# Engineering pathogen-inducible promoters for conferring disease resistance in tomato

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## Abstract

Plant diseases pose a significant threat to global crop production. Most disease resistance genes used in crop breeding programs encode nucleotide-binding leucine-rich repeat receptors (NLRs) that are limited in pathogen specificity and durability. In this study, we leveraged synthetic biology to develop an inducible broad-spectrum resistance in tomatoes. Constitutive expression of autoactive NLRs in plants leads to robust resistance against multiple pathogens but significantly stunts growth. We expressed autoactive NLRs under the control of pathogen-inducible (PI) promoters to mitigate the fitness costs. Taking advantage of extensive, new genomic and transcriptomic resources, we identified PI promoters that responded to multiple pathogens but not abiotic stress. We further validated functionality of predicted elements through a promoter luciferase assay. We generated significant resistance in transgenic tomatoes but we also encountered unwanted expression induction of the native promoter regions in flowers which led to lethal fruit development. Thus, we pursued promoter engineering for fine-tuning the induction. We identified cis-regulatory regions responsible for pathogen-inducibility through promoter bashing experiments and recombined the native promoter with the inducible part and the core promoter. Furthermore, we rationally created synthetic promoters showing a gradient of expression levels, which will allow for selection for transgenic tomatoes with the best performance. We found that the spacing between functional sequences, repeat number of inducible sequences, and core promoters all influence the outcome of engineering. Our study outlines a framework for developing broad-spectrum synthetic immune constructs with reduced fitness cost and provides examples of pathogen-inducible promoter engineering.

## Introduction

Plant diseases contribute to significant crop yield losses worldwide and disease resistance is the second most sought after breeding characteristic after yield.

Developing crop varieties with genetic resistance has been one of the most widely used strategies for controlling plant diseases (Chitwood-Brown et al. 2021; Liu et al. 2021; Karelov et al. 2022; Lin et al. 2022). Many resistance (R) genes that are deployed today encode nucleotide-binding leucine-rich repeat (NLR) immune receptors that recognize molecules (effectors) produced by pathogen strains (Tamborski and Krasileva 2020). However, NLRs often have limitations in both the spectrum and the durability of the resistance. An NLR is typically activated only by a single cognate effector from a few isolates of a single pathogen. Rapidly evolving pathogens can escape NLR surveillance through mutations or losses of the corresponding effector gene, leading to breakdown of the resistance (Ortiz et al. 2022). Thus, there is a need to explore sources of genetic resistance that offer broad-spectrum protection and rely less on gene-for-gene interactions with a pathogen.

One effective strategy is to stack R genes with different effector profiles. However, this approach requires careful selection of the R gene repertoire and poses significant technical challenges in plant transformation due to large sizes of the transgenic constructs (Luo et al. 2021; Zhu et al. 2012). Another strategy involves disabling genes that pathogens manipulate for host susceptibility, which has been successful in generating resistance against various pathogen classes in tomatoes and rice (Fukuoka et al. 2009; Thomazella et al. 2021). Researchers have also discovered that plants constitutively expressing autoactive (or autoimmune) R genes exhibit broad-spectrum resistance to a range of biotrophic pathogens because they can initiate defense responses without being triggered by specific pathogen effectors (Xiao et al. 2001; Clough et al. 2000). This category of R genes includes Arabidopsis *dndl-1* which encodes a cyclic nucleotide-gated ion channel, and truncated or mutated NLRs like *RPW8* (Risk et al. 2013; Xiao et al. 2001). However, constitutive resistance is often accompanied by undesirable fitness costs, such as stunted plant growth and spontaneous lesions (Clough et al. 2000; Xu et al. 2017). For example, the heterologous expression of wheat resistance gene *Lr34* in barley resulted in substantial leaf tip necrosis and reduced growth, indicating constant defense responses (Chauhan et al. 2015; Boni et al. 2018).

Recent studies suggest that regulating the expression of autoactive R genes in a pathogen-inducible (PI) manner could largely reduce fitness costs while maintaining the resistance efficacy (Hutin et al. 2016; Xu et al. 2017; Gallas et al. 2024; Zeng et al. 2015). With the regulation of *Lr34* by the barley PI promoter Hv-Ger4c, robust resistance was achieved against powdery mildew and leaf rust without significant negative effects on growth (Boni et al. 2018). Several studies utilized the nature of pathogen transcription activator-like (TAL) effectors to induce host gene expression to regulate autoactive R genes (Hutin et al. 2016; Gallas et al. 2024; Zeng et al. 2015).

While the strategy is effective, its application is limited to pathogens containing TAL effectors. Successful applications of PI promoters to control autoactive R genes for cross-kingdom broad spectrum immune activation are yet to be developed.

Precise control over gene expression during pathogen attack, abiotic stress, and across different organs and developmental stages is crucial to minimize the fitness cost associated with autoactive R genes. The rapid expansion of RNA sequencing datasets over the past two decades has facilitated the search for promoters that are highly responsive to multiple diseases of interest but not active in other conditions (Tan et al. 2015; Knief 2014; Naidoo et al. 2018; Wei et al. 2022). Finding an ideal promoter in nature is challenging: many defense-related genes exhibit tissue-specific expression patterns, making it difficult to utilize them across other tissues (Sutherland et al. 2024; Emonet et al. 2021; Munch et al. 2018). Moreover, diverse spatiotemporal gene expression patterns commonly lead to pleiotropy in various biological pathways, including immunity (Martin and Tate 2024, 2023; Hou, Wang, and Chen 2022). In other words, genes that could be induced by pathogen stimulation also respond to some plant developmental cues, as shown by the broad-spectrum powdery mildew resistance locus *mlo* genes and the phenylalanine ammonia-lyase (PAL) gene family (Acevedo-Garcia, Kusch, and Panstruga 2014; Huang et al. 2010). This complicates the control of autoactive R genes with native PI promoters alone.

Promoter engineering is a promising strategy to rationally reprogram gene expression patterns. With a better understanding of promoter biology and the development of synthetic biology tools, undesirable pleiotropy of plant engineering could be mitigated by mutating specific cis-regulatory regions (Hendelman et al. 2021; Song et al. 2022). At the same time, synthetic promoters have been created with enhanced attributes for various biotechnological goals in a wide range of organisms (Cazier and Blazeck 2021; Ali and Kim 2019). Promoter engineering studies have also provided insights into fine-tuning the expression of inducible promoters towards various endogenous or environmental signals by using different combinations of well-characterized core promoters and upstream cis-regulatory elements (Rushton et al. 2002; Shabbir Hussain, Wheeldon, and Blenner 2017; Blazeck et al. 2012). We hypothesize that promoter engineering strategies can be utilized to redesign the PI promoters, allowing for better control of autoactive R genes.

In this study, we designed and tested a workflow for using PI promoters to regulate autoactive NLR genes, aiming to confer broad-spectrum resistance in tomatoes without incurring a fitness cost. Our approach is heuristic and involves six steps: a) selecting native PI promoters based on transcriptomic data; b) validating and further selecting PI promoters through transient expression systems; c) testing PI promoters with autoactive

NLRs in transgenic plants; d) engineering the PI promoters in a transient expression system; e) conducting further testing of the engineered promoters in transgenic plants; and finally, f) performing engineering and testing iteratively until a desirable promoter has been achieved. Through this process of promoter engineering, we reduced the occurrence of unwanted gene induction and created synthetic promoters with a gradient of expression levels, suitable for regulating autoactive NLRs with varying sensitivities. Our research outlines a synthetic biology framework for utilizing transcriptional control to enhance plant disease resistance while balancing the trade-off between immunity and fitness.

## Results

### Mining biotic and abiotic stress transcriptomic datasets guided the selection of PI promoters

Identifying a PI promoter that enables both broad-spectrum responses and specificity to pathogen stimuli is critical for our synthetic design. To achieve this, we analyzed transcriptomic datasets of tomato leaves treated with four foliar pathogens from distinct cross-kingdom taxonomic groups: *Pseudomonas syringae* pv tomato DC3000 (Pst) at 9 hour post inoculation (hpi) (GSE64087), *Phytophthora infestans* (Pin) at 12 hpi (GSE33177), *Xanthomonas gardneri* at 6 hpi (Thomazella et al. 2021) and *Xanthomonas perforans* at 48 hpi (Shi et al 2020). These four pathogens commonly cause diseases in tomato production, leading to severe yield loss (Jones et al. 2014). We also examined whether the selected genes were responsive to abiotic stress by investigating additional transcriptomic datasets obtained from tomato plants subject to drought stress (GSE39894), salt stress (GSE43492 and GSE16401) and wounding (GSE14637). In total, 98 genes showed significant expression induction after exposure to at least one pathogen ($\log_2$ fold change > 2 and FDR-corrected p-value < 0.05) but not during abiotic stress ($\log_2$ fold change <1) (Fig. 1a). Although pathogens were chosen from distinct taxonomic groups, 23 genes were commonly activated by two or more pathogens (Fig. 1b; Supplementary table S1). Several unique gene sets were exclusively induced in response to Pst, Pin, Xg or Xp (Fig. 1b). The 23 genes commonly induced by pathogens were selected for further validation.

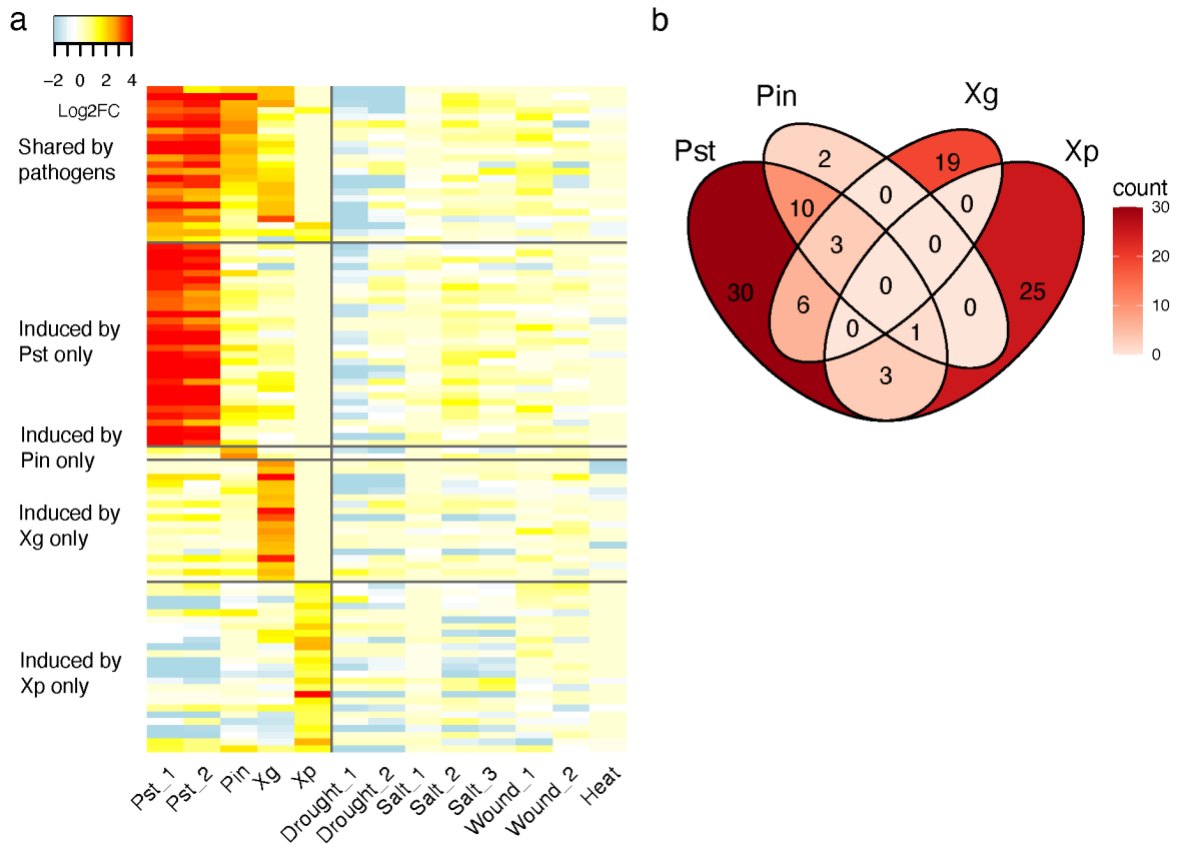


Fig 1. Selection of PI promoters. **a.** The heatmap shows 98 genes that were significantly induced by at least one pathogen ($log_2$ fold change > 2 and FDR-corrected p-value < 0.05) but not by abiotic stresses ($log_2$ fold change <1). The color intensity represents the $log_2$ fold change ($log_2$FC) of the genes (row) under the treatments (column) in comparison with control treatments. The black horizontal lines separate different categories of genes. **b.** The Venn diagram shows the overlaps of significantly induced genes between different pathosystems. Pst, *Pseudomonas syringae* pv tomato DC3000; Pin, *Phytophthora infestans;* Xg, *Xanthomonas gardneri*; Xp, *Xanthomonas perforans*.

**PI promoters predicted from transcriptomic analyses were experimentally tested using transient gene expression reporter assays and *in vivo* pathogen challenge**

An auto-luminescent luciferase-based reporter assay was used to experimentally validate the promoter activity of PI promoters via the *Agrobacterium*-mediated transient expression system on *N. benthamiana* (Garcia and Steinbrenner 2023). Fifteen out of the twenty-three promoters significantly induced by at least two pathogens were successfully cloned and ligated to the luciferase vector. In addition, three promoters of genes known to respond to biotrophic pathogens or pathogen-associated molecular patterns (PAMPs) were included in the assay for comparison: *Arabidopsis* Enhanced Disease Susceptibility 5 (*AtEDS5*), *Nicotiana tabacum* Pathogenesis-Related 1 (*NtPR1*)

and *Nicotiana benthamiana* lysozyme (*LYS1)* (Strompen, Grüner, and Pfitzner 1998; Nawrath et al. 2002; Garcia and Steinbrenner 2023). Promoters of two genes were used as negative controls: the *N. benthamiana* constitutive protein phosphatase 2A (*PP2A*) gene (Pombo et al. 2019) and the synthetic green-tissue specific gene promoter rice $pD_{540-544}$ (Bai et al. 2020). After transient expression of these constructs in *N. benthamiana* leaves, we challenged the infiltrated areas with the following pathogens: PstΔHopQ1, XgΔXopQ, XeΔXopQ, and *Phytophthora capsici* to assess their levels of pathogen induction. We have used bacterial strains without HopQ1/XopQ effector as these are virulent strains on *N. benthamiana* not recognized by endogenous NLRs..

Eight promoters showed significant induction 24 hours after PstΔHopQ1 infiltration, with luminescence differences between the pathogen- and mock-treated leaf areas ranging from 2.1- to 3.4-fold (Fig. 2a and 2c; Supplementary Fig. S1). It is also worth noting that these promoters exhibited different levels of basal expression without pathogen challenge, indicated by the mock-treated leaf areas (Fig. 2c). In addition, the promoter of *AtEDS5* and *NtPR1*, which are known to be involved in basal defense, showed an induction trend but not high enough to reach statistical significance. Compared to PstΔHopQ1, most promoters displayed much weaker responses to XgΔXopQ. Four out of fifteen promoters showed significant inductions, with fold differences from 1.6 to 2.3 (Fig. 2d; Supplementary Fig. S1). Only one gene showed induction by XeΔXopQ (Supplementary Fig. S1). For *Phytophthora capsici* inoculation, increased luciferase activity was seen at the leading edge of lesions where this hemi-biotrophic pathogen was undergoing the biotrophic phase (Fig. 2b), and no luminescence was seen in the middle of lesions due to dead cells. Due to the background induction seen in the negative control promoter $pD_{540-544}$ (Fig. 2e), the fold changes of promoters were compared to that of $pD_{540-544}$ to indicate if they were significantly induced by the pathogen. Eight promoters showed significant inductions, with from 2.2- to 4-fold expression increase by *P. capsici* infection.

Collectively, out of 15 promoters tested by luciferase assay, three promoters were induced by all three pathogens: *Solyc02g086270.4* that encodes a wall-associated kinase, *Solyc01g105070.3* that encodes a peroxidase and *Solyc09g007910.4* that encodes a phenylalanine ammonia-lyase. Two promoters were induced by both PstΔHopQ1 and *P. capsici*: *Solyc02g087070.4* that encodes an alpha-dioxygenase and *Solyc06g009020.2* that encodes a Glutathione S-transferase. These five candidates were selected as PI promoters to modulate the expression of the autoactive NLRs.

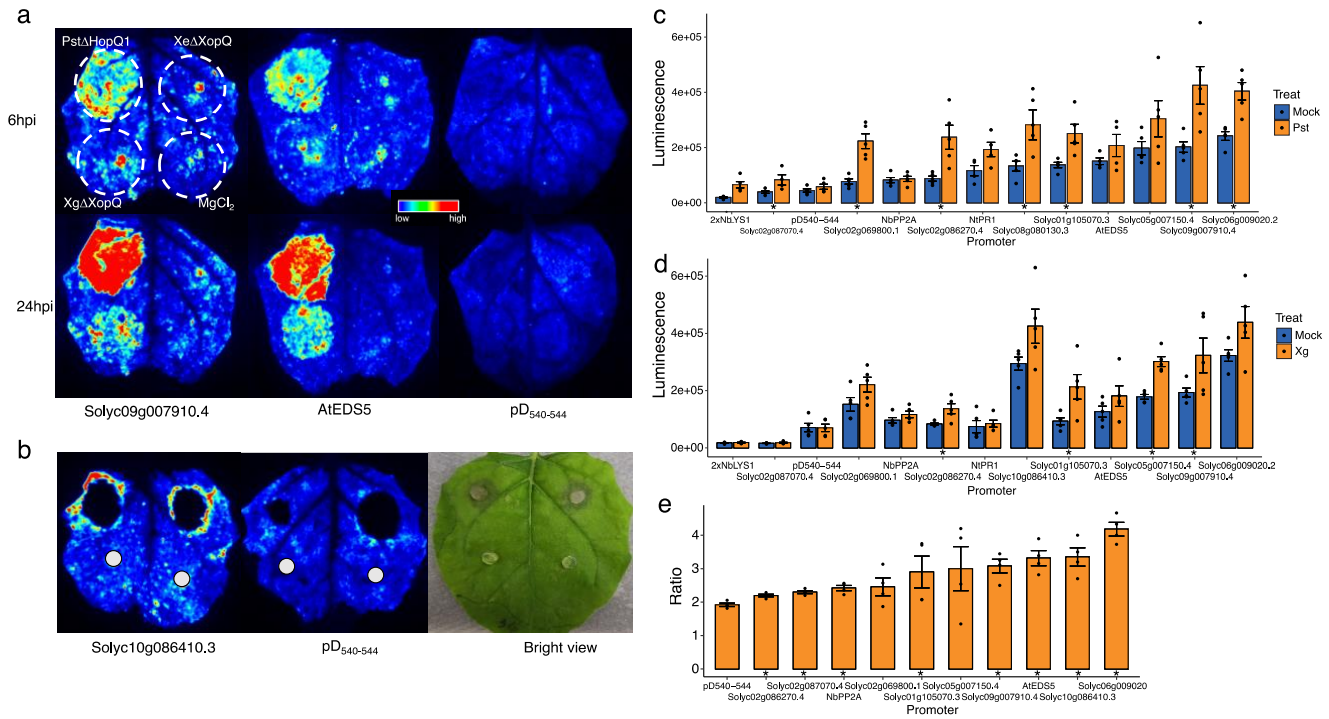


Fig 2. PI promoter validation. **a.** Representative images of leaves infiltrated with selected promoters showed increased luciferase activities of bacteria-infiltrated areas. Agrobacteria alone caused a background level of luminescence, seen as the blue background in these images. The white dashed circles point to pathogen or $MgCl_2$ infiltrated areas. **b.** Representative images of selected promoters showed increased luciferase activities surrounding the necrotic lesion caused by *P. capsici*. The white circles indicate where the clean PDA plugs were put as mock inoculation. **c.** Quantification of luciferase activities driven by various PI promoters with and without PstΔHopQ1 or XgΔXopQ infiltration. Data are represented as mean ± standard error of five biological replicates. P-values were calculated by pairwise t-test, *P<0.05. **d.** The ratio of luciferase activities of *P. capsici*-inoculated area by mock-inoculated area. The statistical comparisons were conducted between the negative control promoter $pD_{540\text{-}544}$ and other promoters. P-values were calculated by pairwise t-test, *P<0.05.

## PI promoters with different strength are useful for regulating NLRs of different autoactivity levels

As seen from the luciferase assay, PI promoters exhibited a wide range of basal expression levels and induction expression levels. To coordinate with the diverse basal expression levels, two autoimmune NLRs of differential autoactivity levels were deployed in our system: $Sr33^{E864V_V840M}$ that shows strong autoactivity and Sr50 that shows relatively weak autoactivity (Tamborski et al. 2023). Combinations of the five selected promoters and two NLRs were tested via Agrobacterium-mediated transient expression on *N. benthamiana* leaves. $Sr33^{E864V_V840M}$ controlled by three promoters

with high basal expression led to significant cell death even without Pst treatment (leaky cell death) (Fig. 3a). When controlled by the other two promoters with lower expression, Sr33$^{E864V_V840M}$ did not cause obvious leaky cell death. By contrast, Sr50 showed leaky cell death only when controlled by the promoter with the highest basal expression. Sr50 controlled by the promoter with the second highest expression caused sporadic leaky cell death in the infiltration spot, and no obvious cell death was seen for three promoters with lower expressions. Collectively, these experiments demonstrate that the coordination between PI promoters of different basal expressions and NLRs with different autoactivities is critical for reducing the leaky cell death. Sr33$^{E864V_V840M}$ with strong autoactivity can be used for PI promoters with low basal level expression while Sr50 with weak autoactivity can be used for those with relatively higher basal level expressions. These results indicate that inclusion of multiple autoactive NLRs can increase the flexibility of PI promoter selection.

**Autoimmune NLRs controlled by native PI promoters showed resistance against *P. syringae* pv. *Tomato* DC3000 but defective reproduction**

Based on the results of the transient expression assay, PI promoters of two genes, *Solyc02g086270.4* and *Solyc09g007910.4*, were used to control Sr33$^{E864V_V840M}$ and Sr50, respectively, and transgenic $T_0$ plants were generated using Agrobacterium-mediated transformation. Most $T_0$ plants transformed with pro*Solyc02g086270.4*::Sr33$^{E864V_V840M}$ grew normally to the flowering stage, like their peer untransformed plants from the same tissue culture process (referred to as “WT” in the following text) achieving similar biomass of vegetative tissues. However, flowers of transgenic $T_0$ tomatoes could not develop fruits, and all of them abscised early and dried out with necrotic sepal and pedicel tissues (Fig. 3c). This suggested that expression of the autoactive NLRs constructs was induced at either flowering or post-pollination stages.

Due to the reproductive defect, clonal propagation was performed for each independent transgenic event to produce transgenic progenies which were further evaluated for disease resistance. Inoculation with Pst revealed that all three independent transgenic events had significantly less bacterial growth after 4 dpi than the WT ($p$-value $< 0.05$), indicating Sr33$^{E864V_V840M}$ regulated by pro*Solyc02g086270.4* could confer effective resistance against Pst (Fig. 3b). The expression pattern of the gene *Solyc02g086270.4* across different tissues on the wild type Heinz tomato cultivar showed that its expression increased by three- to four-fold in fully opened flowers compared to leaves (Fig. 3d), suggesting that the embryo formation could be abolished by high expression of the autoimmune NLR in flowers. The results point to a limitation of using some native PI promoters to regulate autoimmune NLRs that the promoter might also respond to signals other than pathogens such as plant developmental cues. On the other hand,

this construct provides potentially useful embryo lethal gene cassettes that could be used in grafted varieties of vegetatively propagated crops.

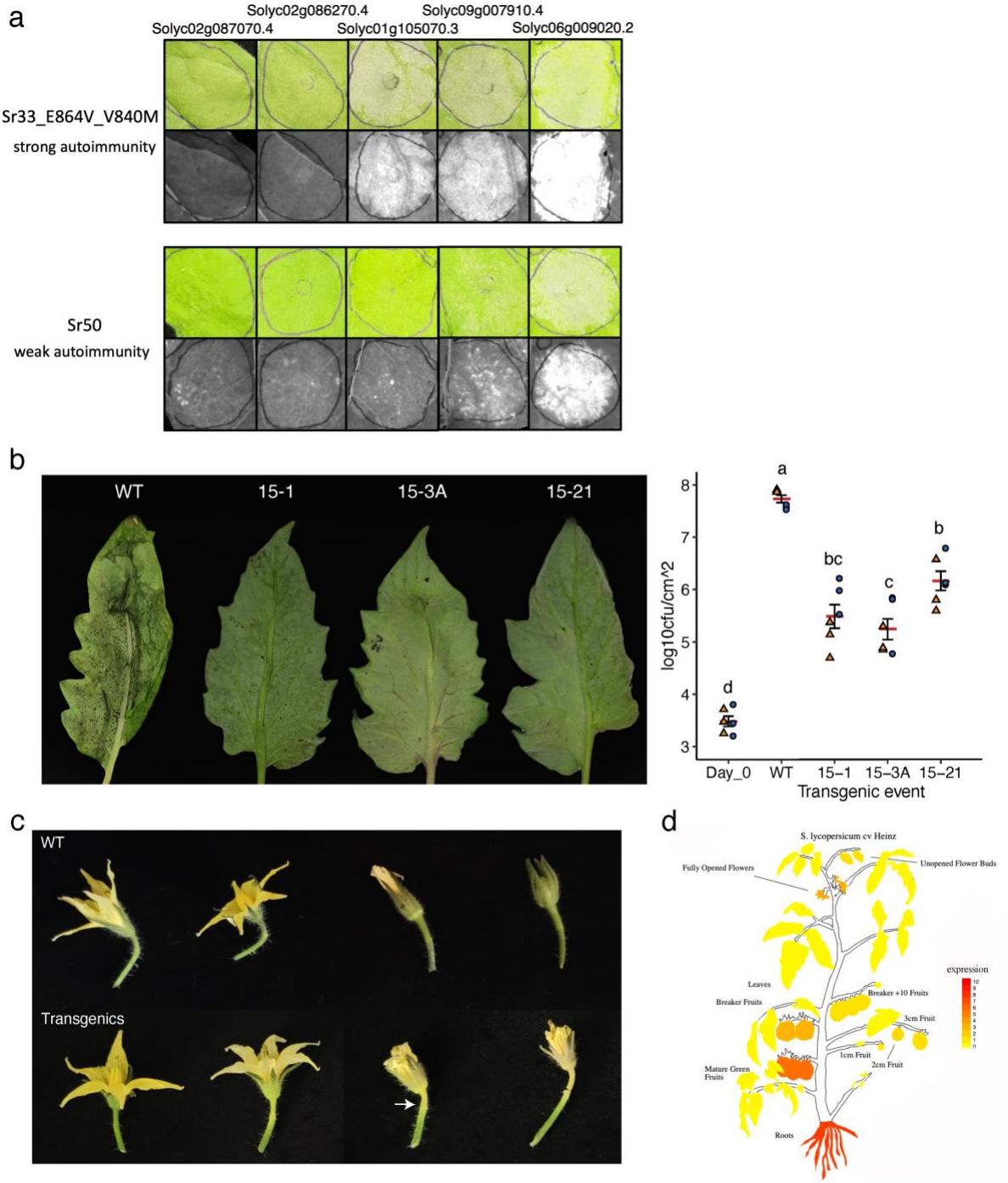


Fig 3. Autoimmune NLR Sr33$^{E864V_V840M}$ by a native PI promoter confers resistance to Pst but has unintended growth defects. **a.** Two autoimmune NLRs of differential autoactivity controlled by diverse PI promoters. The results were observed on *N. benthamiana* leaves two days post *Agrobacteria* infiltration. Photos from the upper row

were taken under natural light and the lower row under UV light to indicate cell death. **b.** Disease symptoms of tomatoes transformed with Sr33$^{E864V_V840M}$ controlled by promoter *Solyc02g086270.4* after *P. syringae* pv. *tomato* DC3000 inoculation. WT, plants generated from the same batch of tissue culture but with no transgene insertion; 15-1, 15-3A and 15-21, three independent transgenic events. Symptoms and bacterial counting were captured four days post inoculation. The letters indicate significant differences between the lines as determined using a one-way ANOVA followed by a Tukey honest significant difference (HSD) test ($P < 0.05$). Symbols (circles and triangles) represent leaves from different plants clonally propagated from the same $T_0$ transgenic event. cfu, colony forming unit. **c.** Flower to fruit setting in wildtype versus tomato transgenics with Sr33$^{E864V_V840M}$ controlled by promoter *Solyc02g086270.4*. The grey arrow points to necrotic pedicels seen for every abscised flower in the transgenics, which led to no fruit setting. **d.** Expression pattern of *Solyc02g086270.4* in different tissues of WT tomato plants. The Fig.  was generated using the Plant eFP https://bar.utoronto.ca/eplant_tomato/ (Waese et al. 2017).

**Promoter bashing identified sequences responsible for pathogen-inducibility**

We hypothesized that the high expression of the PI promoter *Solyc02g086270.4* in the reproductive tissue (Fig. 3d) was caused by cis-regulatory elements independent from the ones responsible for pathogen inducibility. Thus, promoter engineering was conducted on identified native PI promoters *Solyc09g007910.4* and *Solyc02g086270.4* to remove unwanted cis-elements. As the first step of engineering, we identified functional sequences within the promoters responsible for pathogen-inducibility. The 1.8 kb and 3 kb sequences of the two promoters were scanned with NewPlace and a total of 471 and 730 putative transcription factor (TF) binding sites were identified (Supplementary Table S2), including multiple W-box and RAV1 TF binding sites, which are known to be involved in disease resistance and stress responses (Chandan et al. 2023; Javed and Gao 2023). Due to this large number of candidate TF sites, we performed promoter bashing to narrow down the sequences responsible for pathogen-inducibility.

The pro*Solyc09g007910.4* was serially truncated from 5’ end by the positions of W-box and ligated to luciferase for evaluating expression (Fig. 4a). Significant induction by PstΔHopQ1 disappeared when the sequence between -1,408 bp and -1,027 bp were truncated, indicating this region contains regulatory elements largely responsible for pathogen-inducibility. A cluster of five W-box motifs were located within a region of 150 bp from -1,408 bp to -1,027 bp, suggesting that they may be the causal cis-regulatory elements. Our promoter bashing results were supported by the analyses of previously published datasets for transposase-accessible chromatin with sequencing (ATAC-Seq)

that map open chromatin regions and active cis-regulatory elements (Hendelman et al. 2021). A high number of ATAC-seq reads derived from tomato meristem tissues mapped to the 150bp region of the W-box cluster, suggesting that this genomic region is accessible for gene expression regulation (Fig. 4a).

For pro*Solyc02g086270.4*, the same promoter bashing strategy was used. Truncations of the region from -560 bp to -300 bp led to a loss of induction by pathogens and a significant drop in basal level expression (Fig. 4b). More refined serial truncations within this region showed the region from -560 bp to -473 bp was not influential for either pathogen inducibility or basal expression, although it contained a W-box and a TATA box. The region from -473 bp to -400 bp was critical for pathogen inducibility as the induction disappeared largely after truncation. This region was also responsible for basal level expression possibly due to the two TATA boxes located there. The region from -473 bp to -400 bp corresponded to the highest peak in the meristem ATAC-seq data, supporting the open chromatin and active cis-regulatory elements in this region (Fig. 4b).

Several relatively weak ATAC-seq peaks were observed for pro*Solyc02g086270.4* between -560 bp to -944 bp, matching the positions of W-boxes. Truncation of this region led to a slight drop of induction from 1.8 fold to 1.6 fold (Fig. 4b), suggesting it might contain additional cis-regulatory elements responsive to pathogen treatment. However, its pathogen-inducibility is hard to confirm by 5' serial truncation due to the confounding of the large effect element between -473 bp to -400 bp. To validate its pathogen-inducibility without the confounding factors, the sequence between -1,000 bp to -500 bp was directly connected to two sets of core promoters that does not respond to PstΔHopQ1: -400 to 0 bp of pro*Solyc02g086270.4* and a 35S minimal promoter (Fig. 4b and 4c). As a comparison group, we fused the promoter region from -1,500 to -1,000 bp to these two core promoters. The results revealed that only promoters with the region from -1,000 bp to -500 bp could be induced, supporting the presence of additional PI cis-elements in this region (Fig. 4c). Overall, by combining the promoter bashing experiments and ATAC-seq data analyses, we identified functional pathogen-inducible cis-regulatory regions for both promoters *Solyc09g007910.4* and *Solyc02g086270.4*.

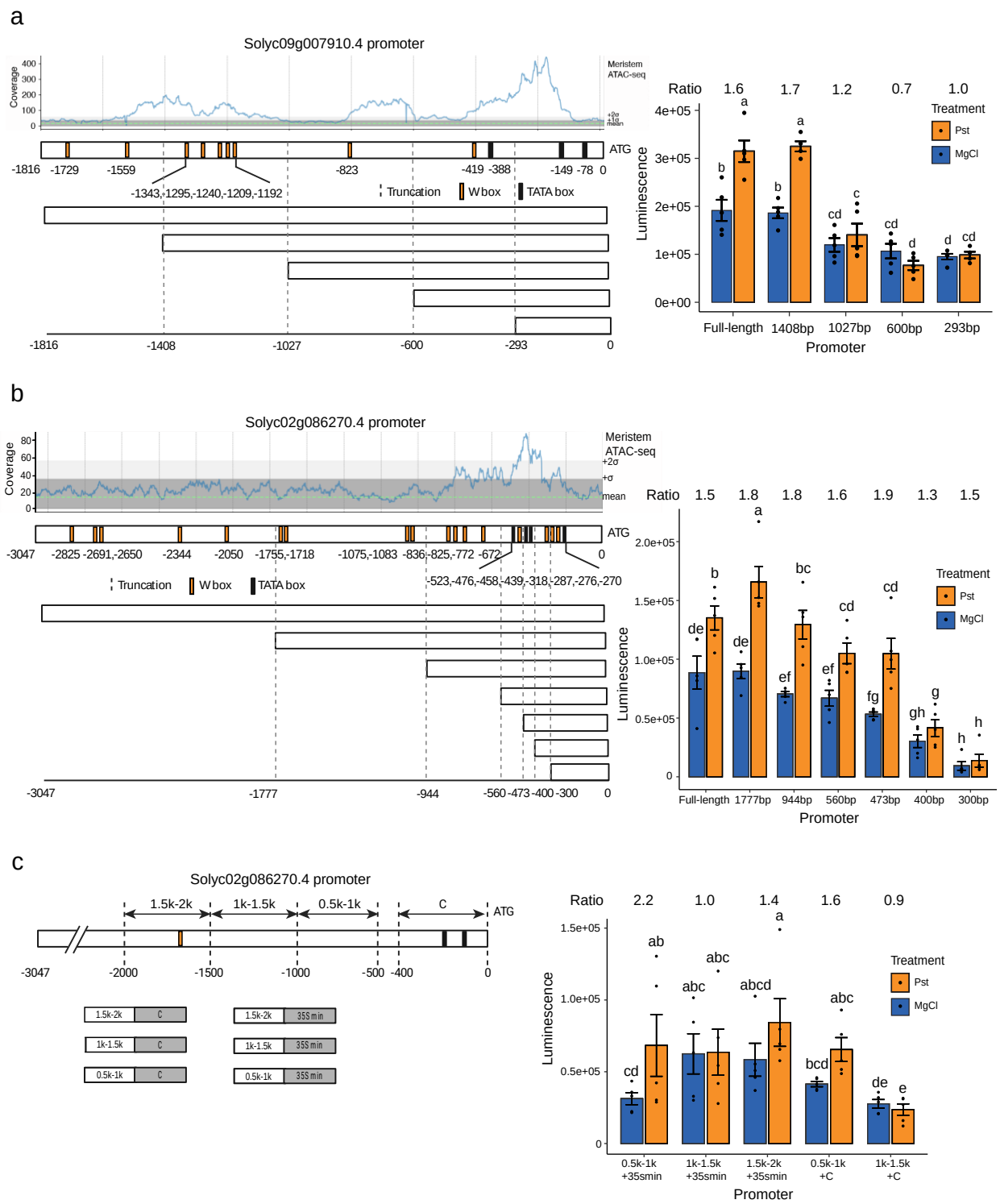


Fig. 4. Promoter bashing to identify pathogen-inducible sequences. **a.** Identification of pathogen-inducible sequences in promoter *Solyc09g007910.4* by 5' end serial truncation. The luminescence was measured via a luciferase assay on *N. benthamiana* 1 day post PstΔHopQ1. The top left shows the ATAC-seq read coverage plot of the promoter, with data from (Hendelman et al. 2021). Mean, the mean read coverage

across the whole genome; s, the standard deviation of the read coverage across the whole genome. The bottom left shows the schematic of the promoter and the truncations. **b.** Identification of pathogen-inducible sequences in promoter *Solyc02g086270.4* by 5' end serial truncation. **c.** identification of additional pathogen-inducible sequences in promoter *Solyc02g086270.4* by recombining the sequence with a core promoter. The luminescence was measured 18 h post Pst infiltration.

**Engineered PI promoters exhibited gradient expressions and lowered the occurrence of unwanted induction**

The data from the promoter bashing experiments and ATAC-seq datasets provided good guidance for rational promoter engineering to remove unwanted cis-elements and modulate pathogen-inducible elements. As seen in Fig 4a and 4b, some promoter regions could contain additional cis-regulatory elements and lead to unwanted induction of gene expressions in the developing tissues. To reduce the unwanted induction of native PI promoters, we recombined the pro*Solyc09g007910.4* by keeping only the pathogen-inducible sequence (W) and the -293 bp to 0 bp non pathogen-inducible region with TATA boxes which we considered as the core promoter (C) (Fig. 5a). At the same time, a variant of the W sequence was created by further truncating W into about 250 bp sequences (called W') more specifically containing the five W-box motifs and recombined in the same way with C. Both the engineered promoters W+C and W'+C could be induced by Pst with similar fold changes to the native promoter, indicating that sequence rearrangements did not disrupt the inducible behavior of the promoter (Fig. 5a). The effect of having different numbers of W and W' repeats were explored to enable fine-tuning of the expression level (Fig. 5a). For W, using two or three W repeats both increased the basal expression level and the induced expression level compared to only one W repeat, and the fold change of induction was improved from 1.7 to 2.0. For W', unexpectedly, one W' repeat gave the highest basal and induced expression of all the engineered promoters tested while increasing the number of W' repeats led to less and less expression. Selected engineered promoters were tested against another pathogen Xg, showing promoter sequence rearrangement did not disrupt the broad-spectrum response either (Supplementary Fig. S2). Overall, we rearranged the promoter to reduce the unwanted induction while maintaining the inducibility. Also, we showed that using various forms of inducible sequences and numbers of repeats can create a panel of promoters with gradient expressions.

**The core promoter is critical for basal level expression but influenced by neighboring sequences**

The core promoter of pro*Solyc09g007910.4* we used (-293bp to 0bp) for engineering corresponds to a wide and tall peak in the meristem ATAC-seq data (Fig 4a). It contained a variety of cis-regulatory elements involved in various pathways, such as light response and secondary metabolism (Supplementary table S2). It might suggest strong basal expression in the meristem tissue that could lead to leaky cell death when connecting with the autoimmune NLR. We further explored whether modulations on the core promoter have any effects on gene expression levels. Serial 5' truncations by 40 bp of the core promoter on the original pro*Solyc09g007910.4* resulted in gradient reductions in the basal expression levels, without affecting the inducibility (Fig 5b). The expression of Pro_C4, where the core promoter was truncated to 133 bp with the first TATA box removed, was almost reduced to the same level as the empty vector, indicating the essential role of the TATA box in primary transcription initiation (Fig 5b). However, when linking core promoter variants directly with the 2xW sequence in the synthetic promoter, the reduction in basal expression was not as obvious as in the original promoter (Supplementary Fig. S3). This result demonstrates that the strength of the core promoter could be rationally modulated but the outcome is influenced by the sequence context in the promoter. Promoters with modulated core promoters can be tested in stable transgenics to select the one with optimal balance between resistance and fitness.

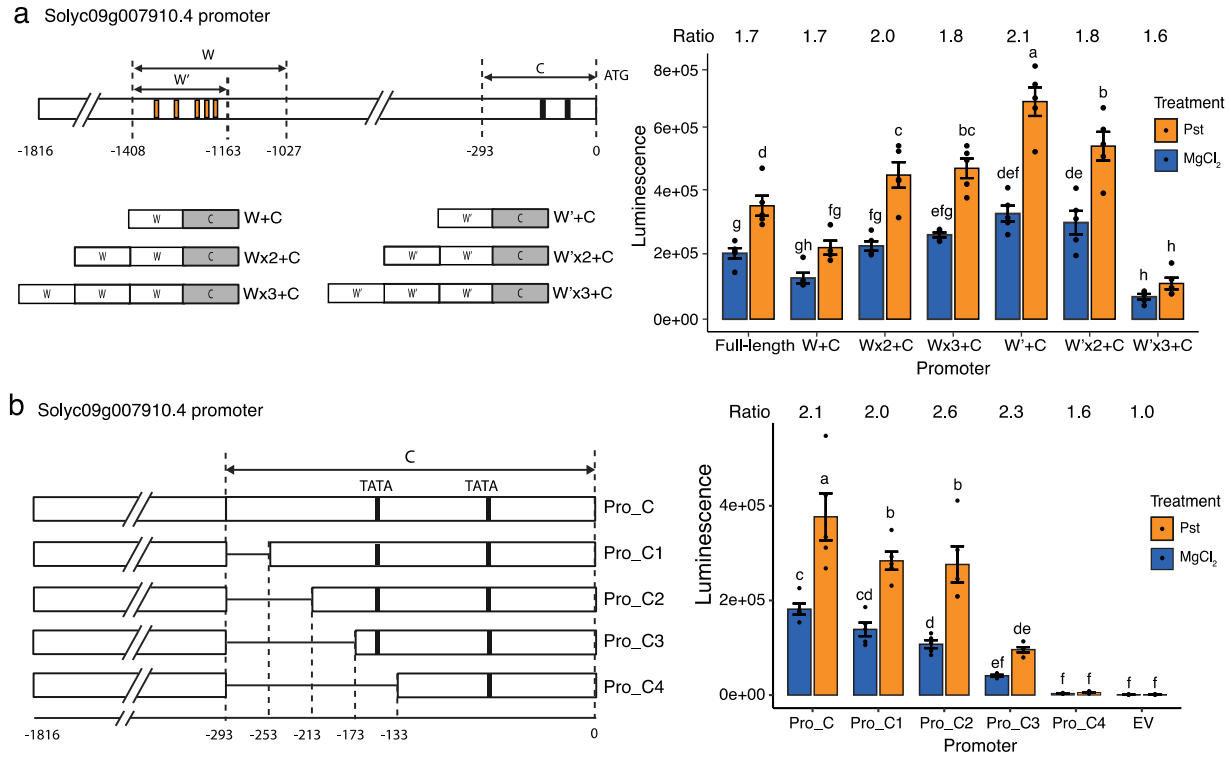


**Fig. 5.** Engineered promoters exhibited gradient expressions. **a.** Synthetic promoters with different repeat numbers of W sequence and W' sequence. W, the ~400bp

pathogen-inducible sequences in pro*Solyc09g007910.4*. W', the ~250bp variant of the W sequence. The luminescence was measured via a luciferase assay on *N. benthamiana* 1 day post PstΔHopQ1. **b.** Synthetic promoters with different versions of core promoter.

## Discussion

Broad-spectrum genetic resistance against pathogens is highly valuable due to the limited target range of native resistance genes used in most crop breeding programs. In this study, we designed a workflow for rational, pathogen-inducible promoter selection and synthetic biology design to develop a novel form of broad-spectrum resistance in tomatoes (Fig 6). After application of this workflow, we developed synthetic constructs comprising an autoactive NLR gene that provides robust resistance, regulated transcriptionally by a PI promoter to prevent constitutive defense activation. One of the PI promoters we identified showed significant induction upon exposure to multiple pathogens. However, when transformed into tomato plants, it caused unintended expression in reproductive tissues, leading to lack of fruit development. To mitigate this fitness cost, we dissected the native promoters and identified the cis-regulatory regions responsible for pathogen inducibility. We recombined the promoter to remove sequences that might cause unwanted induction while maintaining its ability to respond to multiple pathogens. We also employed synthetic promoter engineering strategies to fine-tune the promoter strength for better performance in transgenic plants.

By mining transcriptomic data from tomatoes treated with various biotic and abiotic stresses, we selected PI promoters that were induced significantly by multiple pathogens but not sensitive to abiotic stress. Notably, many conserved defense-related genes, such as PR1, EDS5 or the WRKY70, were not among our selected promoters (Han et al. 2023; Nawrath et al. 2002; Vidhyasekaran 2016). A comprehensive transcriptomic study found that many genes rapidly and commonly up-regulated by PAMPs are also induced by abiotic stresses as part of the plant general stress response (Bjornson et al. 2021). In support of this, all thirteen PR1 genes in tomato were significantly upregulated by drought stress (Akbudak, Yildiz, and Filiz 2020). Therefore, it is advisable to avoid using these genes for our purpose, despite their conservation in response to pathogens. Especially with increasing extreme weather events through climate change, deploying such genes responsive to abiotic stress can lead to fitness costs in regulating autoactive NLRs. It also highlights the importance of mining transcriptomic data of plants in different conditions, not limited to pathogens treatment, as pathogen-responsiveness may not be the sole criterion of determining specificity of pathogen inducibility. The expression of many known PI genes can also be dampened by pathogen effectors (Göhre et al. 2008; Chen et al. 2017), after rapid induction by

PAMPs. This is supported by our luciferase assay results, where these basal defense-related genes showed less significant induction than our selected genes (Fig 2c and 2d). Overall, our results suggest that using transcriptomic data specific to crop-pathogen systems, including both susceptible hosts and abiotic stress treatments, may provide better PI promoters than those of many known conserved basal defense molecular players.

Transgenic tomatoes with the construct pro*Solyc02g086270.4*::Sr33$^{E864V_V840M}$ exhibited significant resistance against Pst in leaves but showed defective fruit formation, likely due to the high promoter activity in flowers. We believe that this synthetic construct could be promising to create an embryo lethal gene inducible cassettes useful for crop variety commercialization, especially when disease resistance and yield can be uncoupled from fruit production, such as in clonal plants or grafted varieties. Although phenotyping for transgenics with pro*Solyc09g007910.4*::Sr50 is pending, similar problems are anticipated based on the multiple high peaks in the meristem ATAC-seq data (Fig 4a). It is common for transgenes to cause undesirable phenotypic changes due to unintended expression. For instance, transforming rice with the *IPA1* gene under its native promoter increased grains per panicle but reduced tillering, which negatively impacted yield (Song et al. 2022). These two traits were governed by the *IPA1* promoter regulation but in different tissues during different developmental stages. By identifying and editing the cis-regulatory elements specific to the tillering stage, the reduced tillering effect was mitigated, breaking the undesirable trait linkage.

Following a similar rationale, we applied promoter engineering to our selected PI promoters to decouple induction upon pathogen infection from induction in developing tissues. Many studies Identify regions controlling unintended expression in specific developmental stages by creating and screening a transgenic tomato library harboring a comprehensive allelic series of cis-regulatory regions (Hendelman et al. 2021; Song et al. 2022), which is a labor-intensive and time-consuming process. Our strategy is distinct in that we recombined the promoter by retaining only the region responsible for pathogen-inducibility and the core promoter region necessary for basal level expression (Fig 5a). Through this rational minimal promoter design, we also intend to minimize the likelihood of retaining unwanted regulatory elements that may be present along the full promoter sequences. Moreover, pathogen inducibility of the recombined promoters can be tested using our transient expression system, with expression levels fine-tuned accordingly. If proven effective in transgenic tomatoes, our strategy could be more efficient and more cost-effective for engineering PI promoters.

The ability to fine-tune the expression of engineered promoters is critical, given the uncertainty of translating results from transient expression systems to stable

transgenics. For inducible promoters, it might be necessary to transform autoactive NLRs with multiple promoters of varying strengths to find the balance between leaky autoimmunity and induced resistance. We explored various promoter engineering techniques to fine-tune the expression of synthetic PI promoters, as it was shown in several synthetic promoter studies that their expression and inducibility could be affected by number, order and spacing of inducible elements (Shabbir Hussain, Wheeldon, and Blenner 2017; Rajkumar et al. 2016; Rushton et al. 2002; Blazeck et al. 2012). We explored the effects of different numbers of inducible sequences, W and W'. We found that increasing the number could either increase or reduce the expression, depending on the inducible sequence used. Both the W and W' sequence harbor a W-box cluster, but W' is a truncated version of W. Thus, the differing effects may result from the spacing between the W-box cluster or between the W-box clusters and the core promoter, suggesting the complexity and dynamics of transcription factor interactions in gene expression regulation. This type of positional effect was also encountered when we were engineering the core promoter. Core promoter variants exhibited different expression levels in the original promoter, but these differences were much weaker when placed adjacent to inducible sequences in the engineered promoter. Several other studies also reported relevant positional effects of cis-regulatory elements, although the mechanisms remain unknown (Murphy, Balázsi, and Collins 2007; Jores et al. 2024).

In conclusion, our study outlines the framework for engineering broad-spectrum resistance in crops using an autoactive R gene regulated transcriptionally by PI promoters. We also established several strategies for making synthetic promoters to reduce unwanted pleiotropic effects and fine-tune expression levels. We used tomato as a proof of concept in this study due to its phylogenetic relatedness to *Nicotiana benthamiana*, so that our findings can be directly translated. Yet, our framework can be applied to other crop systems. This approach could be explored for diseases with no effective genetic resistance resources, such as Huanglongbing in citrus (Alves et al. 2020).

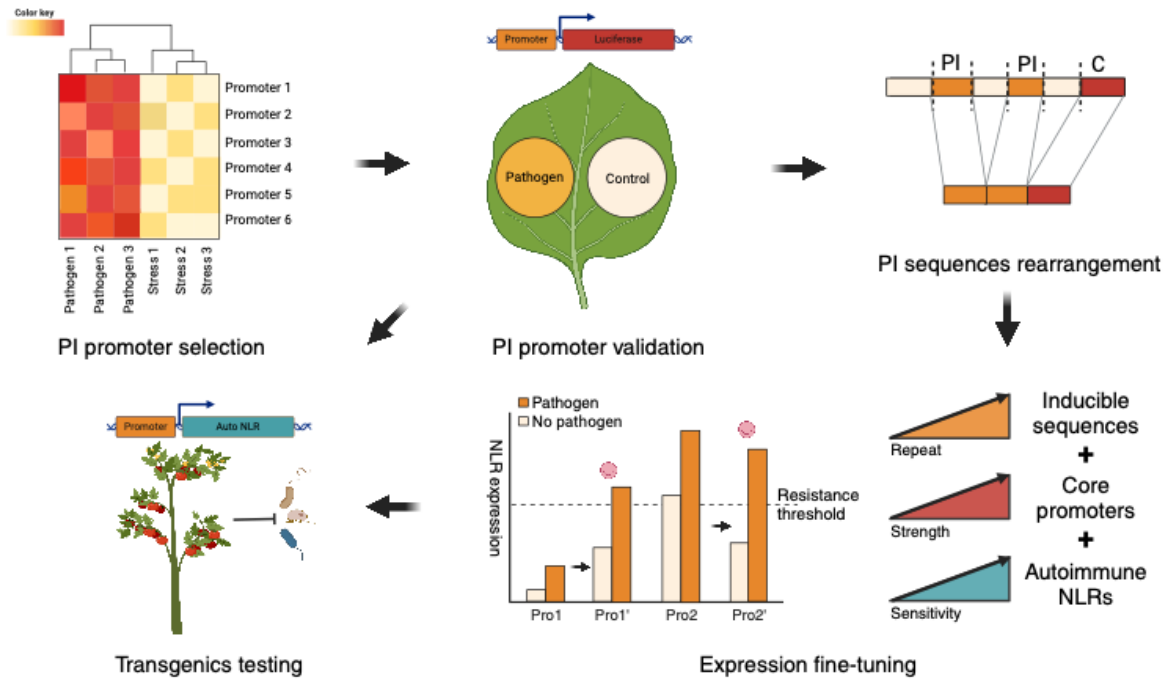


**Fig. 6.** The workflow of generating transgenic crops with autoactive NLRs controlled by pathogen-inducible (PI) promoter to confer broad-spectrum disease resistance with no fitness cost.

## Materials and Methods

### Plant growth

*Nicotiana benthamiana* plants were grown in a Conviron growth chamber under 25°C/23 °C, a 14 h/10 h day/night cycle and 80 µmol/m$^2$/s. The seeds were sowed on the soil surface and covered with a plastic dome to increase humidity until germination. The seedlings were transferred to 3-inch pots 10 days post sowing. Tomatoes were grown in a greenhouse with the condition 28°C/22°C, 14h/10h day/night cycle.

### PI promoter selection

PI promoters were selected using the GeneVestigator (Immunai) that enables cross-comparison between transcriptomic datasets generated with different platforms (RNA-seq or Microarray) and different versions of tomato reference genomes. The promoters of differentially expressed genes (DEGs) after pathogen infection ($\log_2$ fold change >2 and P value<0.05) were selected and filtered by no significant induction by abiotic stress treatment ($\log_2$FC<1).

### Plasmid construction

Constructs in this study were made through GoldenGate cloning or Gibson Assembly cloning methods if relevant restriction enzyme sites were prevalent in the sequence (Engler and Marillonnet 2014; Gibson et al. 2009). DNA parts including 35S promoter, 6xHA tag and heat shock protein 18.2 (HSP) terminator were obtained from the Moclo Plant Toolkit (Engler, et al 2014). PI promoters were cloned from the tomato genomic DNA using the Phusion DNA polymerase (New England Biolabs, USA) with addition of overhangs and the BsaI restriction enzyme site for downstream reactions. Primers were designed based on the *Solanum lycopersicum* ITAG4.0 genome targeting the 1.5kb~3kb genomic regions upstream of the start codon of the gene, including promoter and 5'UTR (Supplementary Table S1). For luciferase assay, promoters were ligated via GoldenGate cloning into the FBP vector (kindly given by the Dr. Steinbrenner's lab) (Khakhar et al 2020; Garcia et al 2023). Synthetic promoters were made by cloning part sequences from native promoters and stitching them together using GoldenGate cloning. For stable transformation constructs, coding region of $Sr33^{E864V_V840M}$ and genomic DNA of Sr50 containing 5'UTR, coding sequences, introns and 3'UTRs were ligated with PI promoters or synthetic promoters and cloned into pCAMBIA2300 vector with a Kanamycin selection marker (Tamborski et al. 2023; Simon, Börner, and Struik 2021).

### ***Agrobacterium*-mediated transient expression**

*Agrobacterium tumefaciens* GV3101:pMP90 transformed with the corresponding construct was grown at 28°C in Luria-Bertani (LB) medium containing with 50 mg/ml Rifampicin, 25 mg/ml Gentamycin and either 100 mg/ml Carbenicillin or 50 mg/ml Kanamycin depending on the vector. The cultures were centrifuged at 4,500 g for 5 minutes and the pellet resuspended in infiltration medium (10 mM MES pH=5.6, 10 mM MgCl2 and 150 uM Acetosyringeone). Optical density was adjusted to a final $OD_{600}$=0.1 as this concentration of Agrobacterium was tested to achieve optimized expression efficiency and single-copy insertion frequency in transient assay (Carlson et al 2023). If co-infiltration, the suspensions of individual constructs were mixed in a 1:1 ratio at a final density of 0.1 for each strain. The top two fully expanded leaves of 4-5 weeks old *N. benthamiana* plants were infiltrated using a blunt syringe. Plants were transferred to dim light (~30 µmol/m2/s) after infiltration for better transient expression (Jutras et al 2021). Phenotypes were recorded 2-3 days after infiltration. For luciferase assay, we noticed that the luciferin will travel outside the *Agrobacterium* infiltration area and cause cross-contamination so we only infiltrated two spots on each side of the main leaf vein.

### ***N. benthamiana* promoter luciferase assay**

Two days post *Agrobacterium* infiltration, leaf areas transiently expressing luciferase were inoculated with pathogens to test the inducibility. Since the wild-type tomato

bacterial pathogens in the transcriptomic analyses are not compatible with *N. benthamiana*, we used the compatible mutants which have specific effector knocked-out. The following pathogen mutants were used: the *Pseudomonas syringae* pv tomato DC3000 HopQ1 knockout mutant (PstΔHopQ1), the *Xanthomonas gardneri* XopQ knockout mutant (XgΔXopQ), and the *Xanthomonas euvesicatoria* XopQ knockout mutant (XeΔXopQ) were obtained from Dr. Brian Staskawicz's lab (Qi et al. 2018). Due to lack of a compatible *X. perforans* strain, the XeΔXopQ was used as a substitute as studies showed they are very closely related (Jibrin et al 2018). Bacterial strains were grown on (LB) medium plate containing 100mg/ml Rifampicin at 28°C for two days. On the night before inoculation, a pellet of bacteria was inoculated into liquid NYG (0.5% peptone, 0.3% yeast extract, 2% glycerol) with 100mg/ml Rifampicin and cultured for 16 h at 28°C. On the next day, bacteria cells were harvested by centrifuging at 4,500g for 5 min and resuspended in 10mM $MgCl_2$ with $OD_{600}$ adjusted to be 0.02 for PstΔHopQ1 and 0.25 for XgΔXopQ and XeΔXopQ (Xu et al. 2017; Thomazella et al. 2021). Bacteria solution was infiltrated into the same infiltration spot as Agrobacterium, with a syringe to make a puncture first to enhance infiltration. The *Phytophthora capsici* strain MP24 was obtained from Dr. Matteo Garbelotto's lab, due to the incompatibility of *N. benthamiana* and *P. infestans*. After growing on the potato dextrose agar (PDA) for 5-7 days, mycelial discs were punched using a 6mm cork borer on the edge of the colony and put on the adaxial side of detached *N. benthamiana* leaves. Mock treatments used PDA punches without *P. capsici*. Detached *N. benthamiana* leaves were put in a humid incubation box covered with wet paper towels.
After 6 hpi and 24 hpi of bacteria infiltration and 40 hpi of *P. capsica* inoculation, luminescence was imaged with a ChemiDoc MP imaging system (Biorad, USA) using the "Chemiluminescence" function with a 60s exposure time. For quantification, the luminescence was measured using an Infinite F Plex Plate Reader (Tecan). Three leaf discs were measured from the same infiltration area as technical replicates and five biological replicates were used. For *P. capsica* quantification, the mean color intensity of the peripheral area surrounding the necrotrophic lesion was measured using the software ImageJ and compared to background intensity.

**Tomato transformation and transgenic plants screening**

Tomato transformation was conducted with the cultivar M82 via *Agrobacterium* at the Plant Genomics and Transformation Facility at Innovative Genomics Institute (IGI Berkeley). T0 plants derived from tissue culture were genotyped for presence of transgene with the Phire Plant Direct PCR Master Mix (Thermo Scientific). Transgene copy number was determined by DNA extraction using the CTAB method (https://opsdiagnostics.com/notes/protocols/ctab_protocol_for_plants.html) and droplet

digital PCR with the QX200™ ddPCR™ EvaGreen Supermix on a QX200 system (Głowacka et al. 2016).

For tomato clonal propagation, young growing shoots with 2-3 expanded leaves were cut. The cutting interface was dipped with Rooting Powder (Bonide Bontone II, Amazon) and planted in soil with 1:2:2 Supersoil:perlite:vermiculite. Transplants were grown in a shaded area for three weeks until new roots grew substantially. The clonally propagated plants were genotyped to confirm the presence of transgene.

**Tomato disease phenotyping assay**

The tomato inoculation with *P. syringae* pv *tomato* DC3000 and *Xanthomonas gardneri* strain 153 were performed mostly following the protocol described previously (Thomazella et al. 2021). Briefly, bacteria were grown in NYG liquid media for 16 h at 28°C. Cells were resuspended in 10 mM $MgCl_2$ and Silwet L-77 at $OD_{600nm}$ = 0.1. Plants were dipped into the cell suspension for five times. Infected plants were put in a growth chamber at 25 °C and 150 µmol/$m^2$/s until disease symptoms developed. For detached leaf assay, leaflets were detached, dipped with bacteria suspension for five times and incubated in a humid transparent box with wet paper towels at the bottom.
For bacterial quantification, three 8mm leaf disks were punched using cork borers, ground in 300 uL 10 mM $MgCl_2$ in a 2 ml Eppendorf tube with two 3.2mm stainless beads using a Biospec mini-beadbeater (1500 rpm, vital distance 3.175 cm). 10x serial dilutions of the homogenate were plated onto LB agar supplemented with 100 µg/ml rifampicin and 50 µg/ml cycloheximide for selecting *P. syringae* pv *tomato* DC3000 and Xanthomonas gardneri. Colonies were observed and counted 72h after plating.

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