HCPPro Suppression of Callose Deposition Contributes to Strain-Specific Resistance Against Potato Virus Y

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ABSTRACT

Potato virus Y (PVY; Potyviridae) is a continuing challenge for potato production owing to the increasing popularity of strain-specific resistant cultivars. Hypersensitive resistance (HR) is one type of plant defense responses to restrict virus spread. In many potato cultivars, such as cultivar Premier Russet (PR), local necrosis at the site of infection protects against the most common PVY^O strain, but the HR often fails to restrain necrotic strains, which spread systemically. Here, we established the role of callose accumulation in the strain-specific resistance responses to PVY infection. We first uncovered that PVY, independent of the strain, is naturally capable of suppressing pathogenesis-related callose formation in a susceptible host. Such activity can be dissociated from viral replication by the transient expression of the viral-encoded helper

component proteinase (HCPPro) protein, identifying it as the pathogen elicitor. However, unlike the necrotic strain, PVY^O and its corresponding HCPPro are unable to block callose accumulation in resistant PR potatoes, in which we observed an abundance of callose deposition and the inability of the virus to spread. The substitution of eight amino acid residues within the HCPPro C-terminal region that differ between PVY^O and PVY^N strains and were previously shown to be responsible for eliciting the HR response, are sufficient to restore the ability of HCPPro^O to suppress callose accumulation, despite the resistant host background, in line with a new viral function in pathogenicity.

Keywords: genetics and resistance, virology

Potato virus Y (PVY) is one of the most important plant viruses affecting potato production (Frost et al. 2013; Karasev and Gray, 2013), and it enters in the list of the top 10 plant viruses in molecular plant pathology (Frost et al. 2013; Gray and Power 2018; Scholthof et al. 2011). PVY is an aphid-vectored virus of the genus *Potyvirus* in the largest family of plant RNA viruses, Potyviridae (Gray and Power 2018). Its 9.7-kilobase monopartite RNA genome has a viral-encoded protein linked to its 5' end and a poly(A) tail at its 3' end. The PVY genome encodes a single large polyprotein (3,063 residues) that is cleaved into 10 functional proteins by three viral-encoded proteases, including P1, the helper component proteinase (HCPPro) and NIaPro, and expresses the highly conserved PIPO protein from a small open reading frame embedded within the genome (Chung et al. 2008; Olspert et al. 2015). Several proteins have multiple functions in the potyviral lifecycle, including HCPPro, which plays a role in aphid transmission, viral movement, pathogenicity determination, and suppression of host gene silencing (Valli et al. 2018). Nine PVY strains are currently known, and each differs at the biological, serological, and molecular levels.

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In the United States, the most dominant PVY strain has been PVY^O. However, PVY^O has been progressively replaced by recombinant necrotic strains, including PVY^{N:O} and PVY^{NTN} (Funke et al. 2017; Karasev et al. 2011; MacKenzie et al. 2018). One of the contributing driving forces of the rapid spread of the necrotic recombinant strains is the popularity of strain-specific resistant cultivars in potato production that show resistance to the common PVY^O strain but not to other strains (Funke et al. 2017). A better understanding of the molecular mechanisms that allow some strains to overcome or suppress host resistance is needed for a long-term solution to control losses owing to PVY.

Plants have developed multiple layers of active resistance responses to defend themselves against viruses (Boualem et al. 2016). Two major classes of pathogen receptors have been identified: extracellular pattern recognition receptors (PRRs) and intracellular nucleotide binding leucine-rich repeat (NLR) immune receptors (Jones and Dangl 2006). The PRRs induce a rapid defense response after recognition of certain characteristic compounds or broadly conserved pathogen-associated molecular patterns in a process known as pattern-triggered immunity (PTI). Successful pathogens rely on effector proteins once in the host cytoplasm to suppress PTI. As a counterdefense response, plants evolved a second line of defense mediated by the intracellular NLR receptors that recognize the pathogen effector either directly or through their activities, which leads to a prolonged effector-triggered immunity (ETI) response (Jones and Dangl 2006). Many classes of plant viral proteins, including the coat protein, viral replicase, suppressor of silencing, the movement protein, or even virus-derived nucleic acids, can act as a pathogen effector (Mandadi and Scholthof 2013; Teixeira et al. 2019). The combined activities of PTI and ETI lead to activation of signal transduction cascades and defense responses, including an oxidative burst, hormonal changes, transcriptional

reprogramming, and programmed cell death, called the hypersensitive response, which results in localized rapid cell death at the site of the pathogen attack to restrict the pathogen spread (Tsuda and Katagiri 2010). One of the earliest cellular responses after pathogen recognition is the rapid deposition of β -1,3-glucan callose on the plant cell walls, which results in the cell wall thickening (Jones and Dangl 2006; Tsuda and Katagiri 2010). Cell wall reinforcement with callose is a frequent plant response to viral infection to act as a physical barrier, and it is thought to block or delay local and systemic movements of viruses (Iglesias and Meins 2000).

In potato, hypersensitive resistance against PVY in several popular potato cultivars is often conferred by strain-specific *Ny* genes (Rowley et al. 2015; Valkonen 1997, 2015; Valkonen et al. 2017). Ten alleles of *Ny* genes against PVY have been described in *Solanum tuberosum* cultivars (Celebi-Toprak et al. 2002; Jones and Vincent 2018; Moury et al. 2011; Szajko et al. 2008, 2014; Tomczyńska et al. 2014). The *Ny* gene induces hypersensitive resistance against the ordinary PVY^O strain, which is accompanied by the induction of necrosis around the site of infection and the restriction of virus spread. However, the strain-specific nature of the resistance and its temperature sensitivity limit its effectiveness (Valkonen 1997, 2015; Valkonen et al. 1998, 2017). This results in the ability, for instance, of the necrotic strains to systemically infect plants (Moury et al. 2011) and the failure to restrict PVY^O at elevated growth temperature (Valkonen 1997; Valkonen et al. 1998). Studies with PVY chimeras containing portions of each non-recombinant virus strain (PVY^O and PVY^N) and site-specific mutants indicate that the avirulence determinant in PVY^O is defined by residues within positions 227 to 327 in the C-proximal part of HCPro (Moury et al. 2011; Tian and Valkonen 2013, 2015). Compared with PVY^O, the necrotic isolates contain eight amino acid polymorphisms in HCPro (N236I, L238K, A2247S, I252V, R262Q, K269R, R270K, and V301I) that determine the strain-specific signature. These residue substitutions likely change the HCPro structural conformation to overcome *Ny* (Moury et al. 2011; Tian and Valkonen 2013, 2015).

Here, we established the role of callose accumulation in the strain-specific response against PVY using potato cultivar Premier Russet (PR) as our model host. PR plants show no systemic virus infection on PVY^O inoculation but fail to control the necrotic PVY strains, in line with a strain-specific type of resistance (Goyer et al. 2015; Novy et al. 2008). We revealed that the failure to restrain PVY^O at elevated temperature or to restrain the necrotic PVY^{N:O} strain, which shares a type O genome except for the N-type P1 and HCPro genes, at either temperature correlated with a suppression of callose deposition. The necrotic PVY strain was naturally capable of suppressing pathogenesis-related induced callose accumulation in PR, which explains the ability of the virus to spread in this resistant host genotype. This strain-specific response is dependent on the PVY HCPro protein. The avirulence activity of HCPro^O in inducing callose deposition in the context of the PR potato cultivar can be dissociated from viral replication. Mutations within HCPro^O of the eight amino acid residues that define the difference between the O-type and N-type viral sequences resulted in the suppression of the callose response. Our results provide insights into mechanisms that allow the ordinary or the necrotic PVY strains to elude or suppress resistance responses in potato.

MATERIALS AND METHODS

Plant material, growth condition, and virus inoculation.

Plants of cultivars PR (Novy et al. 2008) and Katahdin (Nie et al. 2012) were propagated in the greenhouse with 16-h continuous light/8-h dark cycles and temperatures between 20 and 24°C during the day and between 15 and 18°C at night. Two- to three-week-old plants were used for all experiments. Frozen PVY^O-infected (accession number NY090031) and PVY^{N:O}-infected (accession

number NY090029) tobacco leaves were used as a source of viral inoculum. Frozen leaves were crushed in 0.2 M KHPO₄ inoculation buffer, and this was used to rub inoculate two compound leaf sets per potato plant. To ensure efficient viral infection, some assays, as indicated, required double inoculation. In these cases, the same leaf areas were inoculated twice with virus inoculum within an interval of 24 h. At least two plants per treatment were used, and each experiment was performed with at least two independent repeats. Where indicated, mock inoculation consisted of the same treatment using only the inoculation buffer.

Time course assay. PVY^O and mock-inoculated PR and Katahdin plants were initially maintained at 20°C in growth chambers (Percival Scientific) under 14-h light/10-h dark cycles for 24 h. On the second day postinoculation (day 2), plants were either maintained at 20°C (normal growth temperature) or placed at 28°C (elevated growth temperature). For each sample, one leaflet was collected both from the locally inoculated compound leaf and from the systemic compound leaf immediately above the inoculated leaf. The leaf samples were collected from day 1 to day 7 and kept at –80°C until use.

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay. Approximately 100 mg of leaf tissue was ground in 500 μ l of general extract buffer 3 (Agdia) with five to six stainless steel beads (3 mm) added in a 2-ml microcentrifuge tube. The crude mixture was shaken continuously at 1,500 rpm for 5 min using a tissue homogenizer (SPEX Sample Prep 1600 MiniG) and centrifuged at 12,000 $\times$ g for 2 min, and 10 μ l of supernatant was diluted 1:10 in RNase-free water. The total RNA concentration present in the crude extract was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). A final concentration of 100 pg of total RNA in the crude extract was used as a template in a 10- μ l RT-LAMP reaction. The RT-LAMP assay was performed as previously described (Przewodowska et al. 2015; Treder et al. 2018) using the Y4 primer set, which targets the PVY coat protein. The reaction mixture (10 μ l) contained 0.375 μ M each outer primers (F3 and B3), 1.5 μ M each inner primers (FIP and BIP), 0.75 μ M each loop primers (LB), 1 $\times$ Isothermal Master Mix containing proprietary fluorescent dye (Novazym Polska sc.), and 0.25 U of AMV reverse transcription (Superscript III; Invitrogen) with 100 pg of total RNA as a template. The thermal profile in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) included 60 cycles of 30 s at 65°C. The amplification was followed by the analysis of the melting temperature (65 to 95°C at 0.5°C/s). The cycle quantification (Cq) value was considered positive if it was three standard deviations below the average Cq value for mock-treated plants. In our experiments, the average Cq value of mock-treated samples was 30, with a standard deviation of 4. Therefore, the threshold was set at a Cq of 18.

Reverse transcription quantitative PCR assay. Twenty-four hours after mock and PVY^O inoculation of PR and Katahdin potato leaflets, total RNA was extracted by the RNeasy plant mini kit (Qiagen) from three leaves with an equal amount of tissue and treated with the DNase max kit (Qiagen) according to the manufacturer's instruction. The purity and integrity of total RNA were determined by NanoDrop (Thermo Fisher Scientific) measurement and electrophoresis in 1% agarose gel. Complementary DNA (cDNA) was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad) per the manufacturer's instructions. Quantitative PCR (qPCR) was performed for the quantification of the expression level of potato β -1,3-D-glucan endohydrolase (PGSC0003DMT400051528) using forward (CCAGAGCTTGAGAAACATTTTGAT) and reverse (TAGTTTCAGCAGAAATGTCCCAAAC) primers and the internal control, eukaryotic elongation factor 1 α (Chen and Halterman 2011). The qPCR was performed in a 10- μ l reaction volume with Sso Advanced universal SYBR green supermix (Bio-Rad) in a Bio-Rad machine (C1000 touch thermal cycler) under the following conditions: 95°C for 3 min and 39 cycles at 95°C for 10 s and 57°C for 30 s followed by a default melting curve setting in the machine. A melting

curve was generated to verify the presence of a single amplicon of each qPCR product. The change in expression of candidate genes was calculated based on the $2^{-\Delta\Delta CT}$ value (with CT being the threshold cycle for target amplification) as previously described (Livak and Schmittgen 2001). The experiment was repeated twice with three replications of each sample. A significant difference among the samples was calculated by Tukey's test by using SAS (version 9.4; SAS Institute Inc.).

Reverse transcription PCR assay. Twenty-four hours after mock or HCP^o infiltrations of the PR plants, total RNA was extracted by Trizol (Invitrogen) from three leaves with an equal amount of tissue and treated with the DNase max kit (Qiagen) according to the manufacturer's instruction. For each sample, 100 ng of total RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instruction. One microliter of the reaction was used as a template for standard PCR with 55°C as the annealing temperature, and specific primers (forward 5' GATCTCGAGCTTCTCAATGAGATATT 3' and reverse 5' TACACATAATTACCATCTTTTCGAACTCG 3') targeting a conserved region within the HCP^o sequence (base pairs 354 to 890) were used. The expected size of the amplicon was 536 nucleotides. To rule out DNA contamination, we also included total RNA extract as a template for PCR.

Callose assay on virus-inoculated leaves. Two leaflets per plant of PR and Katahdin were rub inoculated separately with inoculum of PVY^o and PVY^{N:O}. The plants were initially maintained at 20°C. After a second inoculation after 24 h, one set of plants was transferred to a temperature of 28°C, and the second set was kept at 20°C. Twenty-four hours later, two leaf discs were collected from each inoculated leaflet using a #4 stainless hole punch. Each set of leaf discs was placed in a single well of a 12-well tissue culture plate with 3 ml of destaining solution (1:3 acetic acid: ethanol). The 12-well plate was shaken at 150 rpm overnight at room temperature. The destaining solution was then replaced with 3 ml of wash solution (100 mM K₂HPO₄) and kept in the rotating table for 30 min. The wash solution was then replaced with staining solution (100 mM K₂HPO₄ with 0.1 mM aniline blue). The leaf discs were stained overnight, and they were visualized with an Olympus BX60 Epifluorescence Microscope. Images of four different microscopic fields of the entire leaf disc were captured with an Olympus DP73 camera. Eight images were taken per leaflet per plant. The optimal excitation wavelength for aniline blue was 370 nm, and the emission maximum was by 509 nm. For documentation, we used the Zeiss AxioVision 3.0 software. The images were acquired with 1,600 × 1,200 resolution and left untreated except for the adjustment of brightness. Generally, we used 30- to 800-ms exposure time. Callose deposited area and number of callose spots were quantified using ImageJ software (Rueden et al. 2017). The experiment was done twice.

For the flagellin 22 (flg22) assay on virus-inoculated leaves, two leaves of PR or Katahdin were double-rub inoculated with either buffer or PVY^o or PVY^{N:O} inoculum with an interval of 24 h and kept at 20°C; 12 h after the last inoculation, the inoculated area was infiltrated with 1 µM synthetic flg22 peptide (catalog number RP19986; GenScript) and placed at 20°C for 24 h. Two leaf discs were collected for the callose assay from each set of plants. The experiment was done twice.

Callose assay on *Agrobacterium*-infiltrated leaves. PVY^o HCP^o (from GenBank accession number JX424837), PVY^N HCP^o (from GenBank accession number X97895), and the mutant PVY^o HCP^o with the eight amino acid substitutions were synthesized by Twist Bioscience, cloned into the pTwist ENTR vector adapted for use with Gateway Cloning Technology (Thermo Fisher Scientific), and recombined into the Gateway-compatible binary vector pGWB2 (Nakagawa et al. 2007) under control of the cauliflower mosaic virus 35S promoter for expression using *Agrobacterium tumefaciens* strain GV3101. The mutant HCP^o bears eight amino acid differences (N236I, L238K, A2247S, I252V,

R262Q, K269R, R270K, and V301I) that constitute the strain group-specific amino acid signature between ordinary and necrotic strains (Tian and Valkonen 2013). The HCP^o construct with an early stop codon was PCR generated using the QuikChange Site-Directed Mutagenesis strategy (Agilent), with the above PVY^o HCP^o cassette as a template and two overlapping primers (forward 5' GTTCAGTTCTAGAATGCTGAT 3' and reverse 5' ATCAG CATTCTAGAACTGAAC 3'), which inserted a stop codon after the second AUG codon located six bases downstream and in frame with the first AUG triplet. Briefly, after PCR amplification using PfuUltra II fusion High-fidelity DNA polymerase (Agilent), the reaction was treated with DpnI (NEB) to remove the original template and directly transformed into competent bacteria.

Two leaflets per 4-week-old plant of PR and/or Katahdin were infiltrated with the indicated constructs. Infiltration with *A. tumefaciens* strain GV3101 alone was used as a control. The agroinfiltration procedure was performed as previously described (Abdullah and Halterman 2018) with the exception that the cells were diluted to an optical density (OD)₆₀₀ of 0.1 with buffer containing 200 µM acetosyringone. After agroinfiltration, the two complete sets of plants were placed at either 20 or 28°C for 24 h. Two leaf discs were collected from the agroinfiltrated leaflets and treated as described above for callose assay. The experiment was repeated for a total of three replicates.

To test the effect of flg22 on callose accumulation, two leaflets per plant of PR and Katahdin were first agroinfiltrated with PVY^o HCP^o, PVY^N HCP^o, mutant PVY^o HCP^o, or *Agrobacterium* alone, and they were kept at 20°C for 24 h. Next, 1 µM flg22 peptide was infiltrated in the agroinfiltrated areas with or without 0.5 µM callose synthase inhibitor 2-deoxy-D-glucose (2-DDG; Thermo Fisher Scientific). Two leaf discs were collected 24 h later for the callose assay.

Statistical analyses. The data from two experimental repeats, which represented at least a total of 16 images, were combined for analysis. Callose area and number of callose spots were subjected to analysis of variance using a completely randomized design, and treatment means were separated using Fisher's least significant differences test ($P \leq 0.05$) using the Agricolae package in R (v2.11.1; <https://www.rstudio.com/>; R core team 2012).

RESULTS

Callose accumulation is involved in PVY resistance in PR, and it is sensitive to temperature. Callose deposition is often proposed to work in concert with earlier defense responses to restrict virus movement beyond the site of infection (Wu et al. 2018). We thus investigated the role of callose deposition in the strain-specific and temperature-sensitive resistance against PVY found in potato PR (Novy et al. 2008). We validated the temperature sensitivity of resistance in PR by inoculating the plants with PVY^o and next moving them from 20°C to an elevated growth temperature of 28°C (Supplementary Fig. S1). At 7 days after inoculation at 20°C, the virus level in the PVY^o-inoculated PR plants, measured by an RT-LAMP assay (Treder et al. 2018), was above the preset threshold (with a C_q value = 18) in both local and systemic leaves, and it was similar to the level in mock-inoculated plants, indicating a resistance phenotype. However, at 28°C, the amount of PVY^o in the PR plants was below the preset threshold 2 days after the temperature shift (day 4) in both local and systemic leaves, indicating a breakdown in resistance. As a control, PVY^o was present in both local and systemic leaves at either temperature in susceptible Katahdin plants that show no resistance against PVY (Nie et al. 2012) (Supplementary Fig. S1).

We assessed the callose response on PVY infection in resistant PR plants by monitoring callose accumulation using aniline blue staining 24 h after double-virus inoculation (Fig. 1). Our results revealed that PVY^o inoculation resulted in a significant accumulation of callose (both callose area and number of spots) in the

resistant PR leaflets compared with the mock treatment when the plants were kept at 20°C (Fig. 1). In contrast, leaflets of susceptible Katahdin did not accumulate callose in response to PVY^O more than the mock-inoculated control (Supplementary Fig. S2). We established that the callose response was strain specific, because the necrotic PVY^{N:O} strain, which shares a type O genome except for the N-type P1 and HCPro genes, failed to induce callose accumulation at 20°C in either PR (Fig. 1) or Katahdin (Supplementary Fig. S2) compared with the mock-inoculated control. An elevated growth temperature (28°C) after PVY^O inoculation significantly reduced callose accumulation in PR plants compared with 20°C. A slight increase in the number of callose spots was observed in response to PVY^{N:O} at 28°C, but the total callose area was not significantly different from the PVY^O- or mock-inoculated control at 28°C.

HCPro is the pathogen effector of callose response. Callose production is typically triggered by pathogen elicitors as part of the early plant defense responses. One of the effector proteins previously implicated in eliciting hypersensitive resistance against PVY is the HCPro protein (Tian and Valkonen 2013, 2015).

To test whether HCPro from PVY^O (HCPro^O) out of the context of viral replication specifically elicited callose deposition in PR, we transiently expressed HCPro^O in PR potato leaflets and assessed callose formation 24 h postinfiltration (Fig. 2). At 20°C, transient expression of HCPro^O induced significantly more callose (both callose area and number of spots) than with *Agrobacterium* alone in the PR plants (Fig. 2). Insertion of an early stop codon to prevent HCPro^O protein synthesis failed to induce callose, in line with a phenotype dependent on protein expression (Supplementary Fig. S3). Consistent with the temperature sensitivity observed with virus infection, callose accumulation was significantly reduced at 28°C upon the overexpression of HCPro^O in PR plants compared with that observed at 20°C (Fig. 2). HCPro^O alone failed to induce callose in susceptible Katahdin plants at either temperature (Supplementary Fig. S4). The strain specificity of the callose response was replicated with the N-type HCPro from a necrotic PVY strain (HCPro^N) (Fig. 2). Consistent with the ability of PVY^N strains to overcome defense response, infiltration of HCPro^N alone failed to induce callose at either temperature in both PR plants and susceptible Katahdin (Fig. 2 and Supplementary Fig. S4). The

Fig. 1. Callose response on potato virus Y (PVY) inoculation in resistant cultivar Premier Russet (PR) plants at different temperatures. **A**, Representative images of callose deposition responses in PR mock-, PVY^O-, and PVY^{N:O}-inoculated plants 24 h after double inoculation at two different growth temperatures. **B**, Boxplots of callose area and spots in the various treatments. The data represent a total number of 16 images per treatment.

expression of the HCPro constructs in planta was verified by reverse transcription PCR (Supplementary Fig. S5). Altogether, the data are in line with a specific recognition of the HCPro^O protein as the effector eliciting defense responses in the resistant host.

PVY^{N:O} and N-type HCPro suppress callose induction in PR plants. The accumulation of callose is primarily determined by a balance between the enzymatic activity of callose synthase, the endogenous enzyme mediating callose synthesis, and β -1,3-glucanases, hydrolytic enzymes that specifically degrade callose (Wu et al. 2018). Mining transcriptional data of susceptible cultivar Russet Burbank potato in response to PVY^O (Goyer et al. 2015) revealed a transcriptional upregulation of a β -1,3-D-glucan endohydrolase (PGSC0003DMT400051528), suggesting that this gene may be responsive to virus inoculation. We thus assessed transcriptional changes of this β -1,3-D-glucan endohydrolase 24 h after PVY^O inoculation of PR and Katahdin plants grown at 20°C

(Table 1). The reverse transcription qPCR analysis revealed a 16-fold increase of the expression level of the β -1,3-glucanase transcript in the PVY^O-infected susceptible Katahdin plants. However, we observed a downregulation of its expression in the PVY^O-inoculated PR plants compared with that in the mock-treated plants (Table 1). Such results suggest the importance of this β -1,3-glucanase during viral infection.

TABLE 1. Change in transcript accumulation of β -1,3-glucanase (PGSC0003DMT400051528) at 48 h after potato virus Y^O inoculation

Cultivar	Fold change ^a	<i>Pr</i> > <i>F</i>
Premier Russet	−3.5 ± 0.3	<0.0001
Katahdin	+4.4 ± 0.3	<0.0001

^a Log twofold change compared with mock inoculation.

Fig. 2. Callose response to the transient expression of potato virus Y (PVY) helper component proteinase (HCPro) in resistant cultivar Premier Russet (PR) plants. **A**, Representative images of callose deposition responses in PR plants 24 h after mock (Agro), PVY^O HCPro (HCPro^O), or PVY^N HCPro (HCPro^N) infiltration at two different growth temperatures. **B**, Boxplots of callose area and spots in the various treatments. The data represent a total number of 16 images per treatment.

Although the lack of callose deposition in the susceptible plants was anticipated to be linked to the absence of host response on PVY inoculation (Supplementary Fig. S2), the above results prompted us to test whether the induction of β -1,3-D-glucan endohydrolase in susceptible plants after PVY^O infection reveals an ability of the virus to actively suppress callose formation. We thus infiltrated Katahdin plants with the synthetic 22-amino acid peptide from the conserved N-terminal part of flagellin (flg22), a known bacterial elicitor and callose inducer (Gómez-Gómez and Boller 2000), after PVY^O and PVY^{N:O} inoculations (Fig. 3). We hypothesized that, with the induced upregulation of the β -1,3-D-glucan endohydrolase in the susceptible plants, PVY^O infection would block flg22-induced callose accumulation. Infiltration of potato leaflets with flg22 alone strongly induced callose deposition in Katahdin plants within 24 h compared with mock inoculation (Fig. 3). flg22 failed to induce callose in susceptible leaflets inoculated with PVY^O, in line with the upregulation of the β -1,3-glucanase expression level. We observed a similar phenotype in leaves inoculated with PVY^{N:O} (Fig. 3), revealing the ability of PVY to suppress callose accumulation in a strain-nonspecific manner. However, callose

formation was not suppressed in the PVY^O-inoculated resistant PR plants (Fig. 4). The level of callose accumulation was not significantly different than that observed with the infiltration of flg22 alone or that induced during the resistance response by PVY^O alone. In contrast, PVY^{N:O} maintained its ability to reduce callose levels in PR leaflets exposed to flg22 to background level (Fig. 4). Together, these data suggest that PVY is capable of suppressing flg22-induced callose accumulation during susceptible interactions in a strain-nonspecific manner. However, PVY^O is not able to suppress callose in PR plants, possibly because of the much stronger activation of defense responses.

Our previous results demonstrated that HCPro^O alone out of the context of viral replication induced callose during a resistant interaction in PR plants. However, no callose was observed in susceptible plants or in response to HCPro^N. To determine whether the ability of PVY^{N:O} to suppress flg22-induced callose was dependent on its HCPro, we repeated the flg22 experiment with transient expression of HCPro in PR plants (Fig. 5). The assay revealed that the expression of HCPro^N limited flg22-induced callose deposition to a similar extent as 2-DDG, a potent callose

Fig. 3. Suppression of flagellin 22 (flg22)-induced callose on potato virus Y (PVY) infection in susceptible cultivar Katahdin (Kat) plants. **A**, Representative images of flg22-induced callose deposition in susceptible Kat plants 24 h after mock, PVY^O, and PVY^{N:O} inoculations. **B**, Boxplots of callose area and spots in the various treatments. The data represent a total number of 16 images per treatment.

synthesis inhibitor. No reduction in callose accumulation was observed on expression of HCPro^O. To determine whether this lack of reduction in callose formation resulted from specific HCPro^O recognition in PR, we tested a mutant version of HCPro^O (HCPro^{mut}), in which we introduced mutations encoding eight amino acid changes that were previously shown to induce an N-type HCPro conformation to presumably avoid recognition by the Ny-like protein (Tian and Valkonen 2013, 2015). Unlike HCPro^O, overexpression of HCPro^{mut} alone did not result in major callose accumulation in PR leaflets. Both callose area and the number of spots were not significantly different than those observed with the infiltration of HCPro^N. These mutations were sufficient to restore the ability to HCPro^O to block flg22-induced callose, despite the resistant genetic background of the plant, indicating functional similarity to HCPro^N.

DISCUSSION

In this study, we have determined that strain-specific resistance to PVY^O in potato PR results in the induction of callose deposition and

restricts both local and systemic spread of the virus. This response is compromised by the virulent necrotic strain and by elevated growth temperature. Furthermore, we found that PVY^{N:O} was naturally capable of suppressing pathogenesis-related induced callose accumulation in PR, which explains the ability of the virus to spread in this resistant host genotype.

The β -1,3-glucan callose is a cell wall polymer and a well-known participant of plant immune responses at a very early stage of pathogen attack. The dynamic turnover of callose is regulated by callose synthases and β -1,3-glucanases that produce or breakdown callose aggregation, respectively (De Storme and Geelen 2014; Wu et al. 2018). Glucanases are divided into different classes based on their sequence similarities, their protein characteristics, their expression level, and localization (Ward et al. 1991). They are also recognized as inducible pathogenesis-related proteins (van Loon et al. 2006). For instance, transcriptional upregulation of different classes of glucanases was reported during early plant responses to susceptible potato–PVY^{NTN} interaction (Baebler et al. 2009; Kogovšek et al. 2010; Otulak-Kozziel et al. 2018; Pompe-Novak et al. 2006). In this study, we provide evidence that interference with

Fig. 4. Suppression of flagellin 22 (flg22)-induced callose on potato virus Y (PVY) infection in resistant cultivar Premier Russet (PR) plants. **A to F,** Representative images of flg22-induced callose deposition in the resistant PR plants 24 h after mock, PVY^O, and PVY^{N:O} inoculations. **G and H,** Boxplots of callose area and spots in the various treatments. The data represent a total number of 16 images per treatment.

callose deposition underlies PVY pathogenicity at least in potato. Our results are consistent with a scenario where PVY induces activity of a class I β -1,3-glucanase in susceptible potato hosts to facilitate infection. Such induction correlated not only with the lack of callose formation in susceptible Katahdin plants but also, with the ability of the virus to block flg22-induced callose. This role of the β -1,3-glucanase in viral infection is in line with previous reports. The overexpression of the class III callose hydrolysis enzyme in resistant cultivar Santé potato lines favored a rapid viral spread of PVY^{NTN} (Dobnik et al. 2013). In mutant tobacco plants deficient in class I β -1,3-glucanase expression, symptomology and spread of tobacco mosaic virus were reduced, and they correlated with increased callose accumulation at the virus-induced lesions (Bucher et al. 2001; Iglesias and Meins 2000).

In the resistant PR plants, recognition of PVY^O resulted in a reduction in β -1,3-glucanase transcription, which correlated with significant callose formation, in line with its importance in PR-mediated resistance. However, we cannot rule out the possibility that callose accumulation is also affected by increased activity of callose synthase during the resistance response. Our results indicate that callose accumulation in PR responds to strain-specific variation within the HCPro effector from PVY. Using a series of chimeric PVY strains, Tian and Valkonen (2013) demonstrated that the genomic region of PVY that elicits resistance maps to a region that encodes the HCPro (Tian and Valkonen 2013, 2015).

In this work, we have found that HCPro from PVY^O, and not from PVY^N, outside of the context of viral replication was sufficient to trigger callose accumulation in the PR plants similar to the full

infectious virus. The functional makeup of HCPro includes the N-terminal region with the putative insect vector binding involved in transmission, the central region with the RNA binding domains as a function in silencing, and the C-terminal region with the viral capsid binding region for movement and the protease function (Plisson et al. 2003; Valli et al. 2018). Eight amino acid residues within its C-terminal region that differ between PVY^O and PVY^N are responsible for changing the HCPro protein structure to render PVY^N avirulent on potato with *Ny* resistance (Tian and Valkonen 2015). Here, we revealed that these same eight amino acids are also essential for the suppression of callose accumulation during a resistance response. It remains to be determined the mechanism underlying this ability of the HCPro protein to block callose accumulation, which could result from a transcriptional regulation of the β -1,3-glucanase.

Temperature sensitivity of disease resistance has been observed in several *R* gene-mediated defense responses, resulting in an accelerated breakdown of the plant defense responses (Venkatesh and Kang 2019). The modulation of pathogen responses by growth temperature adds an additional layer of complexity of plant resistance that needs to be further explored. We validated the temperature-sensitive nature of PVY resistance in PR as reported for several immune responses in plants (Hua 2013) and also, for other *Ny* potato genes conferring hypersensitive resistance against PVY (Valkonen 1997; Valkonen et al. 2017; Venkatesh and Kang 2019). Although local necrotic lesions developed at a 20°C growth temperature in *Ny-1* cultivar Rywal potato on PVY infection, plants were systemically infected when grown at 28°C (Lukan et al. 2018;

Fig. 5. Suppression of flagellin 22 (flg22)-induced callose on helper component proteinase (HCPro) infiltration in cultivar Premier Russet (PR) plants. **A**, Representative images of flg22-induced callose deposition 24 h after mock, potato virus Y^O (PVY^O) HCPro (HCPro^O), PVY^N HCPro (HCPro^N), and mutant PVY^O HCPro (HCPro^{mut}) infiltration. As a control, flg22 was coinfiltrated with callose synthase inhibitor 2-deoxy-D-glucose (2-DDG). **B**, Boxplots of callose area and spots in the various treatments. The data represent a total number of 16 images per treatment.

Szajko et al. 2008, 2014). Similar phenotypes were observed in potato cultivars Exploits (Nie et al. 2012) and Pito (Valkonen 1997) to PVY^O and *Solanum sparsipilum* and *Solanum sucrense* to PVY^N (Valkonen 1997), which failed to develop hypersensitive resistance when the growth temperature was increased from 16 and 18°C to 24°C, resulting in severe symptoms, such as leaf drop and mosaic. The molecular details of temperature sensitivity in resistance responses are largely unknown. Work in 2010 revealed that a single-point mutation in the tobacco *N* gene resulted in the induction of cell death on tobacco mosaic virus inoculation at both low and high temperatures (Zhu et al. 2010). It was concluded that the *N* protein itself or some potential interacting chaperones could be responsible for the temperature sensitivity of the hypersensitive cell death response. However, work on the bacterial elicitor flg22, which is recognized by the flagellin sensing 2 (FLS2), revealed that elevated temperature resulted in a strong inhibition of the flg22-induced response, including a decrease in FLS2 transcription and protein accumulation (Janda et al. 2019). This led to increased susceptibility to the bacterial pathogen. Our results revealed that the failure of induction of callose response on virus infection at elevated temperature might be part of the breakdown of the PR resistance. However, additional experiments are needed to determine if callose accumulation or other resistance responses are affected by elevated temperatures.

Ny is a commonly found PVY^O-associated resistance gene that has been reported in several popular potato cultivars (Rowley et al. 2015). This strain-specific response is dependent on the PVY HCPro protein (Tian and Valkonen 2013).

Our work demonstrates that PR shows strain-specific resistance to PVY^O but does not have the same level of resistance to necrotic PVY strains, which is consistent with previous studies that initially characterized resistance in this cultivar (Novy et al. 2008). We have found that the strain-specific resistance in PR is determined by HCPro, similar to resistance mediated by *N* genes in potato, including *Ny* and *Nc* to PVY^O and PVY^C strains, respectively (Moury et al. 2011; Tian and Valkonen 2013). Resistance in PR resistance is temperature sensitive as reported for *Ny* gene resistance (Valkonen 1997). Together, these data suggest the presence of an *Ny*-like gene in the PR cultivar, although we cannot rule out that multiple active *N* genes are present. This study will allow for a tractable genetic system to study the strain specificity associated with this resistance, and it provides a useful tool to ultimately identify the gene or genes responsible for resistance.

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