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Stacking potato NLR genes activates a calcium-dependent protein kinase and confers broad-spectrum disease resistance to late blight

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ABSTRACT

Late blight, caused by the oomycete plant pathogen *Phytophthora infestans*, is a destructive disease that leads to significant yield loss in potatoes and tomatoes. The introgression of disease resistance (*R*) genes, which encode nucleotide-binding domain leucine-rich repeat-containing receptors (NLRs), into cultivated potatoes, is highly effective in controlling late blight. Here, we generated transgenic *2R* and *3R* potato lines by stacking *R* genes *Rpi-blb2*/*Rpi-vnt1.1* and *Rpi-vnt1.1*/*RB*/*R8*, respectively, in the susceptible cv. Desiree background. The resulting *2R* and *3R* transgenic potato plants showed resistance to highly virulent *P. infestans* field isolates. We hypothesized that stacking *R* genes either resulted in up-regulation of a broader range of immune-related genes, or, more importantly, increase in the fold change of gene expression. To test our hypotheses, we performed transcriptome analysis and identified a subset of core immune-related genes that are induced in response to *P. infestans* in transgenic lines carrying single *R* genes versus lines carrying stacks of multiple *R* genes. In our analysis, stacking *R* genes resulted not only in the induction of a broader range of defense-associated genes but also a global increase in gene expression fold change, caused by the pathogen. We further demonstrated that the *calcium-dependent protein kinase 16* (*StCDPK16*) gene significantly contributed to resistance to a virulent *P. infestans* strain, in the *R* gene background, in a kinase activity-dependent manner. Thus, our data suggest that stacking the *R* genes enhances late blight resistance through modulating the expression of a broader range of defense-related genes and highlights CDPK16 as a novel player in potato *R* gene-mediated resistance.

Keywords: CDPK, gene transcription, late blight, NLR, plant resistance, potato

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INTRODUCTION

Potato (*Solanum tuberosum*), the most important tuber crop, is susceptible to various diseases, especially the highly destructive late blight, caused by the oomycete *Phytophthora infestans*, which is the most serious potato disease worldwide. In the mid-19th century, late blight caused severe destruction of potato crops in Europe, leading to the notorious Irish potato

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famine, which had serious consequences for Ireland's culture and economy, including mass starvation, disease, and emigration (Fry and Goodwin, 1997; Zadoks, 2008). *Phytophthora infestans* can infect potato stems, berries, leaves, and tubers throughout the growth and development stages (Majeed et al., 2017), making it difficult to control late blight in the field. Currently, control of late blight mainly relies on the use of fungicides, excessive application of which can lead to emergence of fungicide-resistant strains of *P. infestans*. Thus, there is an urgent need to explore alternatives to current late blight management strategies. At the moment, growing cultivars that carry *R* genes conferring resistance to *P. infestans* (*Rpi*) remains the most economical and effective late blight control strategy (Paluchowska et al., 2022). *Rpi* genes are usually obtained from wild potato species, which are characterized by great genetic diversity (Vleeshouwers et al., 2011). The identified *Rpi* genes are used in breeding programs to develop potato cultivars resistant to late blight (Spooner et al., 2016).

To date, more than 70 *Rpi* genes, which encode nucleotide-binding domain (NB) leucine-rich repeat (LRR)-containing receptor (NLR), have been cloned from wild potato relatives (Paluchowska et al., 2022). For instance, 10 *Rpi* genes, *R1–R10*, were identified in *Solanum demissum*, and some of them were later introduced into several commercial potato cultivars resulting in good resistance to late blight (Malcolmson, 1969; Rudkiewicz, 1985; Paluchowska et al., 2022). *RB* (also known as *Rpi-blb1*), *Rpi-blb2*, and *Rpi-blb3* were originally mapped in *S. bulbocastanum* (Song et al., 2003; Van Der Vossen et al., 2003; van der Vossen et al., 2005; Lokossou et al., 2009), while *Rpi-vnt1.1*, *Rpi-vnt1.2*, *Rpi-vnt1.3*, and *Rpi-vnt2* were mapped in *S. venturi* (Śliwka et al., 2006; Foster et al., 2009). Recently, by deploying advanced molecular and bioinformatics techniques, several new *Rpi* genes were cloned from *Solanum* species: *Rpi-amr4*, *R02860*, *R04373*, *Rpi-amr1*, and *Rpi-arm3* were cloned from *S. americanum* (Witek et al., 2016; Witek et al., 2021; Lin et al., 2023), *Rpi-chc1.1*, *Rpi-chc1.2*, and *Rpi-chc2* from *S. chacoense* (Haverkort et al., 2016; Monino-Lopez et al., 2021), and *Rpi-ber1.2*, *Rpi-ber1.3*, and *Rpi-ber1.4* from *S. berthaultii* (Monino-Lopez et al., 2021). The R proteins belong to the NLR class and extensive research shows they confer race-specific resistance by recognizing their cognate effector secreted by *P. infestans* (Paluchowska et al., 2022). For example, RB, R8, Rpi-blb2, and Rpi-vnt1.1 recognize AVRblb1, AVR8, AVRblb2, and AVRvnt1, respectively, to trigger plant defense against *P. infestans* (Vleeshouwers et al., 2011; Vossen et al., 2016). Nonetheless, due to emergence and rapid evolution of new virulent lineages of *P. infestans*, a large number of *Rpi* genes, including *RB*, *Rpi-blb2*, *Rpi-vnt1.1*, and *R8*, have been defeated by the pathogen (Witek et al., 2021; Paluchowska et al., 2022), suggesting that a single *Rpi* gene is not sufficient to confer durable resistance over a long time. It was previously shown that stacking several *Rpi* genes in potato could result in durable broad-spectrum resistance against the newly emerged virulent *P. infestans* strains in Africa (Zhu et al., 2012; Ghislain et al., 2019). For instance, stacking *RB*, *Rpi-blb2*, and *Rpi-vnt1.1* in potato cv. Desiree and Victoria conferred durable resistance to local African *P. infestans* strains in the field, although the exact mechanism behind it remains unknown (Ghislain et al., 2019). Notably, a similar example was also recently reported in the case of stacking four or five resistance genes in bread wheat that resulted in broad-spectrum resistance to the fungal pathogen *Puccinia graminis* f. sp. *tritici* (*Pgt*) (Luo et al., 2021; Zhang et al., 2024b). Considering the successful generation of durable resistance to plant pathogens by using *R* gene stacks, as illustrated by the above-mentioned examples, the underlying mechanisms of such resistance deserve to be further investigated.

Upon pathogen attack, plants initiate complex immune responses to restrict pathogen colonization. Plant cell surface-resident pattern-recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) to trigger PRR-mediated immunity, commonly known as pattern-triggered immunity (PTI) (Jones and Dangl, 2006; Couto and Zipfel, 2016; Yu et al., 2017; Kong et al., 2021). Plants have evolved NLRs to directly or indirectly sense pathogen-secreted effectors to activate a set of responses that constitute effector-triggered immunity (ETI) (Ngou et al., 2022a, 2022b). Over the past 30 years, PTI and ETI have been extensively studied that provided many insights into plant immune system mechanisms (Zhou and Zhang, 2020; Ngou et al., 2022a; Jones et al., 2024). PTI and ETI converge into a plethora of overlapping signaling responses, including transcriptional reprogramming of defense-associated genes, calcium influx, reactive oxygen species (ROS) burst, and activation of mitogen-activated protein kinases (MAPKs) (Yu et al., 2017; Yuan et al., 2021b). Recently, several important studies showed that PTI and ETI mutually potentiate each other for an integrated and robust resistance against pathogen infection (Ngou et al., 2021; Yuan et al., 2021a).

In addition, it was reported that activation of PTI induced rapid transient Ca^{2+} influx, in both cytoplasm and nucleus, that plays an essential role in many subsequent immune responses, such as cell death (Yu et al., 2017; Yuan et al., 2017). In plants, three families of proteins are involved in regulating Ca^{2+} signaling, including calmodulin (CAM) and CAM-like proteins, calcineurin B-like proteins (CBL), and calcium-dependent protein kinases (CDPKs) (Cheng et al., 2002; Harper and Harmon, 2005; Luan, 2009). For instance, flg22, a 22-amino acid synthetic peptide derived from bacterial flagellin, could activate several CDPKs, as well as the expression of genes encoding them, in Arabidopsis (Boudsocq et al., 2010). Arabidopsis CPK5, CPK11, and CPK28 play important roles in regulating plant immune responses (Gao et al., 2013; Bredow et al., 2021). In addition to PTI activating Ca^{2+} signaling, recent studies have shown that onset of ETI induces a slower and longer-lasting Ca^{2+} influx as compared to PTI (Yuan et al., 2017; Kim et al., 2022; Wang and Luan, 2024). The calcium channel proteins CNGC2 (cyclic nucleotide-gated ion channel 2) and CNGC4 are known to regulate the AvrRpt2/RPS2-mediated hypersensitive response (HR) as part of the respective ETI

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mechanism (Jurkowski et al., 2004). Of note, NLR ZAR1 (Hopz-activated resistance 1) and helper NLRs ADR1 (Activated disease resistance 1) and NRG1 (N requirement gene 1) function as calcium channels based on the determined protein structures (Bi et al., 2021; Jacob et al., 2021). The calcium channel function of these NLRs is required for cell death in plants, suggesting the importance of calcium in ETI signaling (Wang et al., 2023). However, how ETI activates calcium signaling and whether Ca^{2+} sensors (CAMs, CBLs, and CDPKs) function as part of the ETI mechanism remains unclear.

In this study, we generated *2R* (*Rpi-blb2* and *Rpi-vnt1.1*) and *3R* (*RB*, *R8* and *Rpi-vnt1.1*) transgenic potato plants, carrying indicated *R* gene stacks, which showed broad-spectrum resistance to highly virulent *P. infestans* field isolates. To explore the mode of action of the stacked *Rpi* genes in regulating plant immunity, we performed RNA sequencing (RNA-seq) analysis to identify genes in which the expression is regulated by NLR-induced signaling in potato. RNA-seq data showed that a subset of immune-related genes was up-regulated in potato plants carrying a single *Rpi (Rpi-blb2*, *Rpi-vnt1.1*, *RB*, or *R8)* or combinations of *Rpi* genes in *2R* and *3R* stacks upon *P. infestans* infection, including those encoding key components of PTI and ETI, thus supporting the notion that activation of ETI potentiates PTI signaling. Importantly, stacking *Rpi* genes resulted in the up-regulation of a broader range of immune-related genes and an increase in the gene expression fold change in a global manner, thereby leading to stronger plant immunity against *P. infestans*. Among the immune-related genes, the expression pattern of which differed in the *2R* and *3R* lines, as compared to the transgenic lines carrying single *Rpi* genes, we identified *StCDPK16* as a putative positive regulator of *Rpi*-mediated plant resistance to *P. infestans*. Biochemical experiments revealed that StCDPK16 is a bona fide kinase and its kinase activity is required for *Rpi*-mediated immunity. Thus, these data highlight an important role of CDPKs in regulating NLR-mediated immunity, likely via an action on calcium signaling.

RESULTS

Stacking *Rpi* genes triggers multiple effector-mediated HR and plant resistance to *P. infestans* in *Nicotiana benthamiana*

To avoid digestion of *Rpi-vnt1.1*, *Rpi-blb2*, and *RB* coding sequences (CDSs) by BsaI and BpiI restriction enzymes when using the Golden Gate cloning system, we domesticated the CDSs for these two restriction enzymes sites by introducing synonymous mutations (Figure S1A). The domesticated CDSs of both *Rpi-vnt1.1* and *Rpi-blb2* were fused with their corresponding native promoters and terminators, while for *RB* the CaMV *35S* promoter and NOS terminator were used to produce gene expression cassettes for testing the gene function using HR and disease susceptibility assays. As shown in Figure S1B, expressing domesticated versions of *Rpi-vnt1.1*, *Rpi-blb2*, and *RB* resulted in HR phenotypes similar to those of their wild-type (WT) counterparts in the presence of their cognate effectors (AVRvnt1, AVRblb2, and AVRblb1) in *N. benthamiana*. Furthermore, *Rpi-vnt1.1*^{Domesticated}, *Rpi-blb2*^{Domesticated}, and *RB*^{Domesticate}, but not the *green fluorescent protein* (*GFP*) control, exhibited a robust resistance to *P. infestans* isolate JH19, similar to the WT counterparts (Figure S1C). Western blotting confirmed that Rpi-vnt1.1^{Domesticated}, Rpi-blb2^{Domesticated}, and RB^{Domesticated} protein levels were comparable to the WT versions when expressed in *N. benthamiana* (Figure S1D). Altogether, the data demonstrate that *Rpi-vnt1.1*^{Domesticated}, *Rpi-blb2*^{Domesticated}, and *RB*^{Domesticated} perform as their WT counterparts and are hereafter referred to as *Rpi-vnt1.1*, *Rpi-blb2*, and *RB*.

We then proceeded with building the gene stack construct, carrying *Rpi-vnt1.1* and *Rpi-blb2*, using the Golden Gate cloning strategy for generating *2R* transgenic potato plants in the cv. Desiree background (Figure 1A). At the same time, we made the *Rpi-vnt1.1/RB* two gene cassettes for generating *3R* transgenic plants using the potato cv. Desiree already containing the *R8* gene in the background (Desiree^{R8}; Figure 1A). To validate whether the gene stack constructs could express the corresponding *Rpi* genes *in planta*, we co-delivered them with constructs carrying their cognate avirulence (*Avr*) genes in *N. benthamiana*. As shown in Figure S2A, delivery of the *Rpi-vnt1.1/Rpi-blb2* stack resulted in recognition of AVRvnt1 or AVRblb2, but not AVR8, to trigger HR that is comparable to the HR induced by either *Rpi-vnt1.1* or *Rpi-blb2*, when co-delivered with their cognate effector genes, indicating that the *Rpi-vnt1.1/Rpi-blb2* stack construct could efficiently express *Rpi-vnt1.1* and *Rpi-blb2*. Similarly, the *Rpi-vnt1.1/RB* construct efficiently expressed *Rpi-vnt1.1* and *RB* to recognize AVRvnt1.1 or AVRblb1, respectively (Figure S2B). In addition, *Rpi-vnt1.1/Rpi-blb2* and *Rpi-vnt1.1/RB* gene stacks conferred complete resistance to *P. infestans*, as compared to the *GFP* control, when expressed in *N. benthamiana* (Figure S2C). Altogether, the data suggested that both gene stack constructs were capable of expressing the corresponding *Rpi* genes *in planta*, thus enabling us to use them for generating the *2R* and *3R* transgenic potato plants described above.

Stacking *Rpi* genes triggers multiple *Avr*-induced HR in potato

We introduced the *Rpi-vnt1.1/Rpi-blb2* construct into susceptible cv. Desiree using Agrobacterium-mediated transformation and generated *2R* gene stack transgenic potato lines (Figure 1A). Meanwhile, we introduced the *Rpi-vnt1.1/RB* construct into the Desiree^{R8} background and generated *3R* gene stack lines (Figure 1A). Out of the 52 *2R* stack T_0 plants, two lines (#1 and #2) carried both *Rpi-vnt1.1* and *Rpi-blb2*, as confirmed by genotyping polymerase chain reaction (PCR) and real-time quantitative PCR (RT-qPCR) (Figure 1B, D). Out of the 68 *3R* stack T_0 plants, two lines (#6 and #9) carried both *Rpi-vnt1.1* and *RB* as confirmed by

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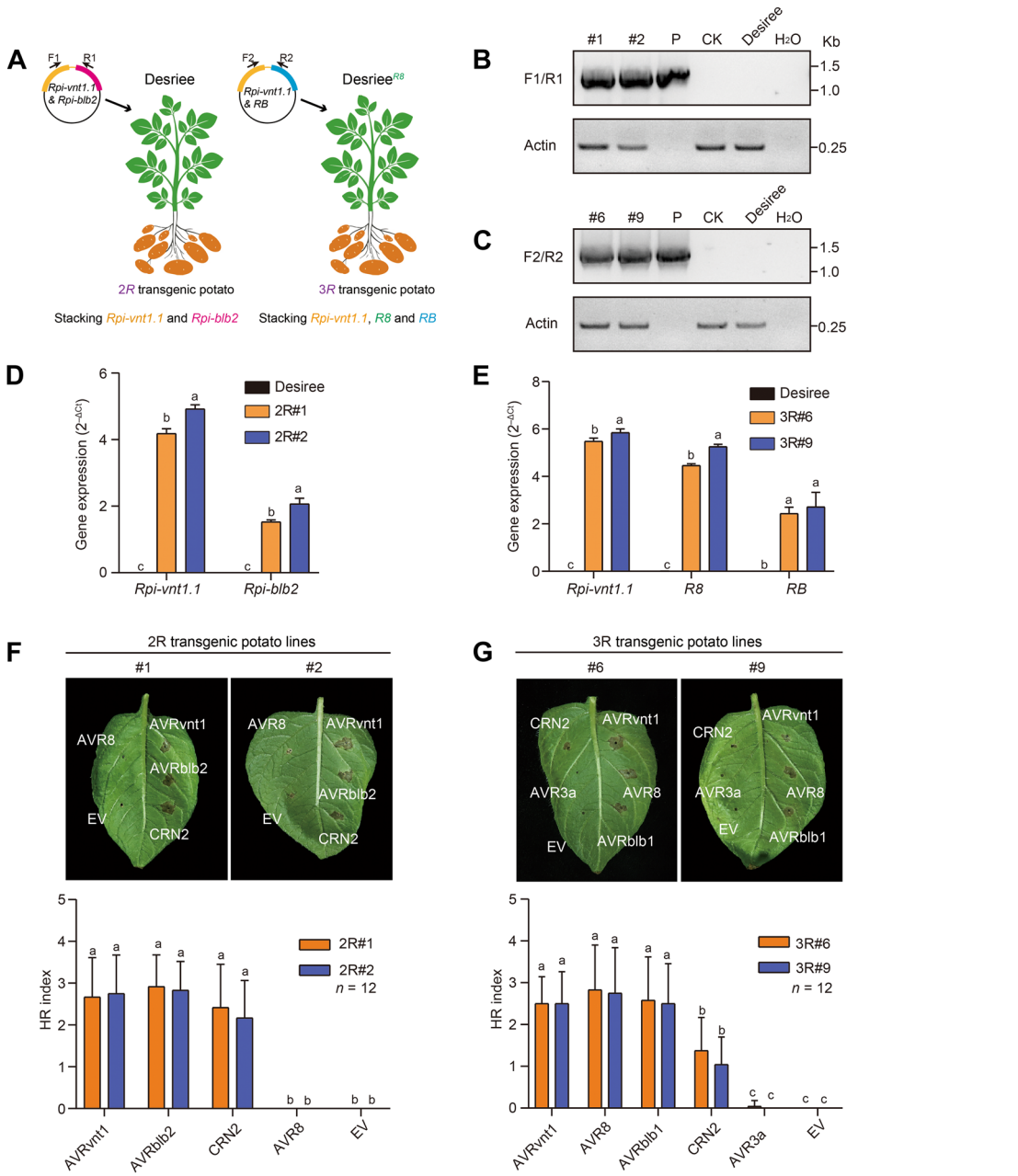

Figure 1. Stacking *Rpi* genes in potato enables recognition of the cognate *Phytophthora infestans* effectors

(A) Cartoon images show the *Rpi-vnt1*/*Rpi-blb2* and *Rpi-vnt1*/*RB* gene cassette constructs and the resulting *2R* and *3R* gene stacked transgenic potato plants in the cv. Desiree and Desiree^{R8} backgrounds, respectively. **(B, C)** Identification of *2R* and *3R* gene stacked transgenic potato plants by polymerase chain reaction (PCR)-genotyping. Genotyping PCR, using genomic DNA, was performed with primers F1/R1 and F2/R2 (Table S3) for screening *2R* and *3R* plants, respectively. The potato *Actin* gene was used to as a positive control. "P" represents the plasmid DNA, "CK" represents the transgenic line, produced using the empty vector, that does not carry any *Rpi* transgenes, and "H_2O" is the negative control. **(D)** Expression of *Rpi-blb2* and *Rpi-vnt1.1* in the *2R* gene stacked potato plants. **(E)** Expression of *Rpi-vnt1.1*, *R8*, and *RB* in the *3R* gene stacked potato plants. Relative expression levels were normalized with the potato *EF1α* housekeeping gene and presented as means ± *SD* ($n = 3$). **(F)** The *2R* transgenic lines recognize AVRvnt1 and AVRblb2 to induce hypersensitive response (HR). *AVRvnt1* and *AVRblb2* were expressed in *2R* gene stacked potato lines using potato virus X (PVX). The HR index was measured at 15 d post-inoculation (dpi). The data are shown as means ± *SD* ($n = 12$). **(G)** *3R* recognizes AVRvnt1, AVRblb2, and AVR8 to induce HR in *3R* transgenic potato lines. The experiments were performed as in **(F)**. For the experiments presented in **(B–G)**, three biological replicates were performed with similar results. "a," "b," and "c" in **(D–G)** denote statistically significant differences according to one-way analysis of variance followed by Tukey's test ($P < 0.01$).

genotyping PCR and RT-qPCR (Figure 1C, E). To determine whether high levels of resistance affect plant growth and development, we conducted a greenhouse experiment in which growth and development of potato transgenic plants carrying single and multiple *Rpi* genes were assessed (Figure S3). *Rpi-blb2*, *Rpi-vnt1.1*, *R8*, and *2R* potato transgenic plants showed similar growth phenotypes compared to WT Desiree plants, while *RB* and *3R* potato transgenic plants

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showed slightly reduced growth phenotypes (Figure S3). Notably, the potato virus X (PVX)-based HR assay demonstrated that the *2R* stack transgenic potato plants (#1 and #2) recognized AVRvnt1 and AVRblb2, but not AVR8, which was used as a negative control (Figure 1F). Similarly, the *3R* stack transgenic potato plants (#6 and #9) recognized AVRvnt1, AVRblb1, and AVR8, but not AVR3a (negative control), to trigger HR (Figure 1G). In both experiments, CRN2, a Crinkler effector from *P. infestans*, which induced common cell death and served as a positive control, triggered HR in all *2R* and *3R* potato plants (Figure 1F, G). Meanwhile, transgenic potato lines carrying a single *Rpi* gene recognized their corresponding AVR factors (Zhu et al., 2014), which otherwise did not cause HR in the non-transgenic cv. Desiree plant (Figure S4). Collectively, these results demonstrate that stacking *Rpi* genes can simultaneously lead to activation of HR in response to multiple AVR factors.

Stacking *Rpi* genes expands the resistance spectrum to distinct *P. infestans* isolates that have overcome resistance mediated by a single *Rpi* gene

To determine whether potato lines carrying stacks of *Rpi* genes are characterized by an expanded resistance spectrum, we performed disease assay by inoculating *2R* and *3R* plants with different *P. infestans* field isolates. Due to the complexity of its genome and the ability to reproduce sexually, *P. infestans* is capable of rapidly evolving into lineages that overcome resistance mediated by a single *Rpi* gene. For instance, the NL07434 strain, by an unknown mechanism, has overcome complete resistance of the potato cv. Desiree carrying *Rpi-blb2*, but not *Rpi-vnt1.1*, *R8* or *RB*, as evidenced by the significantly increased disease lesions seen on the infected potato leaves (Figure 2). Another example is P13626, an *AVRvnt1*-silenced isolate, that has overcome complete resistance of cv. Desiree carrying *Rpi-vnt1.1*, but not *Rpi-blb2*, *R8*, or *RB* (Figure 2), while the JH19 isolate, by a currently unknown mechanism again, has defeated the *R8* gene in cv. Desiree, but not *Rpi-blb2*, *Rpi-vnt1.1*, or *RB* genes (Figure 2). On the other hand, Pi88069 and HB1501, which are standard strains used in the laboratory, could not overcome the complete resistance of Desiree^{R8}, Desiree$^{Rpi\text{-}blb2}$, Desiree$^{Rpi\text{-}vnt1.1}$, or Desiree^{RB} (Figure 2). In these experiments, the Desiree^{RB} line showed resistance to all five above-mentioned strains, whereas the non-transgenic cv. Desiree line, which served as a negative control, proved to be susceptible to them (Figure 2). Importantly, *2R* and *3R* stack transgenic plants exhibited complete resistance to these five strains (Figure 2), indicating that stacking *Rpi* genes in potato confers broad-spectrum resistance to *P. infestans*. In addition, unlike the non-transgenic cv. Desiree control, the *2R* and *3R* stack lines showed complete resistance to eight *P. infestans* field isolates from different regions of China (Figure S5A, B), further supporting the conclusion that stacking *Rpi* genes expands the resistance spectrum to *P. infestans*, and thus has great potential as a strategy for enhancing potato resistance to late blight.

Stacking *Rpi* genes in potato broadens the spectrum of gene up-regulation and increases gene expression fold change in response to *P. infestans*

To investigate the transcriptional reprogramming when multiple *Rpi* genes are simultaneously activated, we performed RNA-seq analysis on transgenic potato lines, carrying multiple or single *Rpi* genes, with or without *P. infestans* inoculations. Samples were collected at 2 d post-inoculation (dpi) (Table S1). During RNA-seq data analysis, gene expression in different samples was first normalized using cv. Desiree non-transgenic control line with or without *P. infestans* infection, respectively, to account for the basal transcription level. Then the differentially expressed genes (DEGs) (P-value ≤ 0.05, and fold change ≥ 2 or ≤ 2) were defined as those specifically up-regulated by the activated Rpi proteins. *Phytophthora infestans* induced 3,353, 2,065, and 822 DEGs in *2R* (*Rpi-blb2*/*Rpi-vnt1.1*), *Rpi-blb2*, and *Rpi-vnt1.1* transgenic potato lines, respectively, with 184 genes overlapping in all three genotypes (Figure 3A; Table S1). Importantly, stacking *Rpi* genes in *2R* plants resulted in a broader range of activated genes and globally increased the gene expression fold change, as evidenced by more DEGs and higher levels of gene up-regulation, as compared to *Rpi-blb2* and *Rpi-vnt1.1* single *R* gene lines (Figure 3B; Table S1). The hierarchical clustering analysis of DEGs suggested that *2R* plants displayed overall increased *P. infestans* infection responses when compared with *Rpi-blb2* and *Rpi-vnt1.1* transgenic lines (Figure 3C). It is noteworthy that, in response to *P. infestans*, the *2R* lines showed specific up-regulation of 2,354 genes, whereas only 1,180 and 374 genes were specifically up-regulated in *Rpi-blb2* and *Rpi-vnt1.1* lines, respectively (Figures 3A, S6A; Table S1), indicating differences in functions of individual *Rpi* genes in regulating plant immunity. Inoculation with *P. infestans* induced expression of 2,579, 717, and 448 DEGs in the *3R* (*Rpi-vnt1.1*/*RB*/*R8*), *RB*, and *R8* transgenic potato lines, respectively (Figure 3D; Table S1). Similar to *2R*, stacking three *Rpi* genes in *3R* lines also resulted in a broader range of activated genes and significantly enhanced the gene expression fold change in a global manner, as evidenced by a higher number of DEGs and higher levels of gene expression when compared with *RB*, *R8*, and *Rpi-vnt1.1* lines (Figure 3E). Similarly, the hierarchical clustering analysis of these DEGs showed that *3R* plants displayed overall increased levels of responses to *P. infestans* infection as compared to *RB*, *R8* and *Rpi-vnt1.1* plants (Figure 3F). Of note, *3R* plants showed specific up-regulation of 2,045 genes in response to *P. infestans*, whereas *RB, R8*, and *Rpi-vnt1.1* showed 274, 111, and 308 genes, respectively (Figures 3D, S6B), further suggesting that different *Rpi* genes play different roles in plant immune responses. Overall, the data suggest that stacking two or three *Rpi* genes in *2R* and *3R* lines, respectively, results in up-regulation of a broader range of genes in response to *P. infestans* and significantly boosts the gene expression fold change in a global manner.

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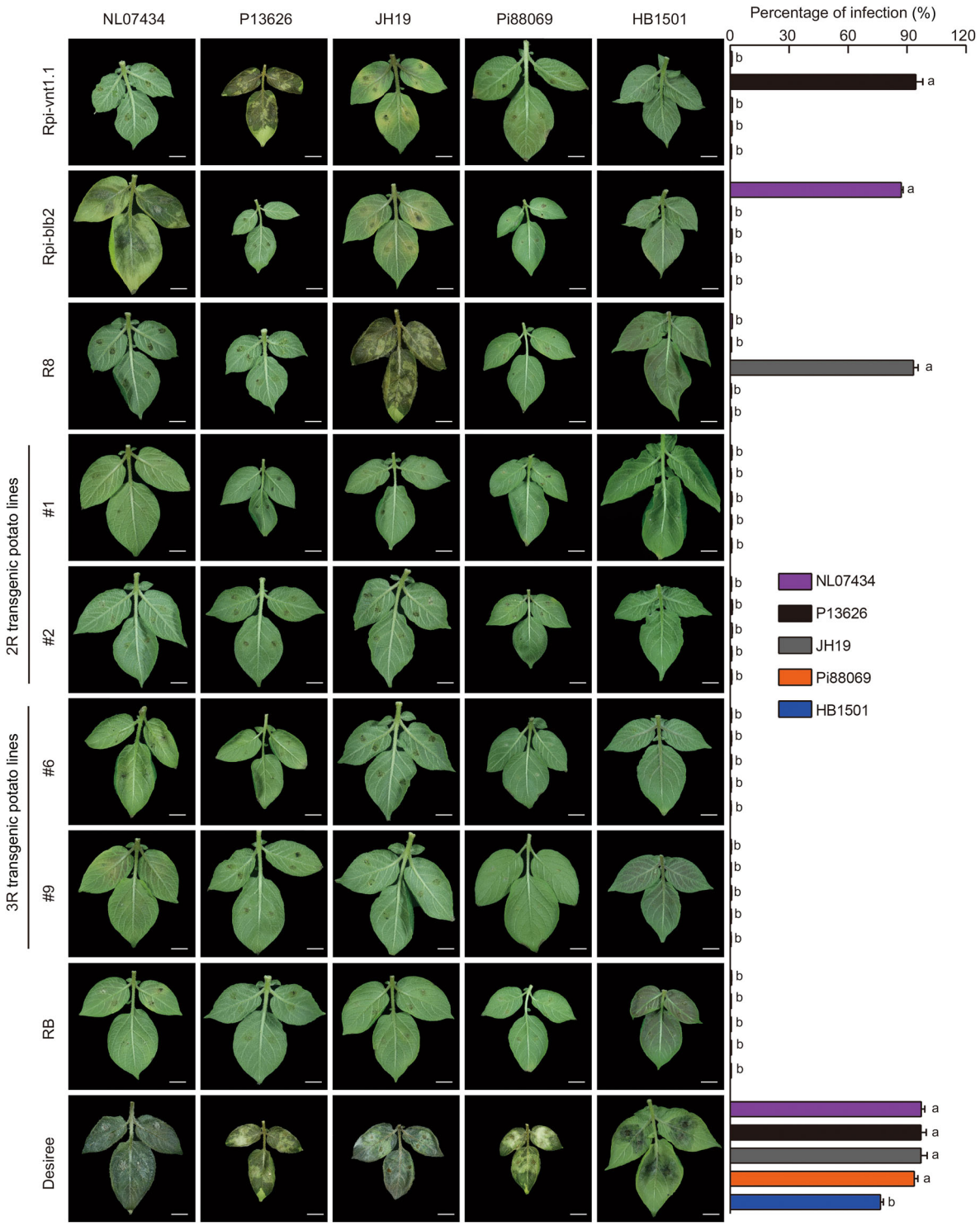

Figure 2. Stacking *Rpi* genes confers broad-spectrum resistance to distinct *Phytophthora infestans* isolates that have overcome single *Rpi*-mediated resistance

Leaves from 4-week-old transgenic potato plants carrying indicated *Rpi* genes were inoculated with different *P. infestans* isolates. NL07434, using currently unknown mechanisms, overcomes *Rpi-blb2*-mediated resistance; P13626, an *AVRvnt1*-silenced isolate, overcomes *Rpi-vnt1.1*-mediated resistance; JH19, using currently unknown mechanisms, overcomes *R8*-mediated resistance. Pi88069 and HB1501, which are standard laboratory-used strains, only infect the susceptible cv. Desiree plants and served as controls. Infected potato leaves were photographed at 5 d post-inoculation (dpi) (left) and the percentage of infection was estimated by measuring the lesion area (right). The data are shown as means ± *SD* ($n = 3$). "a" and "b" denote statistically significant differences according to one-way analysis of variance followed by Tukey's test ($P < 0.01$). Scale bar, 10 mm. For each experiment, three biological replicates were performed with similar results.

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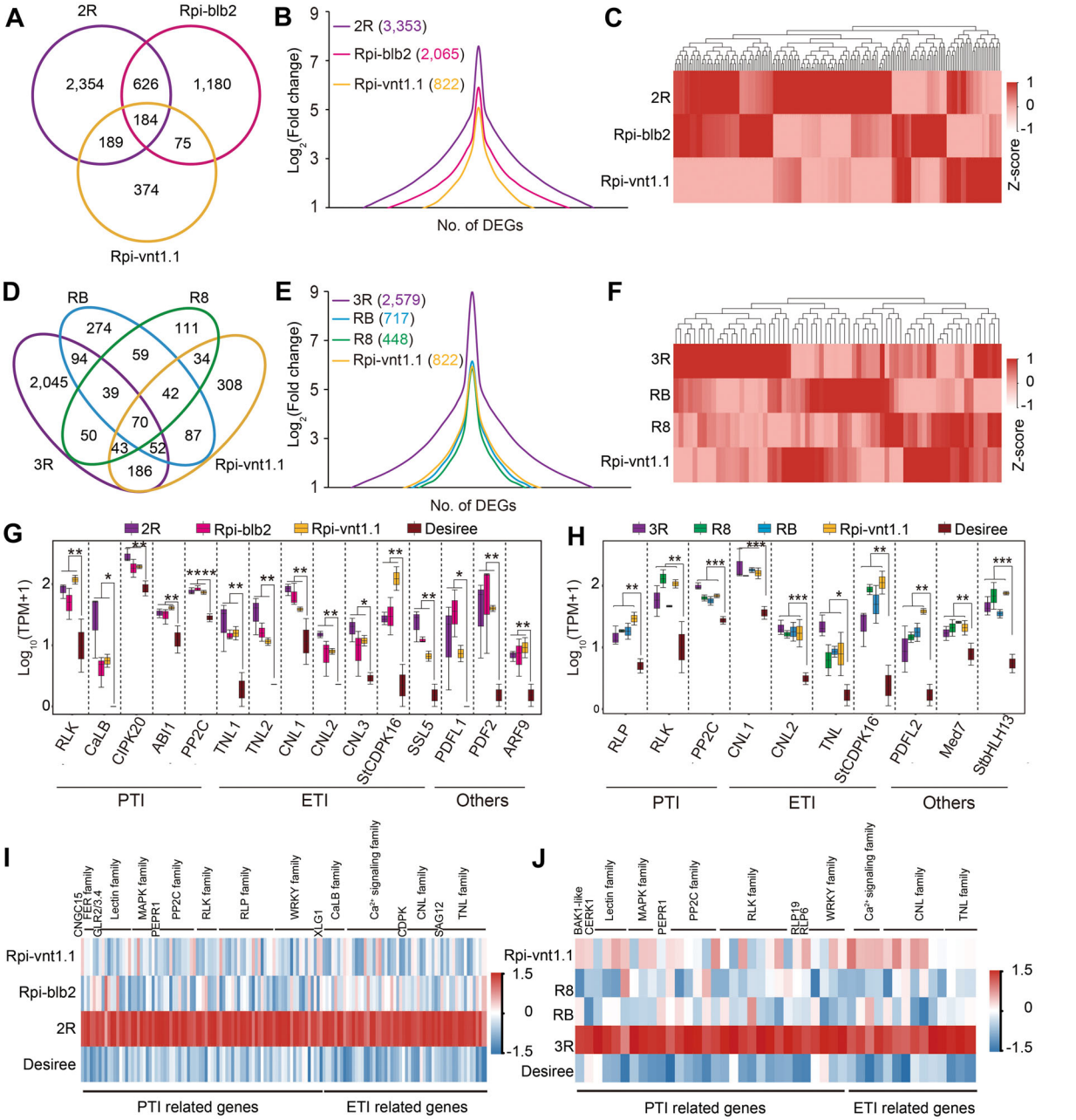

Figure 3. Stacking *Rpi* genes results in induction of a broader range of genes in response to *Phytophthora infestans* and an increase in gene expression fold change in a global manner

(A) A Venn diagram representing genes up-regulated by *P. infestans* in *2R*, *Rpi-blb2* and *Rpi-vnt1.1* plants. A list of up-regulated genes is shown in Table S1. Detached potato leaves carrying the *2R* gene stack, *Rpi-blb2* and *Rpi-vnt1.1* were inoculated with *P. infestans* strain HB1501 and then harvested for RNA sequencing (RNA-seq) analysis at 2 d post-inoculation (dpi). **(B)** Stacking *Rpi* genes (*2R*) induces a broader range of genes and boosts gene expression fold change in a global manner. The graph was generated using the up-regulated differentially expressed genes (DEGs) from *2R*, *Rpi-blb2*, and *Rpi-vnt1.1* plants. The *x*-axis represents the number of DEGs, and the *y*-axis represents $\log_2$(fold change). **(C)** Heatmap of overlapping genes in *2R*, *Rpi-blb2*, and *Rpi-vnt1.1* plants. The heatmap was generated using normalized $\log_2$(fold change) of each gene at the 2 dpi stage, with red indicating relatively high expression and white indicating low expression. A list of DEGs is shown in Table S1. **(D)** A Venn diagram of genes up-regulated by *P. infestans* in *3R*, *R8*, *RB*, and *Rpi-vnt1.1* plants. A list of up-regulated genes is shown in Table S1. Detached potato leaves carrying *3R*, *R8*, *RB*, and *Rpi-vnt1.1* were inoculated with *P. infestans* strain HB1501 and then harvested at 2 dpi for RNA-seq analysis. **(E)** Stacking *Rpi* genes (*3R*) induces a broader range of genes and boosts gene expression fold in a global manner. The graph was generated using the up-regulated DEGs from *3R*, *R8*, *RB*, and *Rpi-vnt1.1* plants. The *x*-axis represents the number of DEGs, and the *y*-axis represents $\log_2$(fold change). **(F)** Heatmap of overlapping genes in *3R*, *R8*, *RB*, and *Rpi-vnt1.1* plants. The heatmap was generated using normalized $\log_2$(fold change) of each gene at the 2 dpi stage, with red indicating relatively high expression and white indicating low expression. A list of DEGs is shown in Table S1. **(G, H)** RNA-seq analysis shows a subset of overlapping genes related to pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) pathways in *2R* **(G)** and *3R* **(H)** plants, respectively. A list of genes and their names are shown in Table S2. **(I, J)** RNA-seq analysis shows a subset of genes related to PTI and ETI pathways in Desiree, *2R*, *3R*, *Rpi-vnt1.1*, *Rpi-blb2*, *RB*, and *R8* plants. Almost all of these genes show a strong induction in *2R* **(I)** and *3R* **(J)** plants.

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Enrichment analysis of Gene Ontology (GO) categories for 184 overlapping genes from *2R*, *Rpi-vnt1.1* and *Rpi-blb2* lines, and 70 overlapping genes from *3R*, *Rpi-vnt1.1*, *RB*, and *R8* samples revealed that PTI- and ETI-related genes, such as those encoding CDPK, RLP, RLK, CNL, and TNL family proteins, were up-regulated in *2R*, *3R*, and single *Rpi* (*Rpi-vnt1.1*, *Rpi-blb2*, *RB*, and *R8*) plants (Figure 3G, H; Table S2). *Rpi* stacking specifically activated a substantial number of PTI- and ETI-related genes to initiate robust immune responses (Figure 3I, J; Table S2). Moreover, GO analysis showed that genes related to calcium signaling, immune responses and photosynthesis were overrepresented among the up-regulated genes in *2R* and *3R* lines (Figure S6C, D). Real-time qPCR further confirmed that *LOX1* (*Lipoxygenase 1*), *RIN4* (*RPM1-interacting protein 4*), *CERK1* (*Chitin elicitor receptor kinase 1*), *PEPR1* (*PEP1 receptor 1*), *PR5K* (*PR5-like receptor kinase*) and *ZAR1* (*HopZ-activated resistance 1*) were up-regulated in *2R* and *3R* lines as compared to *R8*, *RB*, *Rpi-blb2* and *Rpi-vnt1.1* single *Rpi* lines (Figure S6E). All in all, the data suggest that activation of NLR signaling reprograms expression of a subset of key immune-related genes.

The RNA-seq analysis also indicated that expression of single *Rpi* genes *Rpi-vnt1.1*, *Rpi-blb2*, *RB*, and *R8* resulted in specific up-regulation of a subset of genes (Figure 3A, D). For instance, in *Rpi-vnt1.1* and *Rpi-blb2* lines, a specific set of genes were specifically up-regulated when compared to the *2R* lines (Figures 3A, S7A, B). It is noteworthy that more DEGs were induced in *Rpi-blb2* than *Rpi-vnt1.1* plants (Figures 3A, S7A, B), indicating that *Rpi-blb2* might induce a stronger immune response upon *P. infestans* infection. When compared to the *3R* lines, *RB*, *R8*, and *Rpi-vnt1.1* lines showed specific up-regulation of diverse genes, respectively (Figures 3D, S8A–C). Gene Ontology analysis of these *Rpi*-regulated genes showed that distinct GO categories were overrepresented in data sets derived from different *Rpi* lines (Figures S7C, D, S8D–F), further supporting the conclusion that each *Rpi* gene has a specific function in regulating plant immunity, besides the function in co-regulating transcription of the core subset of immune-related genes.

Additionally, the RNA-seq data analyses revealed that *P. infestans* infection down-regulated 1,813, 1,615, and 672 in *2R*, *Rpi-blb2*, and *Rpi-vnt1.1* plants, respectively (Figure S9A; Table S1). Furthermore, *P. infestans* infection resulted in down-regulation of 936, 707, and 679 in *3R*, *RB*, and *R8* plants, respectively (Figure S9B; Table S1). However, *2R* and *3R* plants did not show down-regulation of a broader range of genes or a significant increase in gene expression fold change for down-regulated genes as compared to plants carrying single *Rpi* genes (Figure S9C, D). These data further support our conclusion that stacking *Rpi* genes enhances plant immunity, most likely by inducing a broader range of immune-related genes and globally increasing their expression levels.

StCDPK16 regulates *Rpi*-mediated resistance in a kinase activity-dependent manner

Recent research has shown that PRR and NLR signaling pathways both converge on the cytoplasmic Ca^{2+} influx, leading to activation of calcium responses (Wang et al., 2020b; Kim et al., 2022; Zhang et al., 2024c). It has been reported that CDPKs, CBL/CIPKs (Calcineurin B-like/CBL-Interacting protein kinases), and calmodulin (CAM) are involved in regulating calcium signaling (Tang et al., 2020). However, whether these calcium-related proteins are involved in NLR signaling remains unclear. In our RNA-seq data, stacking *Rpi* genes up-regulated a subset of genes involved in calcium signaling. For example, *CDPKs*, *Calcium-dependent lipid-binding (CaLB domain) family protein* (*CaLB*), and *Calmodulin-binding proteins* (*CBP*) (Figure 3I, J; Table S2). Among them, the expression of *StCDPK16* was highly induced by *P. infestans* at 2 dpi in *2R*, *3R*, or single *Rpi* plants (Figure 4A), suggesting that StCDPK16 is likely to regulate *Rpi*-mediated immune responses. In addition, phylogenetic analysis showed that StCDPK16 is broadly conserved across plant species (Figure S10), indicating a conserved regulatory mechanism by which StCDPK16 regulates plant immunity.

To test whether StCDPK16 has kinase activity, we performed an *in vivo* kinase assay using immunoprecipitated StCDPK16-GFP, the phosphorylation status of which could be tested using α-pThr/Ser antibodies. As shown in Figure 4B, StCDPK16-GFP exhibited auto-phosphorylation, indicating that it possesses kinase activity. Furthermore, an *in vitro* kinase assay showed that StCDPK16 fused with maltose-binding protein (MBP) (MBP-StCDPK16) also exhibited auto-phosphorylation detectable by α-pSer/Thr antibodies (Figure 4C), providing additional evidence for StCDPK16 kinase activity. In both assays, the kinase activity was not detectable in the kinase-inactive mutant $StCDPK16^{D259A}$, which bears an aspartate (D)-to-alanine (A) substitution in the ATP-binding site (D259A) (Figure 4B, C). In this assay, we used the MEK MAPK kinase as a positive control and it also exhibited kinase activity as detected by α-pSer/Thr antibodies (Figure 4B, C). Altogether, these results demonstrate that StCDPK16 is a *bona fide* kinase.

As a next step, we attempted to determine whether StCDPK16 regulates plant basal and/or *NLR*-mediated immunity, commonly known as PTI and ETI, respectively, by inoculating WT and *Rpi*-carrying *N. benthamiana* plants transiently overexpressing StCDPK16 with different *P. infestans* isolates mentioned above. Expression of StCDPK16 or $StCDPK16^{D259A}$ in WT *N. benthamiana* leaves did not affect infection by *P. infestans* isolates JH19, Pi88069, NL07434, and P13626 (Figures 4D–F, S11A), indicating that StCDPK16 does not contribute to plant basal resistance to *P. infestans*.

The *R8* and *Rpi-blb2* plants showed susceptibility to JH19 and NL07434, respectively, because their corresponding effectors AVR8 and AVRblb2 in the respective isolates were not recognized by the NLR receptor (Figure 2). *Rpi-vnt1.1*

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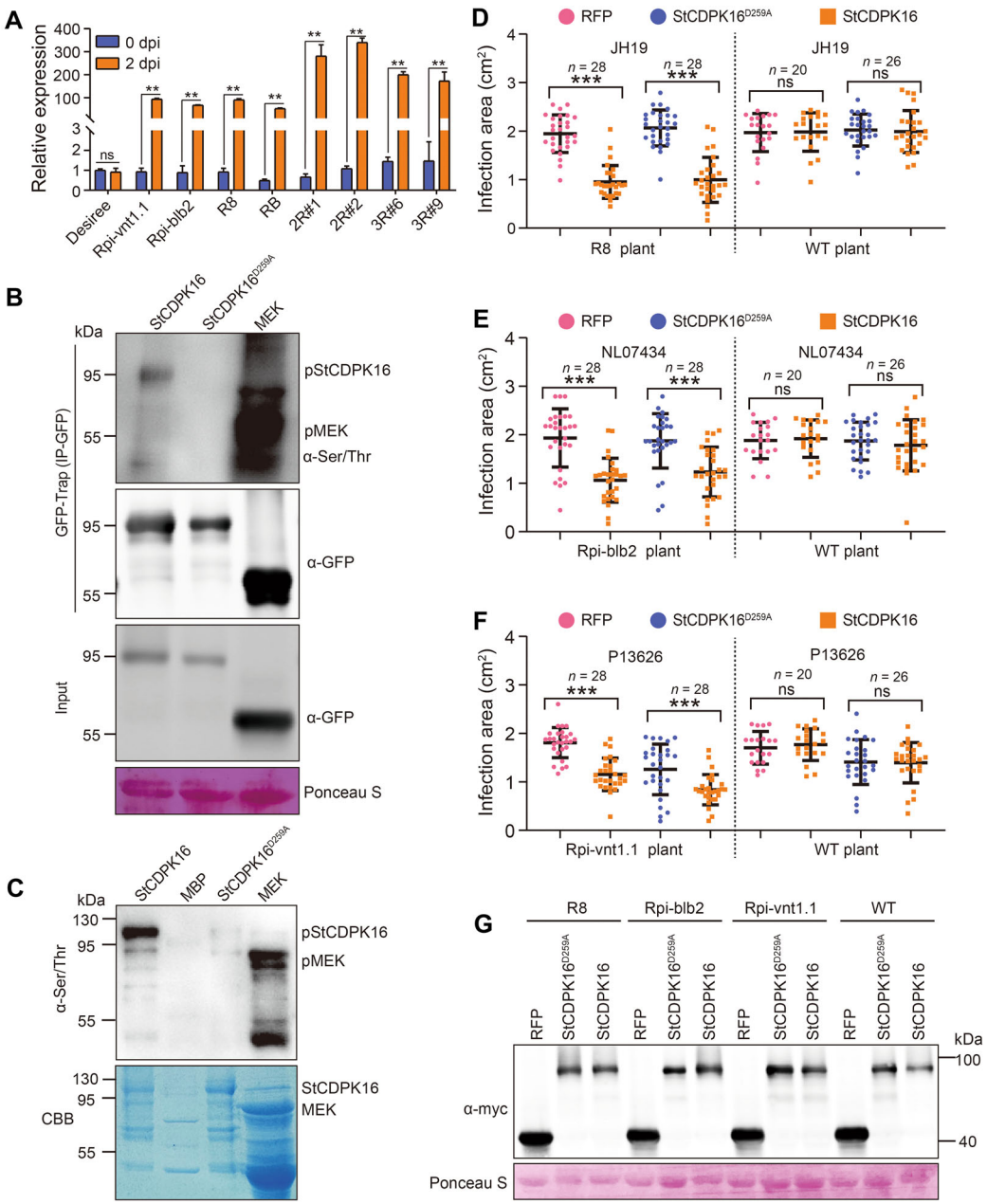

Figure 4. Potato calcium-dependent protein kinase 16 (StCDPK16) contributes to *Rpi*-mediated resistance to aggressive *Phytophthora infestans* isolates

(A) Real-time quantitative polymerase chain reaction (RT-qPCR) shows the relative expression level of *StCDPK16* in leaves of potato plants carrying indicated *Rpi* genes in response to inoculation with *P. infestans* strain HB1501 at 2 d post-inoculation (dpi). Relative expression levels were normalized using the *EF1α* housekeeping gene and data are presented as means ± *SD* ($n = 3$). Double-asterisks (**) denote statistically significant differences according to one-way analysis of variance (ANOVA) followed by Tukey's test (**$P < 0.01$, ns, not significant). **(B, C)** StCDPK16, but not StCDPK16^{D259A}, shows kinase activity *in vivo* **(B)** and *in vitro* **(C)**, as determined by immunoblotting using α-pSer/Thr antibodies. In **(B)**, indicated proteins, tagged with green fluorescent protein (GFP), were expressed in *Nicotiana benthamiana* leaves and then immunoprecipitated (IP) using GFP-Trap agarose beads that was followed by immunoblotting with α-pSer/Thr and α-GFP antibodies (top two). Input proteins are shown by immunoblotting with α-GFP before IP. Ponceau S staining shows protein loading for Rubisco (RBC). In **(C)**, the kinase assay was performed using maltose-binding protein (MBP)-tagged proteins in a kinase reaction buffer as described in the Materials and Methods section. Protein loading is shown using the Coomassie brilliant blue (CBB) staining. **(D)** StCDPK16 regulates R8-mediated plant immunity. *Nicotiana benthamiana* detached leaves carrying *R8* were inoculated with the JH19 strain and infected leaves were photographed under UV light (Figure S9B) at 5 dpi. The lesion areas were measured and are shown as means ± *SD* (n = number of replicates). **(E, F)** StCDPK16 regulates both Rpi-blb2 and Rpi-vnt1.1 mediated plant immunity. *Phytophthora infestans* N07434 **(E)** and P13626 **(F)** infected leaves were photographed under UV light (Figure S9C, D). The experiments were performed as in **(D)**. The data are shown as means ± *SD* (n = number of replicates). Triple-asterisks (***) in **(D, F)** denote statistically significant differences according to one-way ANOVA followed by Tukey's test (***$P < 0.001$; ns, not significant). **(G)** Immunoblot analysis of red fluorescent protein (RFP)-myc, StCDPK16^{D259A}-myc, and StCDPK16-myc in different *Rpi* transgenic *N. benthamiana* leaves using anti-myc antibodies. Total protein was extracted from *N. benthamiana* leaves at 48 h post-inoculation (hpi). Protein loading is shown by Ponceau S staining for Rubisco (RBC). All experiments were performed at least three times with similar results.

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could not confer resistance to P13626, due to its cognate effector gene *AVRvnt1* being silenced in this isolate (Figure 2). Interestingly, when expressed in *R8* transgenic *N. benthamiana* leaves, StCDPK16 caused a significant reduction in the size of JH19-caused disease lesions as compared with StCDPK16^{D259A} and the red fluorescent protein (RFP) control (Figures 4D, S11B), indicating that StCDPK16 positively regulates *R8*-mediated plant immunity in a kinase-dependent manner. Similarly, StCDPK16 reduced levels of NL07434 and P13626 infection in *Rpi-blb2* and *Rpi-vnt1.1* transgenic *N. benthamiana* lines, respectively, compared to StCDPK16^{D259A} and the RFP control (Figures 4E, F, S11C, D). In addition, we silenced *NbCDPK16* and *NbSGT1* (suppressor of the G2 allele of *skp1*), which served as a positive control, in WT and *R8, Rpi-blb2*, *Rpi-vnt1.1*, *2R*, and *3R* transgenic *N. benthamiana* plants using the virus-induced gene silencing (VIGS) method (Figure S12A). *NbCDPK16*-silenced plants showed that the silencing efficiency of *NbCDPK16* reached 50% to 90% compared to *GFP*-silenced plants (Figure S12A). When compared to WT plants, disease symptoms showed that *R8, Rpi-blb2*, *Rpi-vnt1.1*, *2R*, and *3R*-mediated resistance to *P. infestans* HB1501 was compromised in *NbCDPK16*- and *NbSGT1*-silenced plants, but not in *GFP*-silenced plants (Figure S12B). Of note, *NbSGT1*-silenced plants exhibited a larger disease infection area compared to *NbCDPK16*-silenced plants (Figure S12B), indicating that silencing the key co-chaperone involved in stabilization of NLRs (Wu et al., 2017; Zhang et al., 2024a) has a greater impact on plant resistance.

To further support the function of StCDPK16 in regulating *Rpi*-mediated resistance to *P. infestans*, we generated *StCDPK16* and *StCDPK16^{D259A}* transgenic potato overexpression lines in the backgrounds of Desiree^{R8} and Desiree (Figure S13A, B). When infected with *P. infestans* strain JH19, StCDPK16 transgenic potato plants restored the *R8*-mediated disease resistance compared to StCDPK16^{D259A} transgenic plants (Figure S13C). In a control experiment, *P. infestans* strain Pi88069 was unable to overcome *R8*-mediated resistance, as evidenced by the absence of observed infection lesion (Figure S13C). In addition, StCDPK16 and StCDPK16^{D259A} transgenic potato plants with WT Desiree backgrounds showed a similar disease lesion area (Figure S13D), suggesting that StCDPK16 does not contribute to potato basal immunity against *P. infestans* infection. Together, these results corroborate that StCDPK16 regulates *Rpi*-mediated plant immunity in a kinase activity-dependent manner and most likely through modulation of calcium signaling.

DISCUSSION

Over the past 80 years, late blight has been the most serious and destructive disease of potato and continues to cause significant economic losses worldwide (Li et al., 2017). The primary reason for its persistence is that *P. infestans* is undergoing rapid evolution in the field, leading to emergence of new highly virulent isolates that can overcome the *Rpi*-mediated complete resistance of potato (Coomber et al., 2024). To date, approximately 50 *Rpi* genes have been cloned from different wild potato species. However, the resistance mediated by single *Rpi* genes, including *Rpi-blb2*, *Rpi-vnt1.1*, and *R8*, has been almost completely overcome by the newly emerged virulent strains (Vleeshouwers et al., 2011; Paluchowska et al., 2022). That is why breeders have been trying to generate durable and broad-spectrum resistance to *P. infestans* by breeding potatoes with multiple *Rpi* genes using crossbreeding. *RB*, *Rpi-blb2*, *Rpi-vnt1.1*, and *R8* have historically shown high levels of resistance to *P. infestans* and have been used for a long time to breed resistant potato cultivars to control late blight (Vleeshouwers et al., 2011). Genes encoding NLRs undergo strong selection caused by *P. infestans*, which leads to functional diversification of potato NLRs involved in distinct immune pathways. Most potato NLR-mediated immune responses require helper NLRs called NRCs (NLR required for cell death), which serve as a central node of signaling downstream of NLRs (Goh et al., 2024). For example, Rpi-blb2 and R8-mediated immune responses are NRC-dependent, while RB and Rpi-vnt1.1-mediated immune responses do not depend on NRCs. In addition, our previous work showed that RB alternative splicing regulates plant immunity (Sun et al., 2024), and Rpi-vnt1.1 regulates plant immunity in a light-dependent manner (Gao et al., 2020), hinting the important function of these *Rpi* genes in plant immunity. In this study, to determine the downstream common events of *Rpi*-mediated immune responses, RB, Rpi-blb2, R8, and Rpi-vnt1.1 were chosen to generate *2R* (*Rpi-blb2*/*Rpi-vnt1.1*) and *3R* (*RB*/*Rpi-vnt1.1*/*R8*) transgenic potato lines in the cv. Desiree background.

It is well known that the aggregation of multiple disease resistance genes in plants can sometimes lead to undesirable phenotypic consequences, such as growth defects or yield reductions, which could limit their overall performance, especially under field conditions. Therefore, we assessed the growth phenotypes of the transgenic potato plants carrying multiple or single *Rpi* genes. The growth of *Rpi-blb2*, *Rpi-vnt1.1*, *R8*, and *2R* transgenic potato plants was comparable to that of WT Desiree plants, whereas the *RB* and *3R* transgenic lines showed a slight reduction in growth (Figure S3), which is consistent with a previous study showing that transgenic potatoes carrying the genome sequences of *RB* driven by the *CaMV 35S* promoter slightly affect potato growth and yield (Sun et al., 2024), However, potato plants carrying CDS of *RB* show the dwarf phenotype, indicating that the expression of *RB* or its protein levels are precisely controlled in plants to maintain plant growth and immunity. Thus, in plant disease resistance breeding, it is crucial to consider not only the effectiveness of the resistance genes but also their potential impact on plant growth and yield. This requires comprehensive assessments of both agronomic

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performance and disease resistance traits to ensure that the final cultivars are both resilient to pathogens and capable of maintaining high productivity.

Importantly, the *2R* and *3R* stack potato plants showed broad-spectrum resistance to highly virulent *P. infestans* isolates, likely due to the induction of a broader range of immune-related genes and an increase in their expression fold change in response to *P. infestans*, as compared to the single *Rpi* lines. One of such genes, *StCDPK16*, proved to positively regulate *Rpi*-mediated resistance to *P. infestans* in a kinase activity-dependent manner, hinting that StCDPK16-mediated calcium signaling was required for *Rpi*-triggered immunity. Our data demonstrate that stacking *Rpi* genes confers broad-spectrum late blight resistance via inducing stronger gene reprogramming and highlights probable involvement of StCDPK16-mediated calcium signaling in the disease resistance mechanism (Figure 5).

Due to constant exposure to adverse conditions and environmental stresses, *P. infestans* genome undergoes rapid change, resulting in loss, silencing or duplication, followed by diversification, of key virulence genes, particularly those encoding effectors (Chen et al., 2018, 2021; Pais et al., 2018; Coomber et al., 2024). Transgenerational gene silencing and genetic polymorphism in virulence-related genes, such as those encoding effectors, are known to result in gain of virulence and ability to evade host immunity for the Irish potato famine pathogen. For example, *AVR2* exhibits nucleotide diversity, including deletion, insertion, point mutation, and intragenic recombination in different *P. infestans* virulent isolates, leading to evasion of recognition by R2 (Gilroy et al., 2011; Yang et al., 2020). Sequence analysis of *P. infestans AVR3a* from field isolates shows that intragenic recombination is the main reason for AVR3a to escape R3a recognition (Yang et al., 2018). The *AVR4* effector gene, which is under positive selection due to the R4-mediated

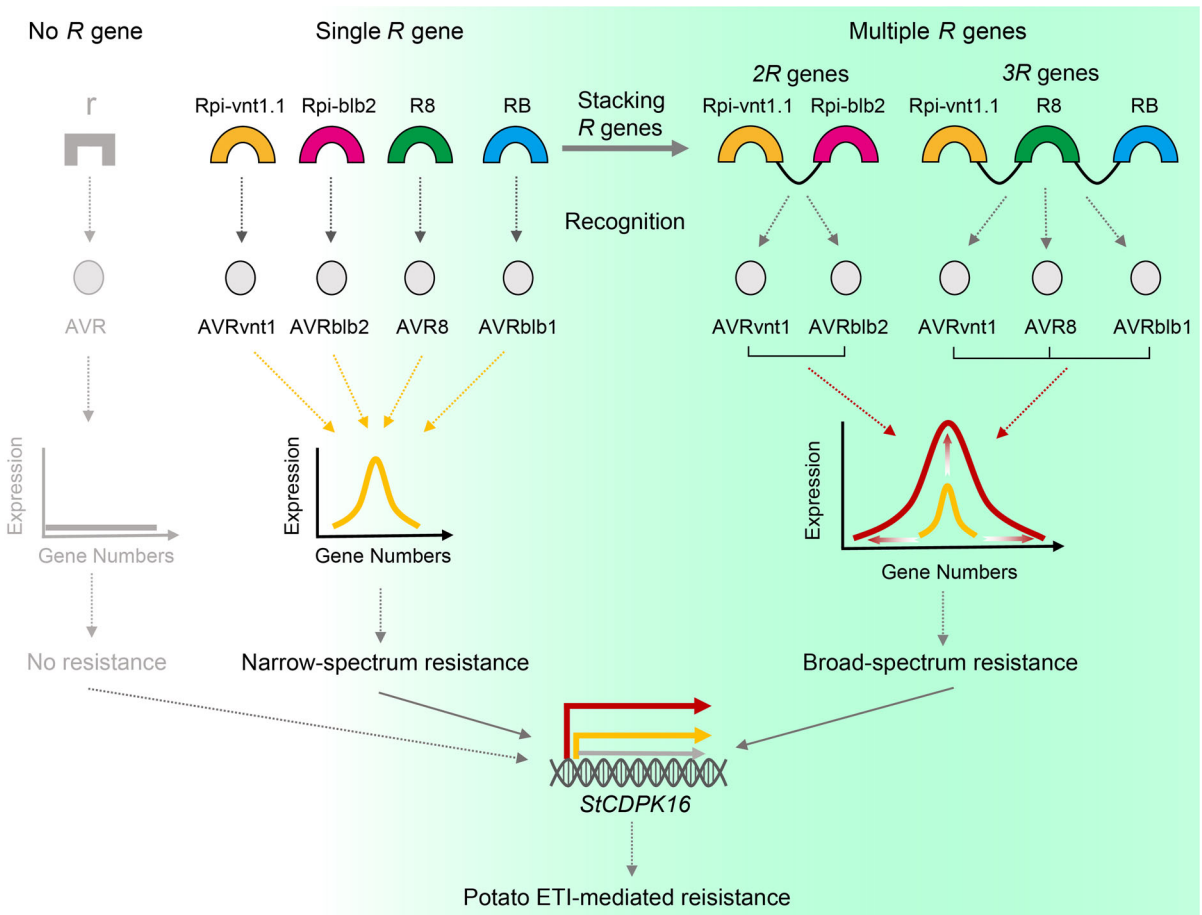

Figure 5. Simple working model showing enhanced potato resistance against late blight through *Rpi* gene stacking
A single *R* gene confers narrow-spectrum resistance due to mutations in the corresponding avirulence (AVR) effector. However, stacking multiple *R* genes expands the resistance spectrum, offering protection against more aggressive strains of *Phytophthora infestans*. Those gene stacks not only induce a broader range of genes but also increase gene expression fold change in a global manner. Additionally, potato calcium-dependent protein kinase 16 (StCDPK16), a key immune regulator, has been identified as contributing to potato effector-triggered immunity (ETI).

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resistance in potato, has mutated into a series of loss-of-function alleles (Waheed et al., 2021). Similarly, transcriptional silencing of *Phytophthora sojae Avr1c*, *Avr1b*, *Avr4/6*, and *Avr3a* helps this pathogen to evade recognition by the NLR genes (Qutob et al., 2009, 2013; Na et al., 2014; Wang et al., 2020a). As another example, *P. infestans* isolate NL07434, which had been collected in the Netherlands in 2007 (Cooke et al., 2012), was found to evade *Rpi-blb2*-mediated complete resistance in this study, albeit the underlying mechanisms remain unknown. In addition, another *P. infestans* strain, P13626, shows no detectable *AVRvnt1* expression during infection and thus it is virulent on plants carrying *Rpi-vnt1.1* (Pais et al., 2018). Interestingly, the P13626 genome still contains the intact AVRvnt1 sequences. Also, JH19, a highly virulent *P. infestans* isolate, was observed to have overcome *R8*-mediated resistance, via currently unknown mechanisms, and we are investigating the regulatory mechanisms by which AVR8 evades recognition by R8. In summary, the above-mentioned *P. infestans* isolates are great experimental tools for studying *Rpi*-mediated plant immunity.

In this study, the pathotests showed that overexpression of *StCDPK16* could increase *Rpi*-mediated resistance to the aforementioned highly virulent isolates. It is noteworthy that StCDPK16 does not contribute to plant basal resistance to late blight, indicating that StCDPK16 specifically regulates *Rpi*-mediated immunity against *P. infestans*. In addition, we isolated eight *P. infestans* strains from different regions of China, including two from Inner Mongolia (NMG), two from Hubei (HB), and four from Chongqing (CQ), all of which could infect potato cv. Desiree. However, none of these isolates were able to infect the *2R* or *3R Rpi* gene stack potato plants, suggesting that each of them carries at least one of the following avirulence genes: *AVRblb2*, *AVRvnt1*, *AVRblb1*, or *AVR8*. In the future, it would be worthwhile to investigate how many effectors are present in these isolates as such analysis could pave the way toward developing new strategies for late blight control in the aforementioned regions of China.

Upon pathogen infection, plants undergo extensive transcription reprogramming, leading to the activation of defense-related genes, including those involved in production of antimicrobial peptides, hormone molecules, and enzymes that reinforce the cell wall (Moore et al., 2011; Li et al., 2016). For example, multiple studies showed that perception of PAMPs triggers rapid and congruent transcriptional outputs at early time points (Bjornson et al., 2021; Winkelmuller et al., 2021). One recent study showed that seven PAMPs (flg22, elf18, Pep1, nlp20, OGs, CO8, and 3-OH-FA) induced similar early transcriptional responses in Arabidopsis (Bjornson et al., 2021). In addition, another study showed that flg22 could induce a subset of common immune-related genes at the early stage of the response in four Brassicaceae species (Winkelmuller et al., 2021). Among these PTI-regulated genes, there were several *CDPK* family members that were significantly induced during the immune response, suggesting that CDPKs play important roles in PRR signaling. In contrast to PTI, which is associated with induction of early immune-related gene expression, activation of NLR signaling (ETI) usually induces expression of the late ones (Tsuda and Katagiri, 2010; Moore et al., 2011; Yuan et al., 2021b). Consistent with the published data, our results show that a large number of such genes are induced in the single *Rpi* potato lines upon pathogen infection, further supporting that the NLR-induced gene regulatory network is conserved across different plant species. Importantly, our transcriptome data show for the first time that *Rpi* gene stacking results in induction of a broader range of immune-related genes and an increase in their expression fold change, suggesting new strategies for improving plant disease resistance through engineering gene regulatory networks.

Several recent studies have shown that activation of ETI could induce PTI-related genes in Arabidopsis, leading to mutual potentiation of PTI and ETI to initiate robust plant immunity (Ngou et al., 2021; Yuan et al., 2021a). Consistent with these findings, our results demonstrate that activation of *Rpi*-mediated immunity induces PTI-related genes, including those encoding RLP and RLK family proteins, as well as MAPK and WRKY transcription factors. Altogether, our data support the notion that plant disease resistance can be improved by strengthening both PTI and ETI responses.

The function of a large proportion of plant ETI-induced genes remains poorly understood. Among the potato genes, which are up-regulated in response to *Rpi* activation, we identified *StCDPK16* as a putative positive regulator of *Rpi*-mediated immunity. *StCDPK16* is not expressed in the plant under resting conditions and its expression is not induced by treatment with elicitors, such as BABA or BTH, or upon infection by virulent *P. infestans* strains that do not induce ETI (Fantino et al., 2017), in agreement with our data (Figure 4A). However, in this study, *StCDPK16* expression was inducible upon ETI activation (Figure 4A), while overexpressing *StCDPK16* did not seem to affect plant basal immunity, based on the pathotests in WT *N. benthamiana* (Figures 4D–F, S9). Consistent with the StCDPK16 function in specifically regulating *Rpi*-mediated immunity, six closely related Arabidopsis CPKs (CPK1/2/4/5/6/7/11) were shown to regulate NLR signaling and plant immunity (Boudsocq et al., 2010; Gao et al., 2013), further supporting the idea that CPKs play important roles in PTI and ETI signaling.

Phosphorylation is a crucial downstream event upon activation of PRR signaling (Kong et al., 2021). Phosphorylation of PRR complexes, including RLK/RLP receptors, co-receptors, scaffold proteins, associated RLCKs, and other immune regulators, is rapidly induced within seconds to minutes upon activation (Couto and Zipfel, 2016; Yu et al., 2017; Saijo et al., 2018; Albert et al., 2020; DeFalco and Zipfel, 2021; Ngou et al., 2022b). For example, a recent study has shown that phosphorylation of BTL2, an RLK, played a role in regulating BAK1/SERK4-mediated cell death (Yu et al., 2023). Another study showed that the BIK1 RLCK and MPK4 phosphorylated DGK5 at distinct sites to maintain phosphatidic acid (PA) homeostasis during the plant immune response (Kong et al., 2024). In contrast to an

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extensive body of knowledge on phosphorylation in PRR signaling, how phosphorylation regulates NLR or NLR components remains largely unknown. To our knowledge, only one NLR named RRS1-R, but not its RRS1-S isoform, is known to be phosphorylated at the C-terminus upon recognition of the effectors PopP2 and AvrRps4, albeit the kinase itself remains unidentified (Guo et al., 2020). The Arabidopsis RRS1-R sensor and RPS4 executor form a complex to recognize two bacterial effectors, PopP2 from *Ralstonia solanacearum*, and AvrRps4 from *Pseudomonas syringae* pv. *pisi*. Phosphorylation of RRS1-R at different sites is required for its autoinhibition and PopP2 responsiveness. In our study, we identified StCDPK16 as a possible positive regulator of NLR-mediated immunity. Our findings suggest that CDPKs might be the kinases phosphorylating NLRs upon recognition of their cognate effectors in plants. StCDPK16 possesses kinase activity, which is essential for its function. Therefore, one could speculate that CDPKs might be involved in phosphorylation of plant NLRs to regulate plant immunity. Overall, our work, combined with previous studies, provides a theoretical basis for breeding durable disease-resistant crops by stacking multiple NLR resistance genes in crop cultivars for controlling plant disease.

MATERIALS AND METHODS

Plasmid construction

AVRvnt1, *AVRblb1*, *AVRblb2*, and *AVR8* tagged with GFP in a *pBIN* vector under the CaMV *35S* promoter for protein expression in *N. benthamiana* were previously described (Gao et al., 2020). *RB*, *Rpi-blb2*, *Rpi-vnt1.1*, and *R8* tagged with Myc in a *pBIN* vector were generated in this study. The fragments of *RB*, *Rpi-blb2*, *Rpi-vnt1.1* were amplified from above-mentioned constructs using primers listed in Table S3, and ligated into the *pAGM4723* vector to generate the *Rpi* gene stack constructs as specified in Figures 1A and S1A using a modified Golden Gate cloning system. We removed the BsaI and BpiI restriction enzyme sites from the *Rpi* gene sequences, for the purpose of domestication, by introducing synonymous codon substitutions to avoid digestion of the amplified PCR products by these two enzymes. The complementary DNA (cDNA) of *StCDPK16* was amplified from a cv. Desiree cDNA library using primers listed in Table S3. It was then ligated into the *pK7WGF2* and *pBIN308* vectors, each containing GFP and the Myc epitope tag at the C-terminus, respectively. This procedure was performed using the ClonExpress II One-Step Cloning Kit (Vazyme, China) according to the manufacturer's protocol. The $StCDPK16^{D259A}$ constructs (in the *pK7WGF2* and *pBIN308* vector backbones) were generated by site-directed mutagenesis with primers listed in Table S3 using the *StCDPK16* constructs as templates. *StCDPK16* and $StCDPK16^{D259A}$ were sub-cloned into a MBP fusion protein expression vector *pMAL-c2x* using the ClonExpress II One-Step Cloning Kit. Primer sequences are listed in Table S3, and all insertions in different vectors used in this study were confirmed by Sanger sequencing.

Potato transformation

For generating potato transgenic plants, 3-week-old potato plantlets (cv. Desiree and $Desiree^{R8}$) gown in half-strength Murashige and Skoog (½MS) medium in a growth incubator at 20–23°C, 50% relative humidity, and 75–100 mE m^{-2} s^{-1} light with a 12-h light/12-h dark photoperiod were used for *Agrobacterium tumefaciens*-mediated plant transformation as previously described with minor modifications (Ducreux et al., 2005). After a 2-d pre-culture period, the explants were co-cultured with Agrobacterium strain GV3101 carrying the indicated constructs with the stacked *Rpi* genes for another 2 d. During this co-cultivation, 2 mg/L of α-naphthaleneacetic acid and 1 mg/L of trans-zeatin were added. Subsequently, α-naphthaleneacetic acid (0.01 mg/L) and trans-zeatin (2 mg/L) were used to promote callus formation and regeneration until visible shoots appeared, and the positive transformants (*2R* and *3R* plants) were selected on Basta (50 mg/L). All potato transgenic plants were further confirmed by RT-qPCR using the primers listed in Table S3.

Potato virus X-mediated HR assay

The binary PVX vector (also named *pGR107*) consisted of genes encoding the replicase, triple gene block, and coat protein as previously described (Torto et al., 2003). *AVRvnt1*, *AVRblb2, AVRblb1*, *AVR8*, and *CRN2* were sub-cloned into the *pGR107* vector and placed under the CaMV *35S* promoter. The resulting plasmids were then electroporated into the GV3101 *A. tumefaciens* strain. The Agrobacterium strains containing indicated *Avr* constructs were grown on Luria-Bertani agar supplemented with kanamycin (50 mg/L) and rifampicin (50 mg/L) for 2 d at 28°C, and then used for plant inoculation. Agrobacterium inoculation and HR observation assays were previously described (Torto et al., 2003; Du et al., 2015). Briefly, 4–6 weeks old potato leaves were pierced at both sides of the mid-vein, and then inoculated with Agrobacterium containing the indicated effectors. The local HR index was observed and scored after 2 weeks.

Phytophthora infestans isolates and infection assays

Potato and *N. benthamiana* plants were grown and maintained in a greenhouse at 22–25°C, 50% relative humidity, and 75–100 mE m^{-2} s^{-1} light with a 12-h light/12-h dark photoperiod. *Phytophthora infestans* isolates were grown on rye sucrose agar (RSA) media plates for 9–12 d at 18°C as described elsewhere (Song et al., 2009). Sporangia were harvested from plates using cold water and zoospores were collected 1–3 h after incubation at 4°C. *Phytophthora infestans* strains included JH19, P13626, NL07434, Pi88069, HB1501, and field isolates NMG-20-29, NMG-20-30, HB-20-26, HB-20-27, CQ-19-01, CQ-19-02, CQ-20-37, CQ-20-38. The zoospore suspension was counted using a hemocytometer and then adjusted to 200 zoospores per

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microliter. Twenty microliters of zoospore suspension were inoculated on 3–4 weeks old detached potato or *N. benthamiana* leaves, and disease symptoms were assessed at 4–5 dpi.

Total RNA isolation and RT-qPCR analysis

Total RNA was isolated from 6-week-old potato leaves infected with *P. infestans* HB1501 as well as uninfected leaves that served as a negative control. Using Plant RNA Kit (R6827; Omega) and quantified using a NanoDrop spectrophotometer (ThermoFisher, USA). One microgram of RNA was treated with gDNA wiper Mix (Vazyme, China) for 2 min at 42°C, and then was reverse transcribed using HiScript II qRT SuperMix II (Vazyme) for 15 min at 50°C. Real-time qPCR was performed using HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme) with the primers listed in Table S3 in a 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The expression of indicated genes was normalized to internal control gene *EF1α*.

Read mapping and differential expression analysis

The read data were analyzed using FastQC, trimmed using Trimmomatic and mapped to the *S. tuberosum* reference genome (The Potato Genome Sequencing Consortium, Nature) (Xu et al., 2011) with HISAT2 (v 2.2.1) and SAMtools (v 1.13). The mapped reads were analyzed by HTSeq (v 0.11.3) with parameters as "-f bam -r name -s no -t exon -i transcript_id -m intersection-nonempty" for counting reads on each gene. All statistical analyses were conducted in the R Environment (v 4.3.2). Statistical analyses and plotting were performed in the Rstudio server using the DESeq. 2, RColorBrewer, gplots, amap, ggplot2, pheatmap, dplyr, ggrepel, and BiocParallel packages. For data exploration (e.g., principal components analysis, correlation, GO term enrichment, and cis-element enrichment), $|\log2(\text{fold change})| \geq 1$, $P_{adj} < 0.1$ was used to obtain a broad landscape of DEGs. To account for time and mechanical stress, we calculated different time points for the WT samples and each transgenic potato sample for combination of DEGs and selected the following criteria: DEGs refer to each transgenic potato sample compared to the same time point of the WT sample. We exclude the genes induced in the WT samples but not induced in each transgenic potato sample. Induced genes refer to the DEGs as compared to the 0 dpi infected stage for each potato sample.

Exploratory data analysis

The visualization of genes induced by various combinations of patterns was done via user-modified adaptations of the ggplot2 and pheatmap R packages. The expression of the core set and induction-specific set of genes up- or down-regulated by pattern treatment was clustered using functionality from Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/). Briefly, the data were filtered to those genes with $|\log2(\text{fold change})| \geq 1$, $P_{adj} < 0.1$ for function cluster (determined empirically to find reasonably specific expression). The GO enrichment analysis was performed using the TBtools-II (v 2.112) (Chen et al., 2023). A documented R-script and the data needed to reproduce figures and analyses are described in the Additional files.

Agrobacterium-mediated VIGS assay

The VIGS assay was performed as previously described (Bachan and Dinesh-Kumar, 2012). Briefly, the VIGS vectors *pTRV1* and *pTRV2* derivatives, *TRV2-GFP*, *TRV2-NbCDPK16*, or *TRV2-NbSGT1* were introduced into *A. tumefaciens* strain GV3101 by electroporation. Bacterial cultures were grown in Luria-Bertani medium containing 50 mg/mL kanamycin and 25 mg/mL gentamicin overnight at 28°C in a shaker with 180 rpm. Cells were harvested by ×500 *g* centrifugation, resuspended in the infiltration buffer (10 mmol/L $MgCl_2$, 10 mmol/L 2-(N-morpholino) ethanesulfonic acid, and 200 mmol/L acetosyringone), adjusted to an optical density at 600 nm of 1.0, and incubated at 25°C for 3 h. Bacterial cultures containing *pTRV1* and *pTRV2* derivatives were mixed at a 1:1 ratio and hand-infiltrated into the leaves of 2-week-old soil-grown *N. benthamiana* plants using a needleless syringe.

In vivo and *in vitro* StCDPK16 kinase activity assay

For *in vivo* StCDPK16 phosphorylation assays, StCDPK16-GFP, StCDPK16^{D259A}-GFP, and MEK-GFP were expressed in *N. benthamiana* leaves for 2 d. The leaf tissue was harvested and used for protein extraction with co-immunoprecipitation buffer (20 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 2 mmol/L dithiothreitol (DTT), 10% glycerol, 0.5% Triton X-100, and protease inhibitor cocktail) by vortexing. Protein extracts were incubated with GFP-Trap agarose beads for 2 h at 4°C with gentle shaking. The beads were spun down and washed three times with washing buffer (20 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 1 mmol/L EDTA, 2 mmol/L DTT, and 0.1% Triton X-100). Immunoprecipitated and input proteins were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel, followed by immunoblotting with the α-GFP(Anti-GFP tag Mouse mAb, Cat#AT0028, Engibody Biotechnology, Inc., Dover, DE, USA) and α-pSer/Thr(α-phosphotyrosine, Cat#61-8300, Thermo Fisher Scientific, Waltham, MA, USA) antibodies.

For *in vitro* kinase activity assays, recombinant MBP-StCDPK16, MBP-StCDPK16^{D259A}, MBP-MEK, or MBP proteins were purified from *Escherichia coli* strain BL21, carrying indicated constructs, using amylose resin (New England Biolabs, USA) according to the manufacture's protocol. The *in vitro* kinase assays were carried out with 1 μg of indicated proteins in a 30 μL kinase reaction buffer (10 mmol/L Tris-HCl, pH 7.5, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 50 mmol/L NaCl, 0.5 mmol/L DTT, 100 μmol/L ATP) at 25°C for 1–2 h. The reactions were stopped by adding the 10× SDS loading buffer. Proteins were then separated on a 10% SDS-PAGE gel and immunoblotted using α-pSer/Thr antibodies to detect phosphorylation activity.

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Data availability statement

RNA-seq datasets were deposited in the National Genomics Data Center (NGDC) database under the accession number PRJCA036635.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

X.Z., F.Z., L.K., V.N. and S.D. designed the experiments, supervised the study, and revised the manuscript; X.Z., X.C., C.Z., H.Z., T.W., J. Z., and X.M. performed most of the research; F.Z. and C.H. carried out bioinformatic analysis; X.Z., X.C., S.W., and X.-J.Z. constructed some of the plasmids and performed some phenotype analyses. All authors read and approved of its content.

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SUPPORTING INFORMATION

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Figure S1. Domestication of *Rpi* gene sequences via synonymous mutations

Figure S2. Stacking *Rpi* genes results in activation of immune responses upon recognition of cognate avirulence (AVR) factors in *Nicotiana benthamiana*

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Figure S3. The growth phenotype of potato transgenic plants carrying a single or multiple *Rpi* genes
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Table S1. List of differentially expressed genes (DEGs) regulated by the single or multiple Rpi genes
Table S2. Annotation information of selected immune-related genes up-regulated in Rpi stacking events
Table S3. Primers used in this study

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