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Evaluation of potato virus X resistance in potato cultivars and identification of an innate immunity-independent resistance phenotype

Jiahui Liu, Ye Liu, Yue Fang, Lili Zhang, Kaixin Yu, Xiaoyun Wu and Xiaofei Cheng*

Abstract

Potato virus X (PVX) is a widely distributed viral pathogen that causes significant losses in potato production by co-infecting with potato virus Y or potato virus A. In this study, the resistance of 23 potato cultivars to PVX was dissected in detail using a PVX infectious clone containing a yellow fluorescent protein (YFP). Among them, four potato cultivars (Longshu-3, Eugene, Atlantic and Waiyin-2) were found to carry an *Rx* gene that confers extreme resistance to PVX; one cultivar (Waiyin-1) displayed partial resistance and was able to delay PVX infection by ~ 5 days; while the rest eighteen potato cultivars were susceptible to PVX. Moreover, we found that the replication but not cell-to-cell or long-distance movement of PVX was inhibited in Waiyin-1. Finally, we determined that the expression of pathogenesis-related (*PR*) genes in Waiyin-1 was not triggered by PVX infection at early infection stage, whereas they were triggered in the *Rx*-carrying cultivar Atlantic during this period of time. In conclusion, our results confirm that *Rx* is a major type of resistance gene in potato cultivars in the Northeast part of China. Furthermore, the possible mechanism underlying Waiyin-1 resistance to PVX is discussed.

Keywords: Extreme resistance, Potato virus X, Potato, Rx, Pathogenesis-related genes

Background

Potato (*Solanum tuberosum* L.) is the world's fourth most important food crop, with a global production of about 388 million tons per year according to the Food and Agriculture Organization of the United Nations (FAO) statistical databases (www.statista.com/statistics/192966/us-potato-production-since-2000). China is the largest potato producer, accounting for 26.3% of global production. Along with the transportation of seed potatoes, potato diseases, especially viral diseases are rapidly spreading across potato planting areas worldwide. At present, potato production is being seriously threatened by virus infection, which not only causes yield and quality decline but also induces seed degradation (Solomon-Blackburn and Barker 2001a). Hitherto, more than 50 viruses have been identified in potato plants (Kreuze et al. 2020a).

Potato virus X (PVX), the type species of the genus *Potexvirus* in the family *Alphaflexiviridae*, is one of the major viruses infecting potato plants worldwide (Kreuze et al. 2020b). Although it does not induce severe symptoms on most potato cultivars, coinfection of PVX with other viruses, such as potato virus A (PVA) and potato virus Y (PVY), can cause serious yield and quality losses in potato production (Kreuze et al. 2020a). For example, coinfection of PVX with potato virus A (PVA) and potato virus Y (PVY) results in

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crinkle symptoms and even plant death (Nie and Singh 2013). The genome of PVX consists of a positive-sense single-stranded RNA (+ssRNA) of about 6.4 kilobase pairs (kb) in length that is encapsulated in flexuous, filamentous and non-enveloped viral particles, and encodes five open reading frames (ORFs) (Kreuze et al. 2020b). The largest ORF (ORF1) on the 5′-end of the genome encodes the viral RNA-dependent RNA polymerase (RdRp), which is absolutely required for viral replication (Betten et al. 2012). The partially overlapping ORFs [2, 3 and 4] comprise a so-called triple-gene-block (TGB) module, encoding three viral movement proteins, namely TGBp1, TGBp2 and TGBp3 (Beck et al. 1991; Tilsner et al. 2012). ORF5 encodes the viral coat protein (CP) that is involved in viral genome encapsulation and cell-to-cell movement (Cruz et al. 1998).

During a long history of coevolution with pathogens, plants have evolved sophisticated resistance mechanisms to combat the invasion of all phytopathogens including viruses (Chisholm et al. 2006). Effector-triggered immunity (ETI) is one of the main antiviral machineries, which is triggered via the recognition of pathogen effectors by intracellular nucleotide-binding domain leucine-rich repeat containing receptors (NLRs) that are historically termed resistance (R) proteins. To date, three *R* genes, namely R_x, N_x and N_b, have been characterized in potato, which confer resistance to PVX (Cockerham 1970; Solomon-Blackburn and Barker 2001b). The R_x gene encodes a coiled-coil NLR (CC-NLR) that specifically recognizes PVX CP and confers an extreme resistance (ER), a specific form of resistance without inducing visible local necrotic lesions (hypersensitive responses) (Bendahmane et al. 1995, 1997, 1999; Moffett et al. 2002). N_x and $N_b$induce necrotic lesions on the infection sites by recognizing viral CP and TGBp1, respectively (Tommiska et al. 1998; Malcuit et al. 1999; Marano et al. 2002). In addition, several atypical dominant viral resistance proteins (ADVRPs) have been characterized. For instance, Jacalin-type lectin required potexvirus resistance 1 (JAX1) from *Arabidopsis thaliana*, putative methyltransferase 1 from *Nicotiana benthamiana* (PNbMTS1) and Remorins (REMs) from potato. Both JAX1 and PNbMTS1 interfere with virus replication by targeting viral RdRp, whereas REMs inhibit the function of TGBp1 in viral cell-to-cell movement (Cheng et al. 2009; Yamaji et al. 2012; Sugawara et al. 2013; Yoshida et al. 2019).

Identification of virus-resistant potato cultivars and dissection of their resistance mechanisms will not only facilitate sustainable potato production but also benefit potato resistance breeding. In this study, we evaluated the PVX resistance of twenty-three potato cultivars and identified a novel resistance phenotype to PVX in a potato cultivar, which will further deepen our understanding of potato resistance to PVX.

Results

Resistance of different potato cultivars to PVX

Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that the tissue-culture plantlets of all 23 potato cultivars were free of PVX (Fig. 1a). These plantlets were then infiltrated with agrobacteria harboring PVX infectious clone pGR107. At 12 days post-inoculation (dpi), Kexin-18, Shepody and Netherlands-15 showed mild yellowing and/or mosaic symptoms, while other cultivars remained symptomless (Fig. 1b). Systemic leaves were analyzed by DAS-ELISA at 12 dpi. The results showed that the optical density values at 405 nm (OD405) of five cultivars, namely Longshu 3, Eugene, Atlantic, Waiyin-1 and Waiyin-2, were close to that of the negative control (healthy Zhongshu-2), whereas those of other 18 varieties were close to that of the positive control (Table 1). These data suggest that Longshu 3, Eugene, Atlantic, Waiyin-1 and Waiyin-2 are probably resistant to PVX infection. We also investigated the presence of PVX genomic RNA in the systemic leaves by RT-PCR at 15 dpi. Consistent with the DAS-ELISA results, an amplicon of the expected size of 1.5 kb was observed in all 18 ELISA-positive cultivars, whereas no band was amplified from Longshu 3, Eugene, Atlantic and Waiyin-2 (Fig. 1c). Unexpectedly, a 1.5-kb band was amplified in Waiyin-1 at 15 dpi (Fig. 1c). To further confirm the infection of PVX in Waiyin-1, we re-examined PVX genomic RNA in the systemic leaves of Waiyin-1 by RT-PCR at 9, 12 and 15 dpi. The results showed that the 1.5-kb band could only be amplified in the systemic leaves of Waiyin-1 at 15 dpi but not at 9 or 12 dpi (Fig. 1d). All amplicons were cloned and Sanger sequenced. The results confirmed that amplicons from the 18 ELISA-positive cultivars and Waiyin-1 were the genomic sequence of PVX. These results suggest that Longshu-3, Eugene, Atlantic and Waiyin-2 are fully resistant to PVX, Waiyin-1 is partial resistant to PVX, while the other 18 cultivars are susceptible to PVX.

To further confirm the resistance of Waiyin-1 to PVX, we constructed a PVX infectious clone that expresses a yellow fluorescent protein (pGR107-YFP) to facilitate the direct visualization of virus infection (Fig. 2a). Infection of *Nicotiana benthamiana* seedlings with pGR107-YFP caused typical symptoms of PVX such as mosaic and leaf scroll-down on systemic leaves at 7 dpi (Fig. 2b, left panel). Under ultra-violet (UV) light, bright yellow fluorescence was observed on symptomatic leaves (Fig. 2b, right panel). Potato seedlings of Longshu 3, Eugene, Atlantic, Waiyin-1, Waiyin-2 and Kexin-1 were then agroinfiltrated with pGR107-YFP. It was shown that yellow fluorescence was only observed on systemic leaves of

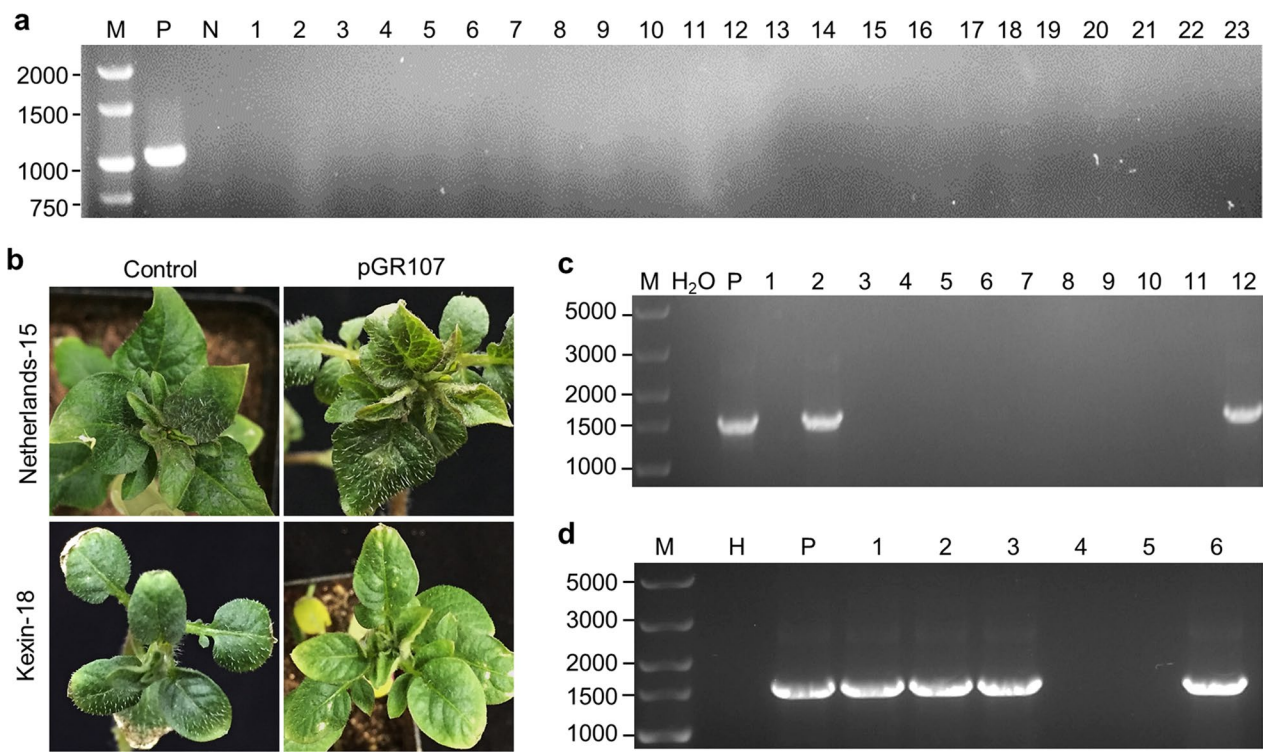

Fig. 1 Symptoms and detection of PVX in different potato cultivars. **a** RT-PCR analysis of the presence of PVX in potato plantlets using primer pair pvx-tgb2-f and PVXcpRT-R. M, DL2000 DNA marker (Vazyme); P, PVX-infected *Nicotiana benthamiana* leaf tissues (positive control); N, healthy *N. benthamiana* leaf tissues (negative control). Potato cultivars were arranged in the same order as in Table 1. **b** PVX symptoms on plantlets of Netherlands-15 and Kexin-18 at 12 dpi. **c** RT-PCR analysis of the presence of PVX in the systemic leaves of potato plantlets using primers PVX5875-F and PVXcpRT-R at 15 dpi. Lanes 1, 3, 5, 7, 9 and 11 are healthy Kexin-1, Atlantic, Eugene, Waiyin-2, Longshu-3 and Waiyin-1, respectively; lanes 2, 4, 6, 8, 10 and 12 are PVX-infected Kexin-1, Atlantic, Eugene, Waiyin-2, Longshu-3 and Waiyin-1, respectively. **d** Detection of PVX genome in Waiyin-1 and Kexin-1 using primers PVX5875-F and PVXcpRT-R at 9, 12 and 15 dpi. H, Healthy Kexin-1; P, positive control (PVX-infected *N. benthamiana* leaf tissues); lanes 1, 2 and 3 are systemic leaves of Kexin-1 agroinfiltrated with pGR107 at 9, 12 and 15 dpi, respectively; lanes 4, 5 and 6 are systemic leaves of Waiyin-1 agroinfiltrated with pGR107 at 9, 12 and 15 dpi, respectively

Kexin-1 at 12 dpi, which was used as a susceptible control (Fig. 2c, left panel). Interestingly, yellow fluorescence also appeared on systemic leaves of Waiyin-1 at 17 dpi (Fig. 2c, right panel), whereas no visible fluorescence signal was observed on the other 4 cultivars throughout the experimental period (data not shown). Together, these results confirm that Longshu 3, Eugene, Atlantic and Waiyin-2 are fully resistant to PVX, whereas Waiyin-1 has a partial resistance.

Identification of the Rx gene in resistant potato cultivars

Atlantic contains the *Rx* gene that confers extreme resistance against PVX (Nyalugwe et al. 2012). Since no obvious necrosis was observed on the inoculated leaves of four resistant cultivars (Longshu 3, Eugene, Atlantic and Waiyin-2), we supposed that the genomes of these four cultivars also contain the *Rx* gene or its paralogs. A specific band of about 1.2 kb in size was successfully amplified from the genomic DNA of Longshu-3, Eugene, Atlantic or Waiyin-2 with *Rx*-specific primers, while no corresponding band was amplified in Kexin-1 (negative control) (Fig. 3a). Interestingly, no *Rx*-specific band was amplified from Waiyin-1. These results suggest that Longshu-3, Eugene, Atlantic and Waiyin-2 may contain *Rx* or its paralog, while Kexin-1 and Waiyin-1 don't carry *Rx*. We further amplified the full-length coding region of *Rx* in Longshu-3, Eugene, Atlantic and Waiyin-2 by RT-PCR. The amplified fragments were then cloned and Sanger sequenced. Multiple alignment of the deduced amino acid sequences revealed that Rx of Atlantic and Longshu-3 is identical to Rx1 (GenBank accession no. AF265664); Rx of Eugene shares 96.48% and 95.31% aa identities to Rx1 and Rx2 (GenBank accession no. AJ249448), respectively; Rx in Waiyin-2 harbors a 32-aa deletion (693–724 aa) as compared to Rx1 (Fig. 3b). In the Neighbour-Joining (NJ) phylogenetic tree, all four

Table 1 ELISA results of potato cultivars infected by PVX at 12 dpi

Number	Potato cultivar name	OD_{405} value
1	Longshu-3	0.1730 ± 0.0990
2	Kexin-1	1.2285 ± 0.0007
3	Xingjia-2	1.3500 ± 0.0424
4	Early white	1.3215 ± 0.0219
5	Zhongshu-2	1.4070 ± 0.0000
6	Kexin-4	1.3140 ± 0.0042
7	Eugene	0.3820 ± 0.0849
8	Atlantic	0.2405 ± 0.0785
9	Waiyin-3	1.2965 ± 0.0177
10	Zhongshu-3	1.4405 ± 0.0516
11	Netherlands-15	1.4855 ± 0.0870
12	Waiyin-2	0.3170 ± 0.0905
13	Kexin-13	1.3945 ± 0.0007
14	Waiyin-1	0.2530 ± 0.1032
15	Plateau-7	1.3200 ± 0.2503
16	Purple Potatoes	1.5070 ± 0.2220
17	Kexin-2	1.4685 ± 0.1902
18	Chunshu-5	1.4245 ± 0.0233
19	Kexin-18	1.1970 ± 0.2447
20	Plateau-5	1.4650 ± 0.1004
21	Qingshu-4	1.4135 ± 0.0021
22	Shepody	1.4450 ± 0.1400
23	Red rose	1.2810 ± 0.0622
Negative control	Zhongshu-2	0.0545 ± 0.0219
Positive control	-	1.1075 ± 0.0262

Five plantlets per cultivar were inoculated and equal amounts of systemic leaf tissues were mixed for ELISA at 12 dpi. Healthy Zhongshu-2 was used as a negative control and the positive control was provided in the ELISA Reagent Set for PVX kit

Rx proteins cluster with Rx1 and Rx2 in the same clade (Fig. 3c). These results imply that the resistance of Longshu-3, Eugene, Atlantic and Waiyin-2 is conferred by the *Rx* gene, whereas there is no this gene in Waiyin-1's genome.

PVX infection is repressed in Waiyin-1

To understand the resistance mechanism of Waiyin-1 to PVX, we sap-inoculated Waiyin-1 and Kexin-1 plantlets with inoculum of pGR107-YFP-infected *N. benthamiana* leaf tissues. Fluorescent infection foci were observed in inoculated leaves of Kexin-1 under UV light at 10 dpi, while no infection spot was found in leaves of Waiyin-1 at the same time point (Fig. 4a). A time course analysis was subsequently performed to monitor PVX infection in the inoculated leaves using RT-PCR from 24 to 96 h post-inoculation (hpi). It was found that the genomic RNA of PVX in Kexin-1 showed a significant increasing trend from 48 hpi onwards, while in Waiyin-1 it just started to increase from 96 hpi onwards (Fig. 4b), suggesting that the early stage of PVX infection could be inhibited in Waiyin-1. To further confirm this hypothesis, we infiltrated Waiyin-1 and Kexin-1 leaves with agrobacteria harboring pGR107-YFP at a low concentration (an OD600 value of 0.0005), so that isolated cells became infected. The inoculated leaves were then observed with a confocal fluorescence microscope from 48 to 72 hpi at a 12-h interval. At 48 hpi, YFP-expressing cells were only observed in the inoculated leaves of Kexin-1, suggesting that PVX replication had occurred in Kexin-1 at this time point (Fig. 4c). Most fluorescent foci in Kexin-1 were expanded into more than two cells at 60 hpi, while only very faint YFP-signal was observed in isolated cells in Waiyin-1 at the same time point (Fig. 4c). At 72 hpi, cell patches with strong fluorescence were observed in Kexin-1, whereas the fluorescence in Waiyin-1 remained in isolated cells (Fig. 4c). Moreover, the fluorescence intensity in Waiyin-1 was significantly lower than that in Kexin-1 at both 60 and 72 hpi (Fig. 4d). Thus, PVX replication occurs before 48 h and starts cell-to-cell movement at about 60 hpi in Kexin-1, while the replication of PVX takes place at about 60 hpi and no cell-to-cell movement was observed even at 72 hpi in Waiyin-1.

To rule out the influence of cell-to-cell movement, a movement-defective PVX infectious clone (pGR107-TGBp2$_{\Delta 52-60}$-YFP) was constructed, in which a nine-amino-acid motif (GGAYRDGTK) between the two transmembrane motifs of TGBp2 was deleted (Fig. 5a). Our previous study showed that this motif plays a critical role in PVX cell-to-cell movement (Wu et al. 2019). Three-week-old *N. benthamiana* seedlings were infiltrated with agrobacteria harboring pGR107-YFP or pGR107-TGBp2$_{\Delta 52-60}$-YFP. Yellow fluorescence was observed in the local leaves of these agroinfiltrated seedlings under UV light from 2 dpi onwards. The fluorescence of pGR107-TGBp2$_{\Delta 52-60}$-YFP was restricted only in the infiltration area, whereas that of pGR107-YFP spread to upper systemic leaves at 6 dpi (Fig. 5b), suggesting that pGR107-TGBp2$_{\Delta 52-60}$-YFP is defective in cell-to-cell and long-distance movement. We then sap-inoculated seedlings of Waiyin-1, Kexin-1 and Atlantic using inoculum of pGR107-TGBp2$_{\Delta 52-60}$-YFP-infected *N. benthamiana* leaf tissues. Kexin-1 and Atlantic were used as susceptible and resistant controls, respectively. The accumulation of PVX genomic RNA in the local inoculated leaves was evaluated by RT-qPCR at 48 and 72 hpi. It was shown that PVX genomic RNA was increased about 1.75 times from 48 to 72 hpi in Kexin-1, whereas it was decreased in Atlantic in the same time period. Interestingly, the relative level of viral genomic RNA was hardly increased in Waiyin-1 from 48 to 72 hpi (Fig. 5c). Together, these

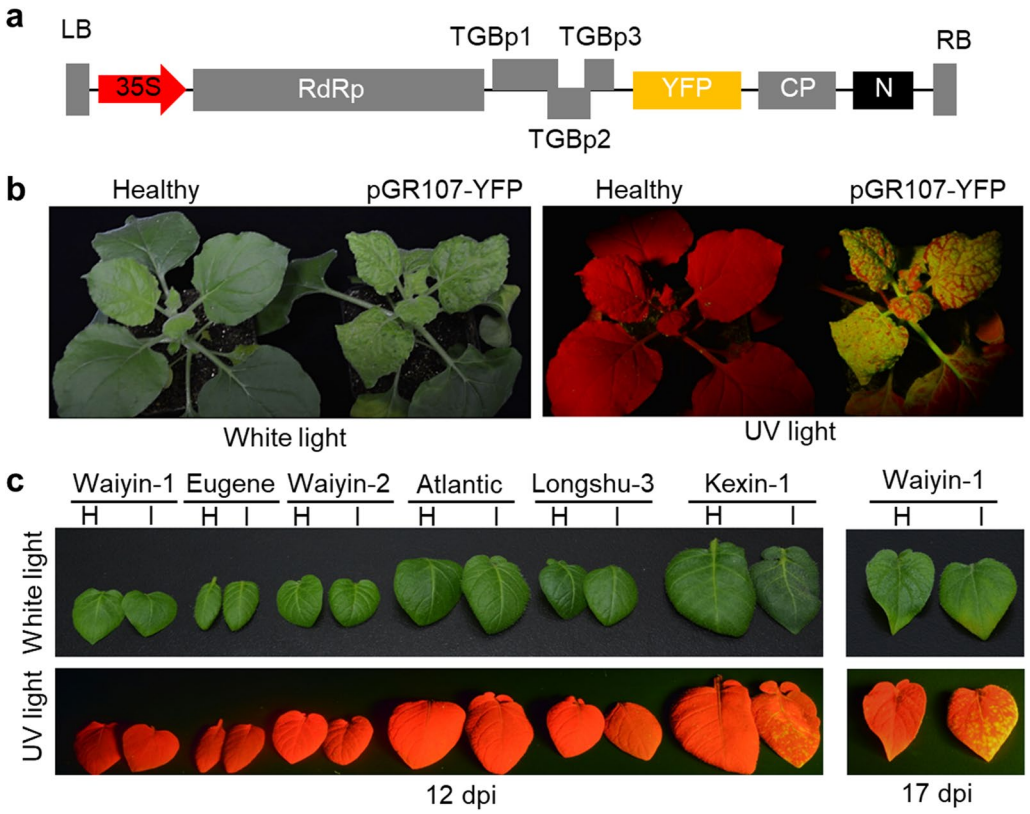

Fig. 2 Evaluation of the resistance of potato cultivars using a YFP-tagged PVX infection clone. **a** Diagram illustration of pGR107-YFP. LB and RB are left and right T-DNA border, respectively. 35S, cauliflower mosaic virus (CaMV) 35S promoter; N, NOS terminator; RdRp, RNA-dependent RNA polymerase; CP, coat protein. **b** Symptoms of *N. benthamiana* infected with pGR107-YFP under white (left panel) and UV light (right panel) at 7 dpi. **c** Symptoms on systemic leaves of potato plantlets infected with pGR107-YFP under white and UV light at 12 and 17 dpi. H, healthy control; I, PVX-infected treatment

results suggest that the replication of PVX is inhibited in Waiyin-1.

Innate immunity is not involved in early antiviral responses in Waiyin-1

To investigate whether the resistance of Waiyin-1 to PVX involves plant innate immunity, the expression of pathogenesis-related (PR) genes, e.g., *PR1*, *PR2, PR3* and *PR4*, was evaluated during PVX infection using RT-qPCR. The expression levels of *PR1*, *PR2*, *PR3* and *PR4* were almost unaltered after PVX infection in Kexin-1, whereas in Atlantic, they were peaked at 48 hpi and decreased at 72 hpi (Fig. 6a–d). These results suggest that the innate immunity triggered by the *Rx* gene typically peaked at 48 hpi. Intriguingly, the expression of *PR1* and *PR4* was not changed in Waiyin-1 at 48 hpi, but significantly increased at later infection stage, e.g., 72 hpi (Fig. 6a, d), while the expression of *PR2* and *PR3* was gradually increased from 48 to 72 hpi in Waiyin-1(Fig. 6b, c). We further investigated the expression of *WRKY1*, a member of the WRKY transcription factor family involved in disease resistance, abiotic stress and some developmental processes, e.g., stomatal movement, light and nitrogen signaling pathways (Qiao et al. 2016; Heerah et al. 2019), during PVX infection. The results showed that the expression of *WRKY1* was slightly increased at 48 hpi and decreased at 72 hpi in Atlantic, while no significant changes occurred in Keyin-1. Interestingly, the expression of *WRKY1* was also significantly increased in Waiyin-1 at 72 hpi but not altered at 48 hpi (Fig. 6e). Together, these results suggest that although the PVX resistance in Waiyin-1 requires the expression of *PR* genes, it is apparently differed from the *Rx*-triggered innate immunity.

Discussion

In this study, we evaluated the PVX resistance of 23 potato cultivars widely cultivated in China. The results suggest that most potato varieties we tested are susceptible to PVX infection and only 4 cultivars (Longshu-3, Eugene, Atlantic and Waiyin-2) are completely resistant to PVX (Table 1 and Fig. 1c). Our results further

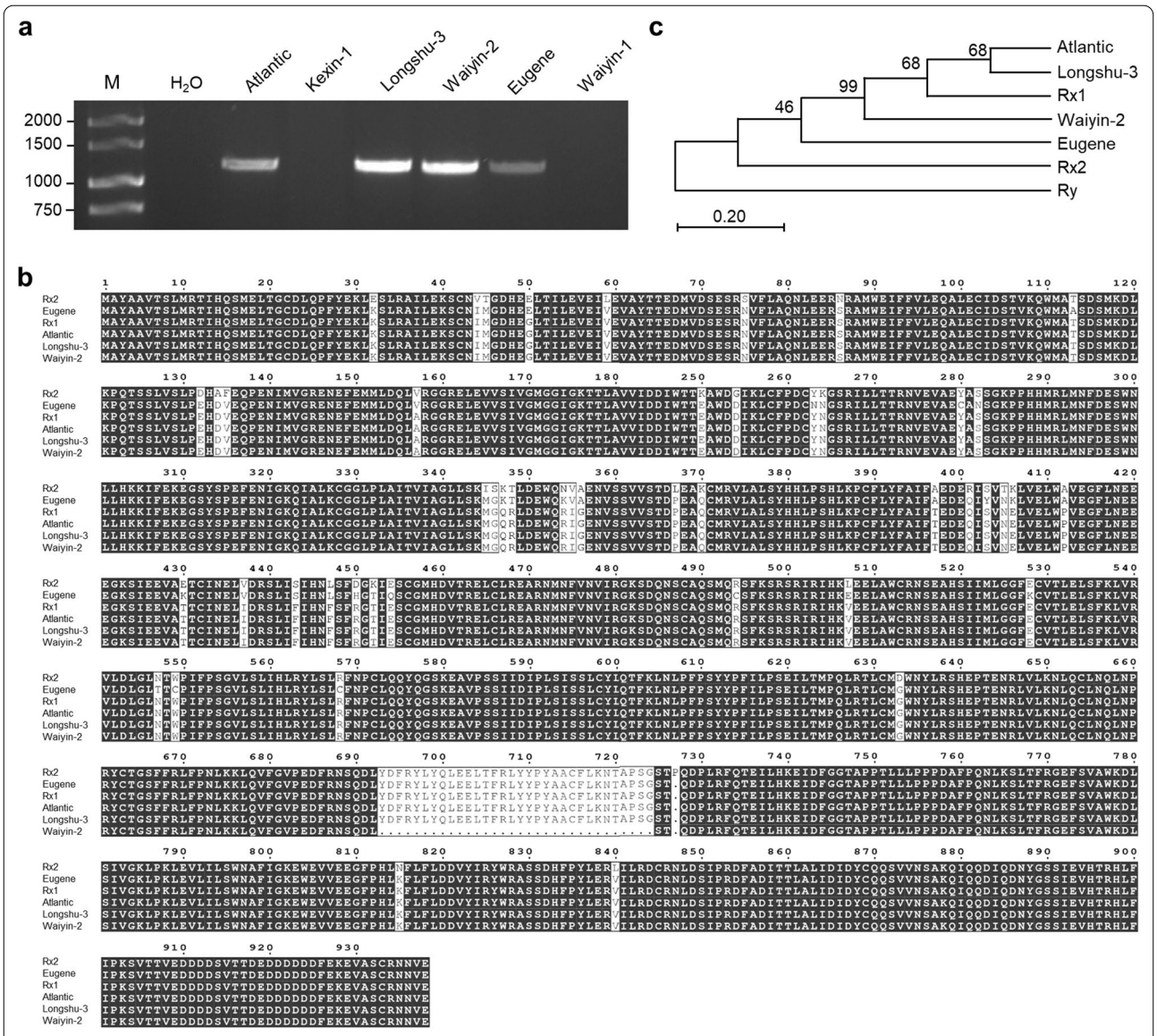

Fig. 3 Analyses of the *Rx* gene in PVX-resistant potato cultivars. **a** PCR detection of the *Rx* gene using primer pair RxSP-S3 and RxSP-A2. M, DL2000 DNA marker (Vazyme). **b** Multiple sequence alignment of the amino acid sequences of Rx1, Rx2 and their paralogs from Longshu-3, Eugene, Atlantic and Waiyin-2. The conserved identical amino acids are highlighted in black. Sequences were aligned with Clustal Omega and displayed by ESPript 3.0. **c** Phylogenetic analysis of Rx1 and its paralogs. The phylogenetic tree was constructed using the Maximum Likelihood method with 1000 bootstrap replicates and rooted to Ry (Genbank accession no. QEL52752)

reveal that the resistance of the 4 cultivars to PVX is conferred by the *Rx* gene, since the deduced amino acid sequences of Rx identified in these cultivars share high identities with Rx1 and Rx2, and all of them were clustered in the same phylogenetic branch (Fig. 3b, c). Noticeably, our bioinformatics analyses indicate that these *Rx* genes share considerable sequence diversity; especially, the deduced amino acid sequence of the Rx from Waiyin-2 lacks 32 aa in the LRR domain. It has been reported that mutations in the LRR domain are capable of altering the specificity of disease resistance conferred by the *Rx* gene (Farnham and Baulcombe 2006; Harris et al. 2013). Therefore, whether these *Rx* varieties have the same resistance spectrum to different PVX isolates is unknown since only the PVX UK3 isolate was evaluated in this study. Nevertheless, our results suggest that the genetic resources of PVX resistance in current potato varieties are considerably narrow, although it is believed that potato cultivars are abundant in genetic variability (Hardigan et al. 2017).

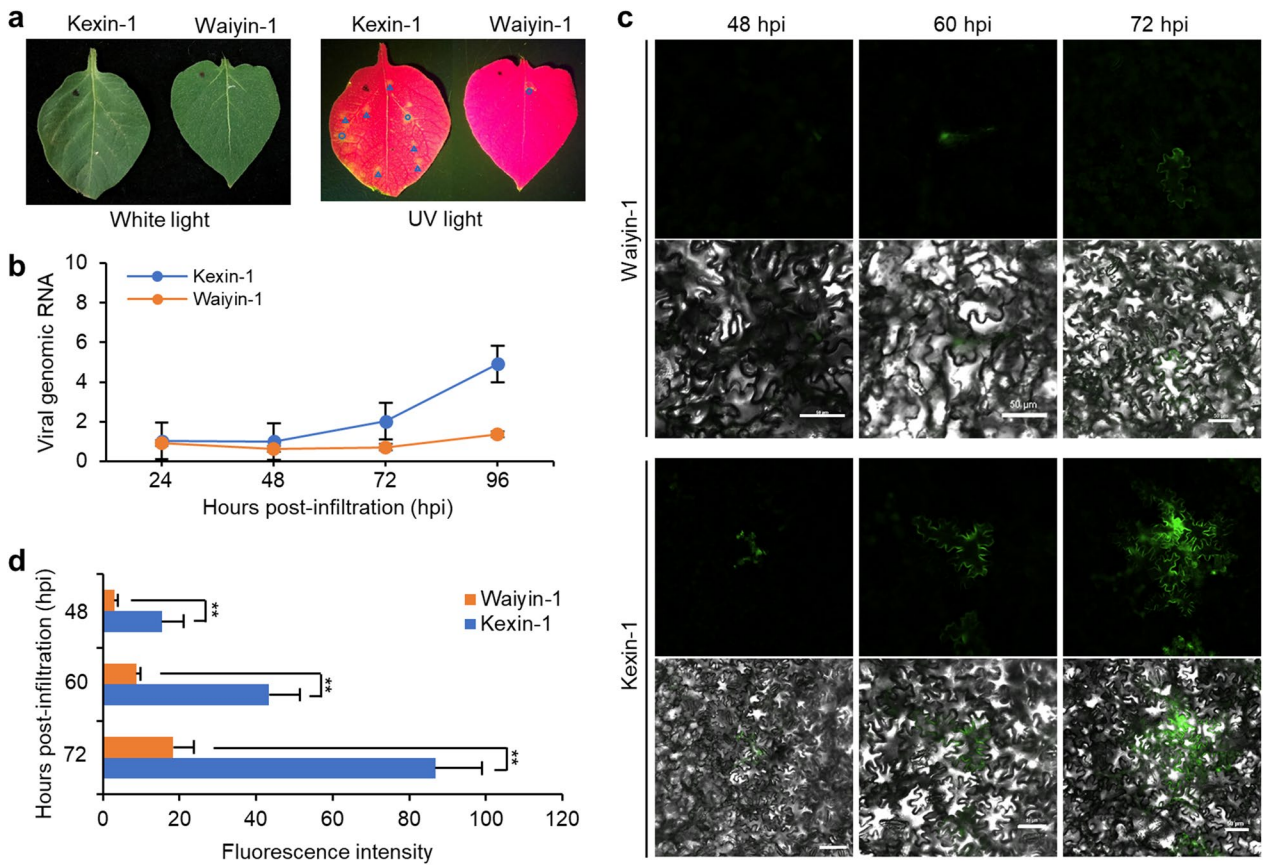

Fig. 4 Waiyin-1 suppresses PVX infection. **a** Visualization of the infection foci on the leaves of Waiyin-1 and Kexin-1 plantlets sap-inoculated with pGR107-YFP at 10 dpi. Circles, mechanical injuries; triangles, the infection foci. **b** RT-qPCR analysis of the accumulation of viral genome RNA in Waiyin-1 and Kexin-1 infected with pGR107-YFP. Potato *ACTIN 7* was used as an internal control. The genomic RNA level of pGR107-YFP in Waiyin-1 was normalized to 1. Bars represent means ± s.d. (n = 5). **c** Microscopic observation of the infection of Waiyin-1 and Kexin-1 by pGR107-YFP. **d** Quantification of fluorescence intensities in the leaves of Waiyin-1 and Kexin-1 infected with pGR107-YFP. Data represent means ± s.d. (n = 8); ** $P < 0.01$ (two-tailed Student's test)

As a consequence of the lack of proofreading activity in RNA-dependent RNA polymerases (RdRp), RNA viruses including PVX have a very high mutation rate (Steinhauer et al. 1992; Duffy et al. 2008), which may allow PVX to easily escape *Rx*-mediated resistance. Therefore, it is necessary to introduce new PVX resistance genes in future breeding projects to avoid any potential damage caused by resistance-breaking PVX isolates.

The most interesting finding in this study is the identification of a partial PVX resistance phenotype in Waiyin-1. In comparison to susceptible Kexin-1, the infection of PVX in Waiyin-1 was delayed by about 5 days. Replication and cell-to-cell movement assays showed that the replication but not movement of PVX was inhibited in Waiyin-1. Moreover, our results reveal that the expression profiles of *PR* genes in Waiyin-1 are apparently different from the *Rx*-triggered transcriptome reprogramming in which *PR* genes, e.g., *PR1*, *PR2*, *PR3* and *PR4*, are upregulated at early stage of PVX infection (Knip et al. 2019). The infection of PVX in Waiyin-1 activates the expression of the four *PR* genes and stress-related *WRKY1* at a later stage. These results suggest that the resistance of Waiyin-1 is not likely associated with a typical R protein. The resistances of most known ADVRPs, such as JAX1, REMs, PNbMTS1, h-type thioredoxin (ZmTrxh) and Tm-1, do not induce the expression of *PR* genes (Ishibashi et al. 2007; Cheng et al. 2009; Yamaji et al. 2012; Sugawara et al. 2013; Yoshida et al. 2019). Recently, a novel class of ADVRP that is able to confer a broad resistance to both positive- and negative-stranded RNA viruses was discovered. This class of ADVRP encodes a sulfotransferase to catalyze the conversion of salicylic acid (SA)

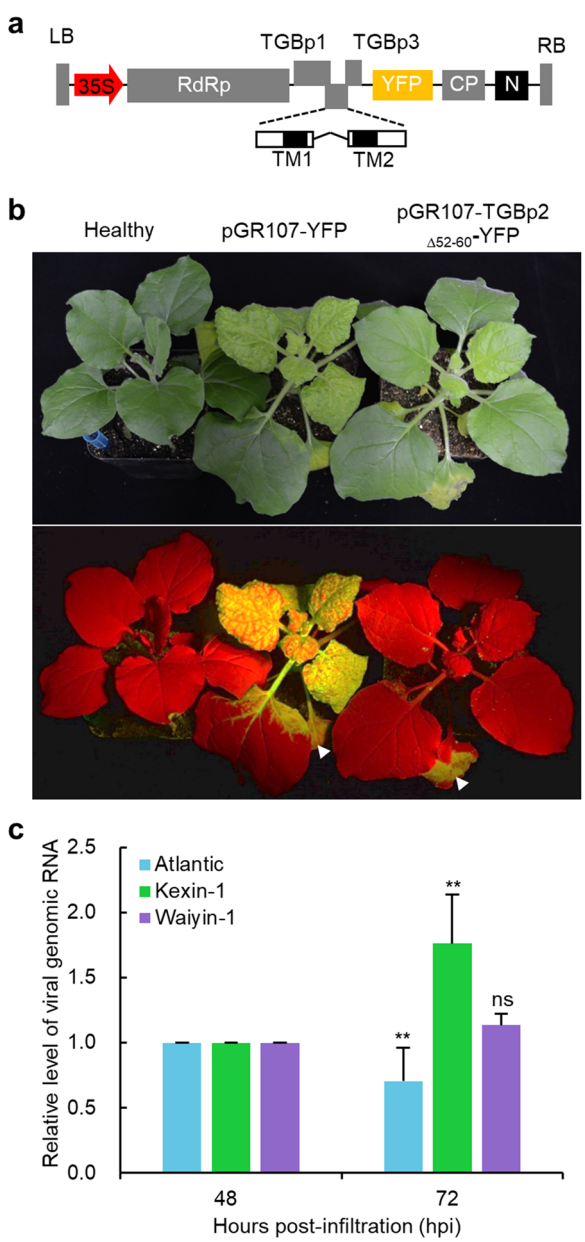

Fig. 5 The replication of PVX is inhibited in Waiyin-1. **a** Diagram illustration of pGR107-TGBp2$_{\Delta 52-60}$-YFP. TM1 and TM2 are the transmembrane motif 1 and transmembrane motif 2 of TGBp2, respectively; other labels are the same as in Fig. 2a. **b** Symptoms of *N. benthamiana* seedlings infected with pGR107-TGBp2$_{\Delta 52-60}$-YFP under white (upper panel) and UV light (lower panel) at 6 dpi. White triangles indicate the inoculated leaves. **c** RT-qPCR analysis of the replication of pGR107-TGBp2$_{\Delta 52-60}$-YFP in leaves of Waiyin-1, Kexin-1 and Atlantic infected with pGR107-TGBp2$_{\Delta 52-60}$-YFP. Data represent means ± s.d. (n = 5); $^{**}P < 0.01$; ns, no significant change (two-tailed Student's test)

into sulphonated SA (SSA), which serves as a signal to trigger SA biosynthesis, resulting in higher expression of *PR* genes (Bake et al. 2010; Wang et al. 2014; Zhao et al. 2021). Whether the resistance of Waiyin-1 is also conferred by a sulfotransferase needs to be investigated in future studies. In conclusion, our results provide new understanding on the resistance to PVX in potato plants.

Conclusions

In short, four potato cultivars (Longshu-3, Eugene, Atlantic and Waiyin-2) were found to contain the *Rx* gene, which confers the extreme resistance to PVX. The partial resistance of Waiyin-1 to PVX is associated with the suppression of viral replication and expression of pathogenesis-related genes. The rest eighteen potato cultivars are susceptible to PVX infection.

Methods

Virus resource, plant materials and growth condition

The infectious clone of PVX UK3 isolate (pGR107) has been described previously (Lu et al. 2003). Potato cultivars were kindly provided by Heilongjiang Academy of Agricultural Sciences. Virus-free potato seedlings were cultivated in fertile soil at 23℃, 60% humidity in a plant growth chamber with a light/dark cycle of 16 h/8 h. *N. benthamiana* plants were grown in pots in a plant growth chamber at 23℃ with a light/dark cycle of 16 h/8 h.

Virus inoculation

For agroinfiltration assay, *Agrobacterium tumefaciens* strain GV3101 harboring pSoup helper plasmid was used. PVX infectious clones were transformed by electroporation. Agrobacteria harboring proper plasmid were collected by centrifuge, washed twice with infiltration buffer (10 mM MES, pH 5.6, 10 mM $MgCl_2$, 100 μM acetosyringone), and resuspended in infiltration buffer at room temperature. After keeping at room temperature for 1 h, agrobacteria were infiltrated into *N. benthamiana* or potato leaves at an optical density at 600 nm (OD_{600}) of 0.4 or as indicated in the context using 1-mL needleless syringes.

For sap-inoculation, potato and *N. benthamiana* leaves were pre-dusted with 600-mesh carborundum powder, and then were rubbed with crude sap of virus-infected *N. benthamiana* leaves in 10 mM of sodium phosphate buffer (pH 7.0). At 2 min post-inoculation, the inoculated leaves were washed with distilled water and covered with a pre-wetted tissue napkin, and were subsequently incubated in a growth chamber at 23℃ with a light/dark cycle of 16 h/8 h for symptom development.

At least five plantlets per cultivar were agroinfiltrated or sap-inoculated. Equal amount of local or systemic leaves of five inoculated plantlets were sampled and mixed for further virus detection at indicated time.

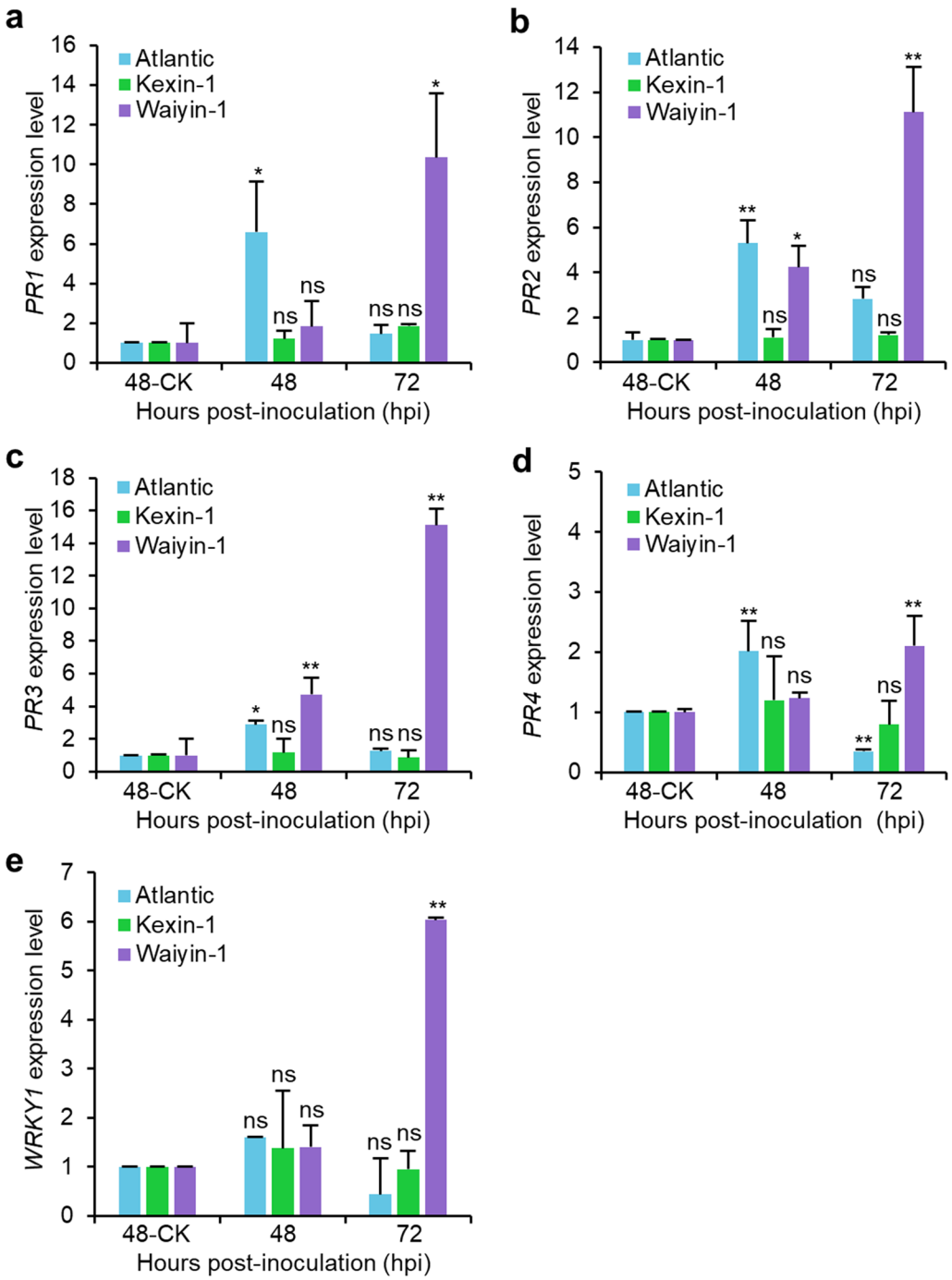

Fig. 6 The resistance of Waiyin-1 is associated with increased expression of *PR* genes. **a-e** The relative expression levels of *PR1* (**a**), *PR2* (**b**), *PR3* (**c**), *PR4* (**d**) and *WRKY1* (**e**) in Waiyin-1, Kexin-1 and Atlantic at 48 and 72 hpi. Data represent means ± s.d. (n = 4); Expressions of *PR1*, *PR2*, *PR3*, *PR4* or *WRKY1* in mock-treated plants at 48 hpi (48-CK) were normalized to 1; ** $P < 0.01$; * $P < 0.05$; ns, no significant change (two-tailed Student's test)

Vector construction

To construct a yellow fluorescent protein (YFP)-tagged PVX infectious clone, the YFP coding region was amplified from pEarleyGate-101 (Earley et al. 2006) using primers 101-yfp-F (5′- ATGGTGAGCAAGGGCGAG GAGCTG-3′) and 101-yfp-sal1-R (5′- CTTGTACAG

CTCGTCCATGCC-3′). The PVX infectious clone pGR107 (Lu et al. 2003) was linearized by PCR at the *Cla*I restriction site located downstream of the duplicated coat protein promoter using primers PVX-Cla-F (5′- GGCATGGACGAGCTGTACAAGTCGACCGCC GATGAACGGTTAAG-3′) and PVX-YFP-R (5′-CAG CTCCTCGCCCTTGCTCACCATCGATGCTAGCTG GTGCTGACCTCTTTC-3′). PVX-Cla-F and PVX-YFP-R have a 21-nt overlap with the 5′ and 3′ ends of YFP coding sequence, respectively. These two fragments were then joined together using the ClonExpress II One Step Cloning Kit (Vazyme Biotech, Nanjing, China). The movement-defective PVX infectious clone, pGR107-TGBp2$_{\Delta 52-60}$-YFP, was constructed using the same strategy with pGR107-TGBp2$_{\Delta 52-60}$ as the template (Wu et al. 2019).

Enzyme-linked immunosorbent assay (ELISA)

ELISA assay was conducted using the ELISA Reagent Set for potato virus X (Agdia, Elkhart, IN, USA) according to the provided protocol. The detailed procedure is as follows: potato leaf tissues were homogenized in general extract buffer (GEB) (0.1 g per 1 mL), and resulting leaf extracts were dispensed in capture antibodies-pretreated ELISA plates (100 µL per well). After incubating at room temperature for 2 h in a humid box, the plate was washed 7 times with 1 × PBST. Alkaline phosphatase-conjugated secondary antibody diluted in ECI buffer was then added to each well at 100 µL per well. The plate was washed 8 times with 1 × PBST after incubating at room temperature for 2 h in a humid box. Each well was added with PNP solution (1 mg/mL in PNP substrate buffer) and incubated for 60 min in the dark. Absorbance values at 405 nm were read using a Microplate reader (Thermo Scientific). All samples were repeated triply.

Nucleic acid extraction, reverse transcription (RT) and polymerase chain reaction (PCR)

Total DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Springer 2010). Total RNA was extracted using the Eastep Super Total RNA Extraction Kit (Promega, Shanghai, China) according to the manufacturer's instructions. First strand complimentary DNA (cDNA) was synthesized using the HiScript III 1st Strand cDNA Synthesis Kit + gDNA wiper (Vazyme) with oligo-d(T)$_{18}$ and 5 µg of total RNA. PCR amplification was conducted as described previously (Liu et al. 2019). For detection, PCR reactions were performed in a 20 µL volume, consisting of 10 µL 2 × *Taq* Master Mix (Vazyme), 0.5 µL of RT product or total DNA, 0.5 µL of each primer (5 mmol), and 8.5 µL distilled water. The primer pair PVX5875-F (5′-GCTGGCTGTGGC TTCGATTTCGAGACCAAC-3′) and PVXcpRT-R (5′-TCTAGGCTGGCAA AGTCGTT-3′) or PVXtgb2-F (5′-GCAGGGCCATAGGCTGACCGCTCCGGTC-3′) and PVXcpRT-R were used for the detection of PVX, which can amplify a 1.5 or 1.2 kb band from the PVX genome, respectively. For identification of the *Rx* gene, a 1.2-kb fragment of *Rx* was amplified by primers RxSP-S3 (5′-ATCTTGGTTTGAATACATGG-3′) and RxSP-A2 (5′-CACAATATTGGAAGGATTCA-3′) (Ohbayashi 2019). For cloning the *Rx* coding sequence (CDS), PCR reactions were performed in a 50 µL volume containing 25 µL 2 × Phanta Master Mix, 0.5 µL of RT product, 1 µL of each primer (5 mmol), and 22.5 µL distilled water. *Rx* CDS was amplified using primers Rx-dorF (5′-GTACAA AAAAGCAGGCTTCATGGCTTATGCTGCTGTTAC-3′) and Rx-dorR (5′-CAAGAAAGCTGGGTCCCGACG CTCGACATTATTGCGGCAAG-3′) designed based on the *Rx2* gene (GenBank accession no. AJ249448). PCR products were analyzed by electrophoresis using 1% agarose gels, and amplicons were purified using the FastPure Gel DNA Extraction Mini Kit (Vazyme) and then ligated into the pEASY-blunt vector (Transgen Biotech). Three independent clones of each fragment were selected for Sanger sequencing (Sangon Bio).

Quantitative PCR (qPCR) was performed in a 20 µL volume using a LightCycler 480 (Roche). The reaction mixture contains 1 µL fourfold-diluted cDNA, 1 µL (5 nM) of each primer, 10 µL 1 × AceQ® Universal SYBR qPCR Master Mix (Vazyme), and 7 µL distilled H_2O. The genomic RNA level of PVX was determined using the primer set PVXcpRT-F1 (5′-GTCAGCACCAGCTAG CACAAC-3′) and PVXcpRT-R1 (5′-AGCCTCAATCTT GCTGAGGTC-3′). The expression of *PR1*, *PR2, PR3*, *PR4* and *WRKY1* was analyzed with primers described by Thomas et al. (2019), with potato *ACTIN* 7 gene (GenBank accession no. DQ252512) as an internal control. All experiments were repeated triply.

DNA sequencing and sequence analyses

DNA sequences obtained via Sanger sequencing were pretreated using the SeqBuilder program to remove the vector backbone and primer sequences and then assembled using the SeqMan program in the Lasergene 7.1 software package (DNASTAR Inc.) (Burland 2000). For multiple sequence alignment, sequences were aligned by Clustal Omega with default parameters and displayed by ESPript 3.0 (https://espript.ibcp.fr/ESPript/ESPript/index.php) (Robert and Gouet 2014; Sievers and Higgins 2018). Maximum Likelihood (ML) phylogenetic tree was constructed using the MEGA X software (Kumar et al. 2018). In detail, sequences were aligned using the Clustal W with default parameters (Chenna 2003), and then

rendered for phylogeny analysis using the ML method. The node significance was evaluated with 1,000 bootstrap replicates.

Microscopic observation

Microscopic observations were performed as described previously (Wu et al. 2018). In brief, the fluorescence signal in the infiltrated leaf area was observed with a TCS SP8 LIGHTNING Confocal Microscope (Leica, Germany). YFP was excited at 496 nm and collected between 520 and 535 nm.

Abbreviations

ADVRPs: Atypical dominant viral resistance proteins; CC-NLR: Coiled-coil NLR; cDNA: Complimentary DNA; CP: Coat protein; CTAB: Cetyltrimethylammonium bromide; dpi: Days post-inoculation; ELISA: Enzyme-linked immunosorbent assay; ER: Extreme resistance; ETI: Effector-triggered immunity; FAOSTAT : FAO statistical databases; hpi: Hours post-inoculation; HR: Hypersensitive response; JAX1: Jacalin-type lectin required for potexvirus resistance 1; kb: Kilo-base pairs; ML: Maximum likelihood; NJ: Neighbor-Joining; NLRs: Nucleotide-binding domain leucine-rich repeat containing receptors; PNbMTS1: Putative methyltransferase 1 of *Nicotiana benthamiana*; ORFs: Open reading frames; *PR* genes: *Pathogenesis-related* genes; PVA: *Potato virus A*; PVX: *Potato virus X*; PVY: *Potato virus Y*; qPCR: Quantitative PCR; RdRp: RNA-dependent RNA polymerase; REMs: Remorins; RT-PCR: Reverse transcription-polymerase chain reaction; SA: Salicylic acid; SSA: Sulphonated salicylic acid; TGB: Triple-gene-block; YFP: Yellow fluorescence protein.

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Authors' contributions

XC conceived the study and wrote the manuscript. JL, YL and YF carried out the experiments. LZ and XW contributed to the materials and analysis procedure. KY analyzed data. XW revised the manuscript. All authors have read and approved the final manuscript.

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Not applicable.

Declarations

Ethics approval and consent to participate

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Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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