mutants and the expression of all proteins was confirmed by western blot analysis (Figs 2c, S2). No significant differences in protein expression levels were observed between CP variants, regardless of the HR phenotype induced (Fig. 2c).

Changes in HR triggered by CP mutants correlate with ion leakage and defence-related marker gene expression

To quantify $R_{yst}$-dependent HR triggered by CP mutants, all CP versions were transiently expressed in $R_{yst}$-expressing tobacco leaves. Electrolyte leakage and expression of defence-related marker genes ($HSR203J$ and $HIN-I$; Chichkova et al., 2004) were measured after 18, 21, 24, 27 and 30 h post infiltration (hpi). Full-length PVY CP was used as a control. All single and double mutants followed the conductivity pattern of the wild-type CP, with the maximum conductivity detected at 27 hpi. However, the ion leakage was lower than that of the control at every time point, especially in the single and double mutants with the $D_{201}R$ mutation. As expected, the triple $S_{125}A_R_{157}D_D_{201}R$ mutant activated no ion leakage (Fig. 2d).

Relative expression levels of defence markers measured by qPCR were consistent with the levels of ion leakage. In $R_{yst}$ tobacco infiltrated with CP$_{WT}$, the highest expression levels of both genes were seen at 18 hpi and gradually decreased at later time points. All single and double mutants showed a significant decrease and delay in $HSR203J$ and $HIN-I$ expression, with maximum observed at 24 or even 30 hpi. The observed changes were less pronounced in $R_{157}D$ and $S_{125}A_R_{157}D$ in comparison with other mutated CP versions (Fig. 2e). The triple mutant was unable to induce $HSR203J$ and $HIN-I$ expression (Fig. 2e).

Structure-based mutations in the CP protein–RNA interaction pocket perturb binding to $R_{yst}$

To determine whether the loss of $R_{yst}$-dependent HR correlated with the lack of binding to $R_{yst}$, we performed Co-IP assays in nontransgenic tobacco using a GFP-tagged $R_{yst}$ construct and full-length CP-RFP or CP triple mutant $S_{125}A_R_{157}D_D_{201}R$-RFP. As expected, CP$_{WT}$ associated with $R_{yst}$ in planta, whereas no association between the triple CP mutant and $R_{yst}$ was detected (Fig. 2f).

Taken together, these results supported the hypothesis that the proper structure of the PVY CP central core region is necessary for $R_{yst}$-mediated recognition and HR development.

The EDS1-SAG101-NRG1 node is indispensable for $R_{yst}$-dependent immunity

TNLs utilise a network of helper NLRs for a fully effective immune response. Two distinct complexes (NRG1/EDS1/SAG101 and ADR1/EDS1/PAD4) are required to execute TNL-initiated immunity (Sun et al., 2020); $R_{yst}$ is no exception (Grec-Baran et al., 2020). To further investigate whether PAD4 or SAG101 were involved in $R_{yst}$-dependent signalling pathway, we transiently expressed $R_{yst}$-GFP, PVY CP-RFP or a combination of both in a set of $N. benthamiana$ knockouts. Single knockouts $pad4$, $sag101a$ and $sag101b$ (two SAG101 isoforms present in $N. benthamiana$ that form heterocomplexes with EDS1), double $pad4/sag101b$, as well as triple $pad4/sag101b/eds1$, alongside wild-type control, were tested and HR was quantified (Fig. 3).

$R_{yst}$-CP co-expression in single $pad4$ and $sag101a$ knockouts or wild-type (WT) $N. benthamiana$ resulted in clear HR, whereas $sag101b$ and double and triple knockouts remained symptomless (Fig. 3a). No statistically significant reduction in the size of HR lesions, nor differences in $R_{yst}$ and PVY CP protein accumulation between WT control and single $sag101a$ or $pad4$ mutant was observed (Fig. 3b,c).

We concluded that $sag101b$ but not $PAD4$ was necessary for CP-triggered and $R_{yst}$-dependent HR.
Rysto-mediated immune response towards potato virus Y (PVY) coat protein (CP) is abolished in sag101b but not in sag101a or pad-4 knockout lines. (a) Representative images of cell death phenotype after transient expression of Rysto-GFP, PVY CP-Strep or both proteins together in single: pad4, sag101a, sag101b, double: pad4/sag101b, or triple: pad4/sag101b/eds-1 Nicotiana benthamiana knockouts alongside the wild-type control. The leaves were photographed under UV light and cell death was scored at 5 d post infiltration (dpi). (b) Cell death phenotype quantified at 5 dpi. The results are presented as dot plots in which the size of a dot is proportional to the number of samples with the same score (count) within the same biological replicate. The statistical analysis was performed using analysis of variance (ANOVA) with post-hoc Tukey’s honestly significant difference (HSD) test. Letters correspond to statistically homogeneous groups ($P < 0.001$). The experiment was independently repeated at least three times. (c) Accumulation of Rysto and PVY CP proteins in N. benthamiana knockouts. Total protein extracts were prepared at 3 dpi and analysed by protein gel blotting with anti-GFP or anti-Strep antibody. Staining of Rubisco with Ponceau S was used as a loading control.
Determination of salicylic acid role in Rysto signalling

SA is a crucial signalling molecule in the activation of plant immunity (Durner et al., 1997). To determine whether SA is involved in the initiation of the local Rysto-mediated response, free SA levels were measured in Rysto tobacco plants after transient expression of CP-RFP or its inactive triple mutant at different time points (18, 24 and 30 hpi) and temperatures (22 and 30°C). SA levels were significantly elevated from 24 hpi in leaves expressing WT CP at both temperatures, compared with the CP inactive triple mutant and mock-treated plants (Fig. 4a). Interestingly, the SA increase was less pronounced at 30°C.

We have shown previously that transiently expressed Rysto can trigger cell death in P Vy-infected SA-deficient (NahG) N. benthamiana plants (Grech-Baran et al., 2020). To further elucidate the relationship between SA and Rysto-activated immunity, CP-RFP and Rysto-GFP constructs were co-expressed transiently in NahG or control tobacco plants. SA-deficient plants developed cell death symptoms similar to those of the nontransgenic controls (Fig. 4b). Furthermore, no significant differences in plasma membrane integrity or macroscopic tissue collapse were observed (Figs 4c, S5).

We next examined the subcellular localisation of Rysto-GFP and CP-RFP in NahG or control tobacco plants. Regardless of the plant background used, Rysto showed solely cytoplasmic localisation, whereas CP was localised to both the cytoplasmic and nuclear compartments (Fig. 4d). No changes in subcellular localisation of both proteins were observed when Rysto was co-expressed with CP (Fig. 4d).

These results suggested that, even though CP of PVY triggers SA accumulation in Rysto-expressing tobacco plants, the Rysto-CP-related cell death is independent of SA accumulation. Similar results were obtained in N. benthamiana plants (Fig. S6).

Rysto recognises a range of CPs from multiple potyviruses

To determine whether CP of PVY shared structural similarities with other members of the Potyvirus genus, we constructed a phylogenetic tree of CPs from 65 potyviruses, which revealed a considerable divergence in their amino acid sequences (Figs 5a, S7; Notes S1). Despite the low similarity at their N- and C-termini, the central subdomain and the amino acids predicted to interact with ssRNA are conserved among all studied potyviruses (Figs 5b, S8, S9).

To test whether the structural similarities of CPs resulted in their recognition by Rysto, we selected 10 economically important potyviruses from distantly related clades: PRSV, papaya ringspot virus; PVM, potato virus M; SMV, soybean mosaic virus; PepSMV, pepper severe mosaic virus; BCMNV, bean common mosaic necrosis virus; MDMV, maize dwarf mosaic virus; TuMV, turnip mosaic virus; PShMV, pea-seed born mosaic virus; SPFMV, sweet potato feathery mottle virus and PPV. The sequences encoding these viral CPs were cloned, FLAG-epitope tagged and transiently expressed in transgenic Rysto or control tobacco plants. Within 3 d, cell death development was observed in Rysto plants for all tested CPs, whereas the controls remained symptomless (Fig. 5c). The expression of the viral proteins was confirmed by immunoblotting (Fig. 5d).

These results demonstrate that Rysto has a broad recognition spectrum and therefore has considerable agronomic potential to control many economically significant potyviruses.

A conserved potyviral CP core architecture is necessary for Rysto-mediated recognition

To assess the range of the Rysto-mediated viral CP recognition, we tested the CP of Ugandan CBSV from Ipomovirus genus of the Potyviridae family (Monger et al., 2001). Structural modelling of CBSV CP revealed its low homology to the P Vy CP structure, with significant differences in the central core region (Fig. 6a,b). As CBSV CP does not have the strongly conserved aspartic acid (D) in LARY (A/G) FDFYE sequence, which is present in all CP cores recognised by Rysto, it cannot form the proper RNA-binding pocket structure, resulting in a different core folding pattern (Figs 2a, 6a). To test Rysto-mediated recognisability, we performed two sets of Co-IP assays. To check whether Rysto interacts with CBSV in planta, tobacco plants were infected with CBSV (DSMZ no.: PV-0957), followed by transient expression of Rysto-GFP or N-GFP as a negative control. In the second set of experiments, a CBSV CP-Strep construct was co-expressed transiently with Rysto-GFP. Although we could detect both CBSV virus accumulation and CBSV CP expression in planta, no HR was observed either after CBSV infection, or when Rysto-GFP was co-expressed transiently with CP CBSV (Fig. 6c). No binding of Rysto to CBSV or to CBSV CP was detected in immunoblots after Co-IP (Fig. 6d,e), which confirmed that recognition was dependent on the highly conserved CP core architecture.

Rysto restricts PPV and TuMV spreading

To test whether Rysto-mediated recognition of the range of potyviral CPs also enabled resistance against potyviruses other than P Vy, we focused on two economically important viruses: PPV and TuMV. PPV is a causative agent of plum pox disease ‘Sharka’, the most important viral disease of stone fruit crops worldwide (Maejima et al., 2020), while TuMV damages various Brassicaceae and other crops (Palukaitis & Kim, 2021).

Although PPV is unable to infect tobacco systemically, it can replicate in inoculated leaves (Sáenz et al., 2002). We site-inoculated leaves of transgenic Rysto and control tobacco plants with a severe PPV isolate (PV-0001). At 7 d after infection, Rysto plants exhibited a local cell death response, while no HR was detected in controls (Fig. 7a). To investigate the specificity of Rysto-mediated recognition, Rysto and control tobacco leaves were site inoculated with two other PPV isolates (PV-0001 and PV-2233). Western blot analysis did not detect PPV proteins in samples from the neighbouring leaf zones of Rysto plants at 7 and 14 dpi whereas, in nontransgenic control samples, viral proteins were detected (Fig. 7b). To further validate Rysto-mediated PPV resistance, we delivered a full-length PPV cDNA clone tagged with GFP into transgenic Rysto and control tobacco plants by...
Salicylic acid (SA) and coat protein (CP)-triggered immunity in Rysto plants. (a) Changes in free SA levels in Rysto-transgenic plants challenged by potato virus Y (PVY) CP under standard or elevated temperature conditions. Triple CP mutant and mock inoculation were used as controls. At selected time points, tested plants were sampled and SA levels were measured by high-performance liquid chromatography (HPLC). Values are the mean ± SD (n = 8). Statistical analysis was performed using a one-way analysis of variance (ANOVA) with Tukey’s honestly significant difference (HSD) test (P < 0.01). (b) Rysto and CP co-expression results in tissue collapse in SA-deficient plants. Rysto or CP, or a combination of both, were transiently expressed in control or NahG Nicotiana tabacum plants. Empty vector was used as a negative control. The photographs were taken at 3 d post infiltration (dpi). Expression of the NahG transcript was confirmed via semi-quantitative RT-PCR (Supporting Information Fig. S10). (c) Quantitative ion leakage assay in NahG and control tobacco plants co-expressing CP and RySto. Statistical analysis was performed using Student’s t-test. Significance codes are assigned based on the statistical analysis per timepoint and should be read columnwise. Graph presentation of the same data is provided in Fig. S6. (d) Cellular localisation of CP and RySto in representative NahG or control tobacco leaf epidermal cells transiently expressing CP, RySto or both. Confocal images were obtained at 2 dpi. For each variant, c. 50 transformed cells were examined. Bar, 10 µm.
agrofiltration. No GFP fluorescence was detected in the infiltrated leaves of Rysto plants, whereas a strong signal was observed in control leaves (Fig. 7c).

Unlike PPV, TuMV can infect N. tabacum systemically (Modarresi et al., 2019). Therefore, after agroinfiltration of transgenic Rysto and control plants with a TuMV-GFP clone, GFP fluorescence and viral protein accumulation were monitored in upper, noninfiltrated leaves. At 21 dpi, no signal was detected in the Rysto plants, while control plants showed intense fluorescence (Fig. 7d). Similarly, western blot did not detect the viral protein in transgenic Rysto plants (Fig. 7e).

Furthermore, we investigated whether Rysto was able to confer resistance to TuMV in plant families other than the Solanaceae. For this purpose, we infected transgenic 35S-Rysto Arabidopsis plants with TuMV-GFP. At 21 dpi, no GFP signal was detected in systemic leaves of transgenic Arabidopsis, whereas strong GFP fluorescence was observed in nontransgenic controls (Fig. 7f).

Consistently, no TuMV protein accumulation was detected by immunoblotting in transgenic Rysto plants (Fig. 7g).

Taken together, our results demonstrated that Rysto immune receptor can trigger resistance against multiple potyviruses in different pathosystems. We propose that Rysto–CP interactions may represent one of the possible universal models of perception in which a single NLR receptor can recognise diverse, but related, pathogen virulence factors by recognising an evolutionally conserved structure.

Discussion

How NLRs sense pathogens and activate immunity is a fundamental question in plant immunity and disease resistance (Jones et al., 2016; Hu et al., 2020). In this study, we showed that the Rysto immune receptor directly recognises conserved structural elements of the CPs of multiple economically important...
Fig. 6 Variation in potyviral coat protein (CP) core domain architecture results in a lack of recognition by Rysto. (a) Overall structure of cassava brown streak virus (CBSV) CP (blue), PVY CP (grey) and structural alignment of both CP stacked together. (b) Amino acid sequence alignment of the predicted core region of CBSV CP (red frame) with the CPs from representative potyviruses recognised by Rysto. (c) CBSV CP is unable to elicit cell death in Rysto-transgenic and control tobacco plants were infiltrated with Agrobacterium expressing PVY CP-Strep-tag, CBSV CP-Strep-tag or RFP-Strep alone as a control. The image was taken under UV light. (d) Rysto does not interact with CBSV in planta. Total proteins were extracted from Nicotiana plants infected with CBSV PV-0957 strain followed by transient expression of Rysto-GFP or N-GFP as a control. Immunoprecipitation (IP) was performed with an anti-GFP antibody and immunoblots were probed with anti-GFP or anti-CBSV antibodies. (e) Rysto does not interact with CBSV CP in planta. Total proteins were extracted from Nicotiana plants expressing Rysto-GFP together with PVY CP-Strep-tag (positive control) or CBSV CP-Strep-tag. RFP-Strep-tag was used as a negative control. IP was performed with an anti-GFP antibody and immunoblots were probed with anti-GFP or anti-Strep antibodies.
potyviruses. This system provides a useful model to understand how NLRs convert recognition to defence activation and cope with the rapid evolution of viral pathogens.

The specific functions of viral proteins (i.e. RNA binding) are often executed by the defined structural motifs conserved in proteins from virtually all organisms (Zamora et al., 2017; Navarro et al., 2020). Potyviruses, the largest group of plant viruses (Gadhave et al., 2020), share a CP folding pattern that is widely distributed in eukaryotic viruses, including influenza viruses (Agirrezabala et al., 2015). As the CP features in virion assembly, RNA silencing and viral translation, it constitutes a potential universal target for NLR immune receptors (Zamora et al., 2017).
Recent cryo-EM studies of PVY CP revealed that the N- and C-terminal ends are likely to be disordered and a conserved folding of its core. The deletion of the N-terminal region of PVY CP results in monomeric CP unit formation, while the C-terminal region was not required for CP’s filament assembly (Keizard et al., 2019). Our results suggested that a single-CP core domain unit is sufficient for Ryso-mediated recognition and CP filament assembly is not necessary for this type of response. The fact that the CP core domains of potyviral proteins are well conserved structurally despite having high amino acid polymorphism might enable escape from host resistance responses and expand host specificity (Charon et al., 2018). Our results provide evidence that the intact conserved CP core region is sufficient for Ryso perception, which is consistent with the preservation of its structure among the majority of potyviruses. Similarly, a minimal fragment of 90 aa of PVX CP, encompassing four alpha helices, is sufficient to trigger Rx-mediated resistance (Nemykh et al., 2008). This suggests that the Rx sensing machinery also targets conserved structural elements rather than any particular sequence motif(s) (Baurès et al., 2008).

Nevertheless, a single or double mutation in tobamoviral CP can disrupt R gene-mediated resistance (Antignus et al., 2008), suggesting that certain residues and sequence motifs are crucial for triggering the HR. A similar scenario has been described for the Ns gene that confers resistance to PVX through the recognition of PVX CP aa 62–78 (Santa & Baulcombe, 1995). The S125, R157 and D201 residues that bind to siRNA of potyviruses are crucial for proper tertiary folding of PVY CP (Zamora et al., 2017). We showed that a single or double substitution of these amino acids impairs the cell death phenotype in Ryso plants, whereas the triple mutant completely abolished it by losing its ability to bind to Ryso. Consistently, the macroscopic HR phenotype was found to correlate with changes in the expression of defence-related markers and ion conductivity.

Structure-guided mutations in the triple CP mutant were based on the reversion of the salt bridge between arginine (R) and aspartic acid (D). R157 is predicted to interact with RNA, whereas aspartic acid stabilises this interaction spatially and, through the formation of a salt bridge, is responsible for maintaining the three-dimensional structure of the CP core (Fig. 2a). Notably, disrupting salt bridges are energetically less favourable than reversing them and may result in decreased protein stability (Bossard et al., 2004). Surprisingly, the potentially less stable CP derivative (S125A_R157D) was still able to trigger cell death, unlike the presumably more stable triple mutant variant (S125A_R157D_D201R) with a reversed salt bridge. As both mutant proteins undergo expression, we propose a model in which the phenotype of the triple mutant might be caused by compromised RNA binding, which in turn affects CP conformation, rather than the stability of the molecule. This agrees with a previous report in which the mutations in the conserved R and D residues of the CP were shown to impair the in vitro assembly of the potyvirus Johnsongrass mosaic virus (Jagadish et al., 1993) and to block the assembly and cell-to-cell movement of the potexvirus pepino mosaic virus (Agirrezañaba et al., 2015).

Activated plant intracellular NLR receptors form oligomers that are required for immunity signalling that broadly resemble mammalian NLR inflammasome scaffolds (Ma et al., 2020; Martin et al., 2020). NRG1 and ADR1, helper NLRs of the RPW8-NLR (or Helo-NLR) class, and the EDS1 family of plant-specific lipase-like proteins (EDS1, PAD4 and SAG101), mediate defence signalling downstream of sensor TIR-NLRs (Lapin et al., 2019). Two distinct modules (NRG1/EDS1/SAG101 and ADR1/EDS1/PAD4) are required for TNL-mediated immunity (Sun et al., 2020). Here, we provide evidence that Ryso activates a recognition-dependent HR in an EDS1-SAG101-NRG1 but not in a PAD4-dependent manner. A similar observation was reported for Roq1 (Sun et al., 2020).

TIR-NLR activation also promoted SA biosynthesis and activated SA-dependent defence responses (Gantner et al., 2019). We found that Ryso mediated HR to transient CP expression is independent of SA accumulation. Although transient CP expression in Ryso plants resulted in a moderate increase in SA levels, the retained cell death phenotype after the co-expression of Ryso with CP in NahG plants suggested that the increase in SA was not required for activation of Ryso-triggered HR. Recently, it was reported that the NLR-dependent cell death response was strongly enhanced by the activation of surface receptors (Ngou et al., 2021). As viral coat proteins (CP) can act as PAMPs (Teixeira et al., 2019), and Agrobacterium transient expression floods the apoplast with bacterial PAMPs, we cannot exclude the idea that transient expression of PVY CP boosts PTI and causes a local increase in SA levels in an Ryso-independent manner. Alternatively, the dying cells might release signals that condition adjacent cells to become CP responsive and activate SA biosynthesis and immunity throughout the entire plant (Radojičić et al., 2018).

Achieving complete and durable resistance is the ultimate goal of resistance breeding. We report here that the PVY CP core region architecture is highly conserved throughout the genus Potyvirus. Moreover, we show that the conserved core structure may enable prediction of viral targets of Ryso. Therefore, it is not surprising that a gene targeting such a conserved motif is both durable and broad spectrum (Grech-Baran et al., 2020). Ryso recognises the CPs of at least 10 economically important potyviruses, including PRSV, PVM, SMV, PepSMV, BCMNV, MDMV, SCMV, TuMV, SPFMV and PPV, which makes it a desirable candidate to engineer potyviral disease resistance into a range of crops worldwide.

To date, only a few sources of resistance to PPV have been identified, and no R genes against PPV have been cloned so far (Ilardi & Tavazza, 2015; Zuriaga et al., 2018). We show here that Ryso-expressing tobacco plants display resistance to both severe and mild PPV isolates, suggesting that Ryso is a valid alternative to RNAi-mediated resistance (Hily et al., 2007). Similarly, several resistance genes (including Tu, TuRB01, TuRB02, TuRB04) have been described for TuMV, but none have been cloned so far, and several resistance-breaking strains have been described (Palukaitis et al. 2021). Like TuRB04 (Jenner et al., 2002), Ryso confers ER to TuMV. Our in silico analysis suggests that Ryso might resist TuRB04-breaking strains, therefore providing a valuable additional resistance gene for stacking to obtain truly broad-spectrum resistance. Additionally, we proved that Ryso confers resistance in systems other than Solanaceae. Collectively, our
study not only provides new insights into the mechanism of the R gene surveillance system, but also offers solutions for crop resistance against potyviruses, which are emerging as one of the most serious challenges of current agriculture, especially as the use of insecticides is becoming more restricted.

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Author contributions

MG-B, performed most of the experimental work, data analyses and writing, JH supervised work and edited the article; KW, JTP performed bioinformatical analyses, TM, AG-U performed the research; ML performed confocal scanning, JDGJ and KW edited the article.

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Data availability

The data that support the findings presented in this publication are available from the corresponding authors upon reasonable request.

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**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Structural features of PVY CP subdomain core region.

**Fig. S2** The effect of the single amino acid residue replacement on the stability of the CP–RNA complex.

**Fig. S3** Related to Fig. 2(d). Boxplot presentation of CP mutants-triggered ion leakage for Rysto plants.

**Fig. S4** Cell death rate scale.

**Fig. S5** Related to Fig. 2(c). Barplot presentation of ion leakage of NahG and control tobacco after CP and Rysto co-expression.

**Fig. S6** SA deficiency does not impact the subcellular localisation of Rysto and CP proteins or HR activation.

**Fig. S7** Phylogenetic analysis of potato virus Y CP protein and other Potyviridae CP proteins.

**Fig. S8** Structure of coat proteins cores of various potyviruses modelled by homology.

**Fig. S9** Sequence alignment of conserved regions of potyviruses CP’s core proteins.

**Fig. S10** Semiquantitative RT-PCR of the NahG gene transcript.

**Notes S1** Amino acid sequences of potyviral CPs that were used for phylogenetic analysis performed in Fig. S7.

**Table S1** Primers used to clone potyviral CPs.

**Table S2** Primers used for site-directed mutagenesis of PVY CP.

**Table S3** Primers used for PVY CP truncations.

**Table S4** Primers used for Rysto cDNA cloning.

**Table S5** Primers used for qPCR and RT-PCR reactions.

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