Evolving a Plant Pattern Recognition Receptor to Gain Resistance to a Pathogen-Derived Effector by Using a Novel Reverse Yeast Two-Hybrid System

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Abstract

As the first layer of plant immunity, plants use pattern recognition receptors (PRRs) to perceive the existence of pathogens. To counteract PRRs, pathogens secrete effectors that inhibit PRR's functions. In this study, we aim to develop a new strategy to enhance crops' disease resistance by engineering PRRs to elude the attack of effectors, using a directed evolution approach. As a proof of concept, we worked on disrupting the interaction between pathogen effector AvrPtoB and plant immune receptor CERK1. To achieve this, we developed a novel reverse yeast two-hybrid (rY2H) system, TRUST-rY2H (Truncation Resistant and Universal Self-cleaving peptide Technology reverse yeast two-hybrid), which is resistant to a high rate of truncation mutations and can be universally applied to different genetic protein protein interaction assays. Combining this novel rY2H system with a computational prediction method, we successfully identified mutations disrupting AvrPtoB-CERK1 interaction. An *in planta* screening system was also developed to identify *bona fide* AvrPtoB-resistant CERK1. This method not only has potential applications in PPI disruption and pathogen resistance but also opens avenues for future explorations in other realms.

Introduction

Plant pathogens pose a global burden on agriculture, reducing harvest by up to 40% (American Phytopathological Society, 2023). Diseased crops not only lead to reduced yield and quality but ultimately impact the economy and environment (Martins et al., 2018; Savary et al., 2017). Currently, pesticides are used to protect crops, however, they negatively impact the environment and pose health risks to humans (Pathak et al., 2022). Therefore, developing pathogen-resistant crops remains a challenge that demands innovative solutions and research.

Plant innate immunity relies on pattern recognition receptors (PRRs) that detect pathogen associated molecular patterns (PAMPs) (Boutrot & Zipfel, 2017; Zipfel, 2014). Phytobacteria

evade this immune response by introducing virulence proteins called effectors into plant cells via e.g. the type III secretion system to promote pathogenesis (Macho & Zipfel, 2015). As a result, residues on the interaction interfaces find themselves under high selective pressure, facilitating the fixation of interaction-disrupting mutations (Bishop et al., 2000; Bishop et al., 2005; Shabab et el., 2008).

Among the phytobacteria, *Pseudomonas syringae* ranks as the scientifically and economically most important bacterial pathogen worldwide (Mansfield et al., 2012). Strains of *P. syringae* are categorised into at least 50 pathovars and express a variety of effector proteins. One well characterised effector of *P. syringae* is AvrPtoB. Besides *P. syringae*, members of the AvrPtoB protein family are also present in many other strains of *Pseudomonas*, and at least two other genera of bacterial phytopathogens, *Xanthomonas* and *Erwinia* (Oguiza & Asensio, 2005). In *Arabidopsis thaliana*, AvrPtoB targets the receptor kinase CERK1, which is responsible for chitin elicitor signalling and resistance to fungal and bacterial pathogens (Gimenez-Ibanez et al., 2009). CERK1 consists of an extracellular domain with three tandem-LysM motifs, a transmembrane domain, a juxtamembrane domain, and an intracellular Ser/Thr kinase domain (Yang et al., 2022). AvrPtoB can repress chitin responses and overcome CERK1-mediated resistance by inhibiting the kinase activity and targeting CERK1 for degradation (Gimenez Ibanez et al., 2009).

The yeast two-hybrid (Y2H) system is a molecular technique used to detect protein-protein interactions, like those between effector and PRR. In traditional Y2H, the bait protein is fused to a DNA-binding domain (DBD), and the prey protein is fused to an activation domain (AD) (Fields & Song, 1989). For reverse Y2H (rY2H) the roles are switched; the bait is fused to the AD, and the prey to the DBD. Interaction between wild-type proteins in rY2H is selected against, leading to no growth. However, if certain mutations disrupt interaction, the cells will grow on the selection media (Leanna & Hannink, 1996; Vidal et al., 1996). Unfortunately, truncation of proteins is very common in rY2H colonies, e.g. more than 97% of 5-fluoroorotic acid-resistant colonies are expected to contain alleles coding truncation mutations (Gray et al., 2007). To overcome this, we developed the Truncation Resistant and Universal Self-cleaving peptide Technology reverse yeast 2 hybrid (TRUST-rY2H).This system not only avoids truncation mutation but also allows for the AD and DBD to be switched.
By applying our novel TRUST-rY2H system, we evolved CERK1 to gain resistance to AvrPtoB. Identified mutations can be integrated into the plant genome using gene editing. The resulting crop variety may be naturally resistant against *P. syringae*, without relying on bacteriocides.

Results

AvrPtoB interacts with CERK1 and inhibits its function

CERK1 was validated as a direct target of AvrPtoB by Y2H assay (Fig. 1C). The interacting pairs CERK1 and AvrPtoB, as well as positive control antigen T and p53, were fused to DBD and AD and expressed in yeast. In -LWU medium the combination of CERK1-DBD with AvrPtoB-AD shows a considerably higher level of growth compared to all other combinations, demonstrating a strong interaction between CERK1 and AvrPtoB. Interestingly, the interaction between CERK1-DBD and AvrPtoB-AD is not replicated when the DBD and

AD are switched between the proteins, implying the DBD and AD could hinder this interaction if they are not fused favourably. This indicates a need for a Y2H system where DBD and AD can be freely switched between proteins of interest to avoid false negatives.

In order to confirm the impaired immune response caused by CERK1-AvrPtoB interaction, a ROS burst assay was carried out (Fig. 1D). We found that the plants infiltrated with AvrPtoB-producing bacteria showed a significantly decreased ROS production. The results from both Y2H and ROS burst assays recapitulate that AvrPtoB interacts directly with CERK1, inhibiting its function and leading to immune suppression. As such, disrupting the CERK1-AvrPtoB interaction should allow for proper immune function in the presence of pathogenic phytobacteria.

To this end, we applied directed evolution to introduce mutations to CERK1 that prevent interaction with AvrPtoB while maintaining function (Fig 1A). This was done by generating a library of variants, both through error-prone PCR (epPCR) and site-directed mutagenesis for variants generated *in-silico*. Non-interacting mutants will then be selected and transiently expressed in plants. A ROS burst assay of these variants would then confirm their retained function (Fig. 1B).

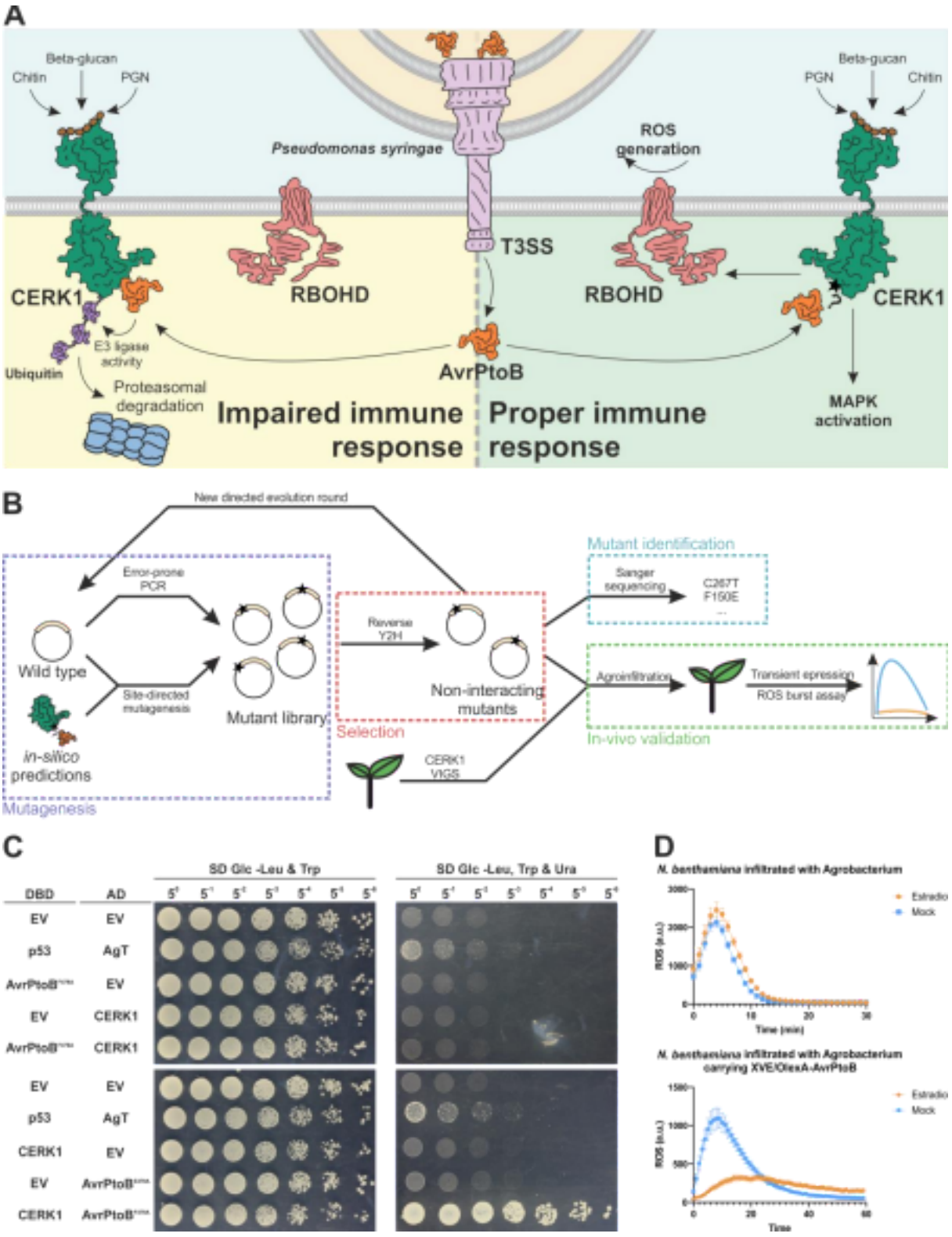

Figure 1. AvrPtoB interacts with CERK1, inhibiting its function and leading to impaired immune response *in planta*.

A) Schematic representation of molecular events in a plant cell harboring an AvrPtoB susceptible (left part) and an AvrPtoB-resistant CERK1 (right part).

B) Workflow for the development of AvrPtoB-resistant CERK1 mutants using directed evolution.

C) CERK1 and AvrPtoB strongly interact when only fused to AD and DBD, respectively.

D) Infiltration with AvrPtoB-producing agrobacteria attenuates the ROS burst in *N. benthamiana* leaf disks exposed to chitin.

Development of a novel reverse yeast two-hybrid system

As the CERK1-AvrPtoB interaction is only visible when CERK1 is fused to the DBD (Fig. 1C), currently available reverse Y2H methods would not be able to select against truncating mutations during the directed evolution workflow (Table 1). To solve this problem, we developed a novel reverse Y2H system, the TRUST-rY2H, which allows for the fusion of CERK1 to either AD or DBD, while still retaining the selection against truncating mutations (Fig. 2A). The system is based on the protein-protein interaction activating the *URA3* gene and can thus be run in both forward and reverse directions using -Ura and +5-Fluoroorotic acid (5- FOA) media, respectively. The anti-truncation system was constructed by fusing BleoR, which confers resistance to zeocin, downstream of the CERK1-DBD fusion. As such, only full-length, in-frame translations of CERK1 support growth in the presence of Zeocin (Fig. 2A & B).

We originally used the $(GS)_3$ linker for BleoR fusion, but this approach notably disrupted the CERK1-AvrPtoB interaction (Fig. 2C last row). We hypothesised that this disruption is likely due to the proximity of BleoR, a problem that could be solved by switching the $(GS)_3$ linker for a self-cleaving E2A peptide linker, physically separating BleoR from the CERK1-AvrPtoB complex. With the E2A peptide, we observed only a slight disruption of the interaction. Nonetheless, we tried to further optimise the system by checking two additional self-cleaving peptides, P2A and O2A. Both the plate (Fig. 2D) and flow cytometry Y2H assays (Fig. 2E) have shown that the E2A peptide interferes with the CERK1-AvrPtoB interaction the least.

In its current state, the system exhibits leaky behaviour, allowing even truncated mutants to grow in the presence of Zeocin (Supplementary Fig. 2). However, this behaviour is only present when the yeast are allowed to grow for >48h post-transformation and seems to disappear when they are plated immediately after the transformation, as would be the case in our directed evolution workflow (Fig. 2B).

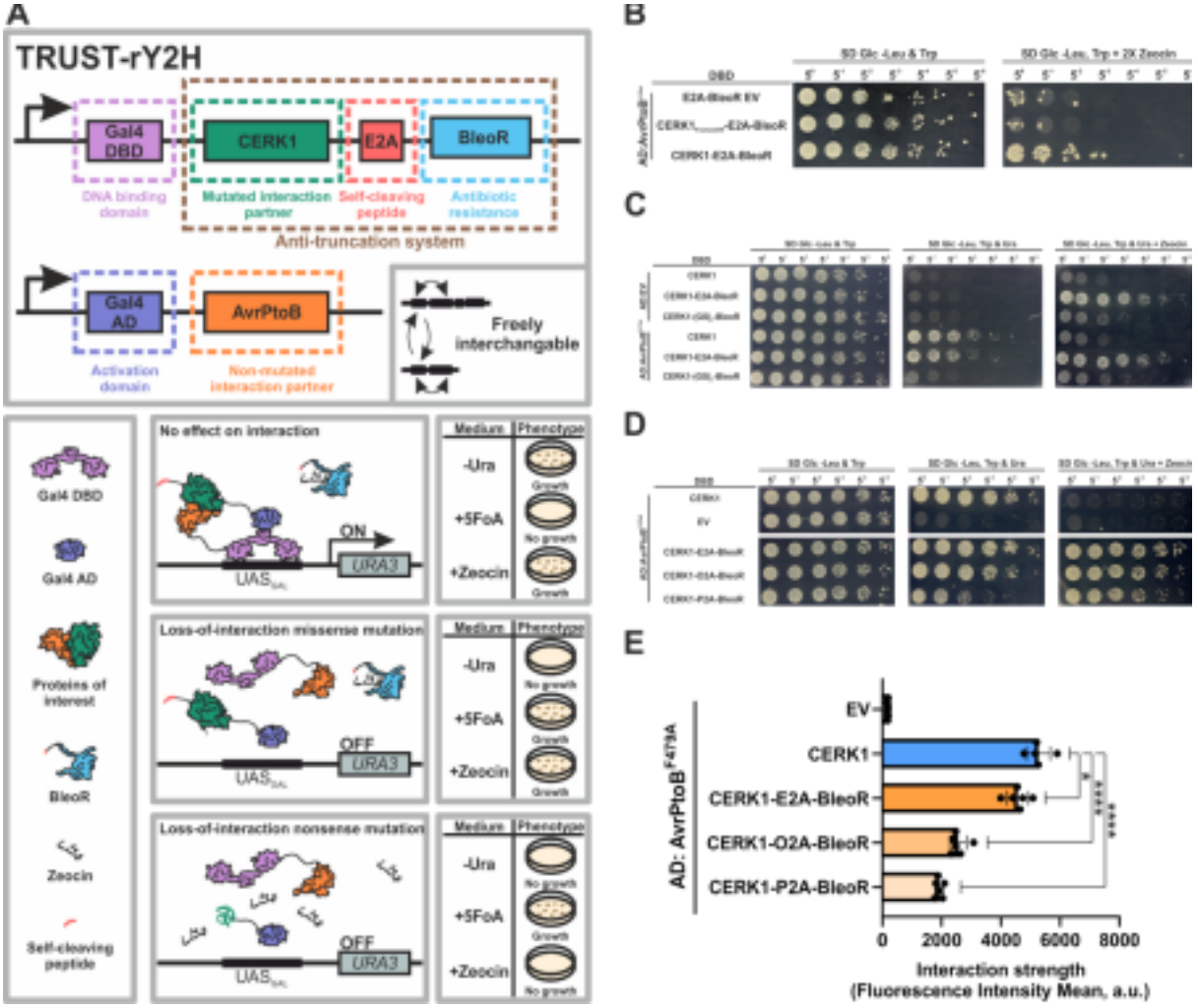

Figure 2. Development and validation of the Truncation-Resistant and Universal, achieved by Self-cleavage peptide Technique reverse yeast two-hybrid (TRUST-rY2H).
A) Schematic explanation of the TRUST-rY2H system (upper panel) and its behaviour depending on the interaction state and selective media used (lower panels). B) When plated immediately post-transformation, truncation mutations are selected against by the BleoR anti-truncation system.
C) Using an E2A self-cleaving peptide instead of the $(GS)_3$ linker reduces the negative impact the BleoR anti-truncation system has on the CERK1-AvrPtoB interaction. D) & E) Compared to other self-cleaving peptides, the E2A peptide causes minimal disruption of the CERK1-AvrPtoB interaction. Differences between sample means in the flow-cytometry data were assessed using one-way ANOVA with Dunnett's posthoc test; <0.01 (*), <0.0001(****)

Table 1. Comparison of TRUST-rY2H to previous methods

	System avoids truncations	Single step selection	Protein of interest can be fused to both the activating domain and DNA binding domain	Compatible with membrane systems (split ubiquitin system)	Fusion does not interfere with protein-protein interaction	Permutable position of AD/DBD to both C and N terminal domain
Original rY2H	-	-	-	-	-	-
rY2H (Vidal et al.)	+	-	-	-	-	-
rY2H (Leanna et al.)	-	-	+	-	-	-
rDY2H (Vincent et al.)	+	+	-	-	-	-
One-plus Two Hybrid system (Kim et al.)	+	+	+	-	-	-
TRUST-rY2H	+	+	+	+	+	+

Table 2. Summary of CERK1 variants obtained from the reverse yeast two-hybrid assays. Each line shows mutations found in a single epPCR colony. Notation R415G means R to G substitution in 415th amino acid. Star denotes a stop codon. Mutations in the interaction regions are underlined.

epPCR rY2H:	**epPCR TRUST-rY2H:**
S510P L398P G502E S444F G461R F267L, L363Q G569R C381W	R415G, A521T Y337C F533S F359S K349R
W412* L388*	K495R, K345* Q451* E575E, K364*

We generated a library of CERK1 mutants using error-prone PCR and subjected it to TRUST-rY2H in order to select for mutants that disrupt CERK1- AvrPtoB interaction. For

comparison, we also selected mutants using the rY2H method. Ten colonies from each screen were sequenced to identify mutations (Table 2). Three out of ten colonies TRUST-rY2H contained were false positives with truncation mutations, while rY2H screen produced two
such false positives. Interestingly, only two mutations (S444F and G461R) were located in or close to the predicted interaction interface. Other mutations were dispersed throughout the whole protein sequence, suggesting a diverse array of mutations impacting the interaction beyond the predicted interaction surface.

In silico mutation discovery

The first round of CERK1-AvrPtoB predictions was done using AlphaFold-Multimer (Evans et al., 2022; Jumper et al., 2021), resulting in an unsatisfactory model confidence of 0.63 (pTM = 0.75 and ipTM = 0.6) (Yin et al., 2022). As the accuracy of the binding complex is pivotal in the discovery of interaction-disrupting mutations, we explored other prediction tools, namely ColabFold (Mirdita et al., 2022). Additionally, the preliminary model showed that both CERK1 and AvrPtoB contain large, intrinsically disordered regions that are not involved in the interaction but are poorly predicted and restrict model confidence (Fig. 3A). The second prediction using the processed input sequences and ColabFold resulted in improved model confidence of 0.74 (pTM=0.83, ipTM=0.72).

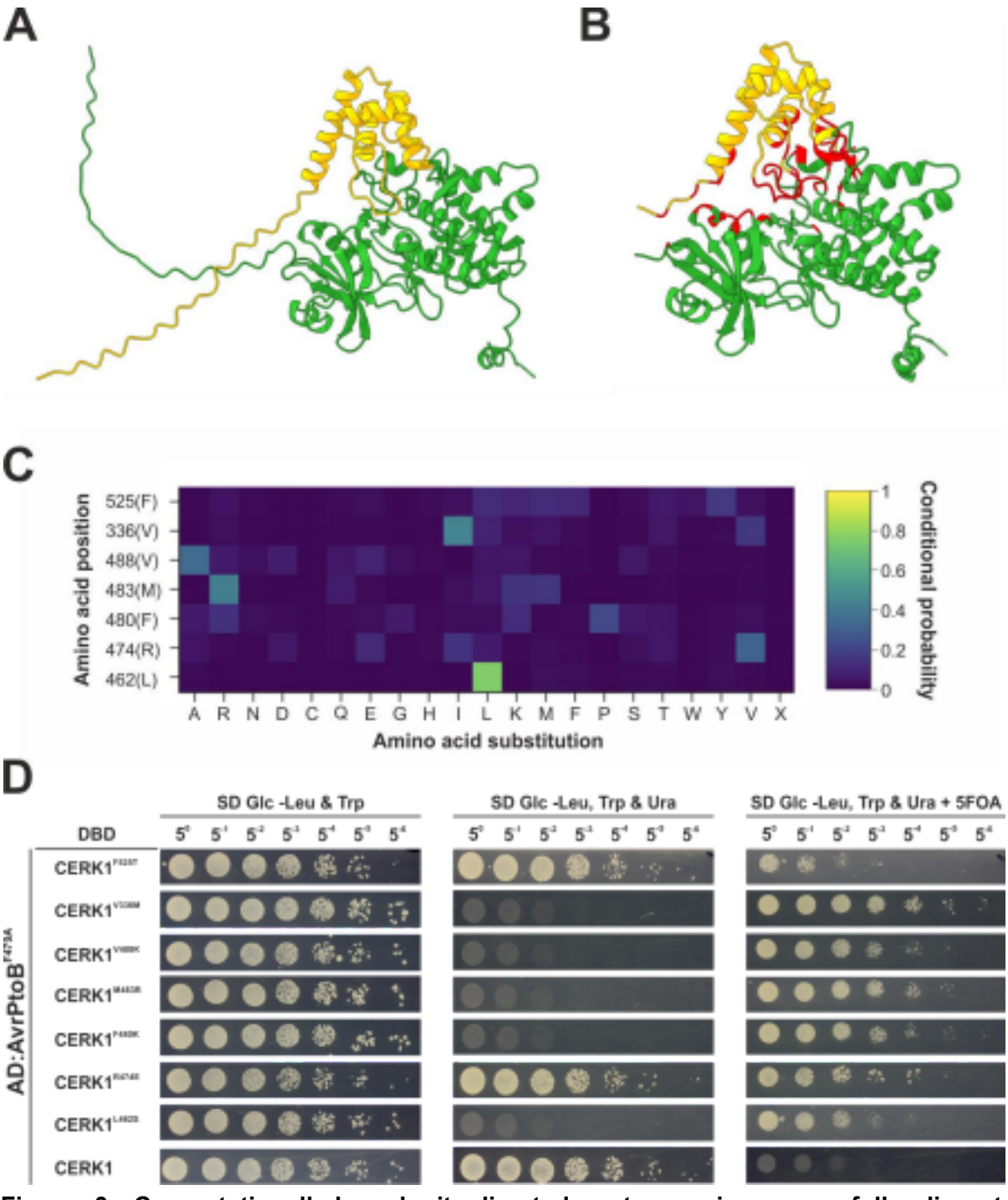

Figure 3. Computationally-based site-directed mutagenesis successfully disrupts CERK1-AvrPtoB interaction.

A) Prediction of AvrptoB-CERK1 binding complex from AF2: The CERK1 is colored green, and the AvrptoB is yellow. Both are attached with a large disordered region.

B) Prediction of cropped AvrptoB-CERK1 binding complex from Colabfold: The CERK1 is colored green, and the AvrptoB is yellow. The binding interface is labeled with red patterns. C) Conditional probabilities of 20 amino acids at selected positions calculated by ColabFold. D) Experimental validation of binding interactions between CERK1-mutants and AvrPtoB-F479A.

In PyMol we roughly determined the binding interface of the best-predicted model by finding all interactions between AvrptoB and CERK1 within 3 Å (Fig. 3B). We further narrowed the scope of our mutation search by excluding potential ATP-binding and

substrate-binding domains (Kobe & Kemp, 2003). From the CERK1 binding interface, we selected residues with the highest number of interaction partners for the site-directed mutagenesis. When choosing the specific substitutions, we opted for those most disruptive for the interaction between CERK1 and AvrPtoB, while still preserving the global protein structure, and in turn the kinase function of CERK1. Therefore, we calculated the conditional probabilities for all 20 amino acid substitutions at the seven most interactive residues using Protein MPNN (Dauparas et al., 2022). Taking the chemical properties of amino acids into account, we settled on the following substitutions: L462S, F480K, V488K, F525T, R474E, M483R, and V336M.

To validate the efficacy of the *in silico* mutations in disrupting the interaction between CERK1 and AvrPtoB, site-directed mutagenesis was employed. We expressed and analysed 7 CERK1 variants in yeast cells, using the wild-type strain as a control (Fig. 3D). As expected, yeast with wild-type CERK1 demonstrated growth on SD gluc -LWU medium, implying an interaction with AvrPtoB. Out of the seven computationally predicted mutants, five (L462S, M483R, F480K, V488K, V336M) successfully disrupted this interaction as evidenced by a lack of growth on the -LWU medium.

In planta screening of AvrPtoB-resistant CERK1 variants that retain kinase activity via transient expression in *N. benthamiana*

Loss of interaction with AvrPtoB can be accompanied by loss of kinase activity. This concern arises from the site-directed mutagenesis analysis of AvrPtoB-BAK1 interaction, where many structure-informed BAK1 mutants that lost interaction with AvrPtoB also lost their kinase activities (Cheng et al., 2011).

For CERK1, we found that two frequently used kinase-dead mutants, D441V and K350N, either lost or exhibited a reduced interaction with AvrPtoB, respectively (Fig. 4A), suggesting that CERK1's kinase activity and its interaction with AvrPtoB might be also closely correlated. Consequently, an additional round of screening for CERK1 variants that retain kinase activity is necessary.

To perform the screening in a rapid and high-throughput manner, we overexpressed CERK1 in *N. benthamiana* using the strong, constitutive 35S promoter, and checked for a cell death phenotype (Fig. 4B). This is based on the fact that cell death triggered by overexpression of CERK1 is dependent on CERK1's kinase activity (Pietraszewska-Bogiel et al., 2013; Suzuki et al., 2018), i.e., only kinase-active CERK1 variants can trigger cell death. Indeed, expression of wild-type CERK1 and not the D441V kinase-dead mutant caused cell death in *N. benthamiana* (Fig. 4C). This strategy is very straightforward and does not need a cerk1 mutant of *N. benthamiana*, which takes a considerable amount of time to construct. However, the cell death phenotype is not solely dependent on the kinase activity, but also the juxtamembrane domain. As such, the lack of cell death does not mean the variant is kinase-dead, so this strategy might generate some false negatives (Zhou et al., 2020).

Active CERK1 variants will be subjected to a second round of *in planta* screening designed to screen for *bona fide* AvrPtoB-resistant CERK1 variants (Fig. 4D). In this round of screening, virus-induced gene silencing (VIGS) is used to silence the native CERK1 of *N. benthamiana* (NbCERK1) (Gimenez-Ibanez et al., 2009). The AtCERK1 variants are expressed using the native promoter, ensuring that AtCERK1 expression remains at a low

level, insufficient to trigger cell death, which allows for the measurement of a ROS burst in response to chitin. At the same time, AvrPtoB is also expressed in an estradiol-inducible manner. By comparing the ROS bursts of mock and estradiol treatment groups, we can determine whether the CERK1 variant is resistant to AvrPtoB.

Due to time constraints, we were unable to perform *in planta* screening of variants obtained from the rY2H and computational prediction. However, our preliminary test using wild type CERK1 and CERK1 D441V kinase-dead mutant proved our method of *in planta* screening to be adequate for testing kinase-active variants (Fig. 4C), and the successful silencing of NbCERK1 created the chassis to screen AvrPtoB-resistant CERK1 variants (Fig. 4E)

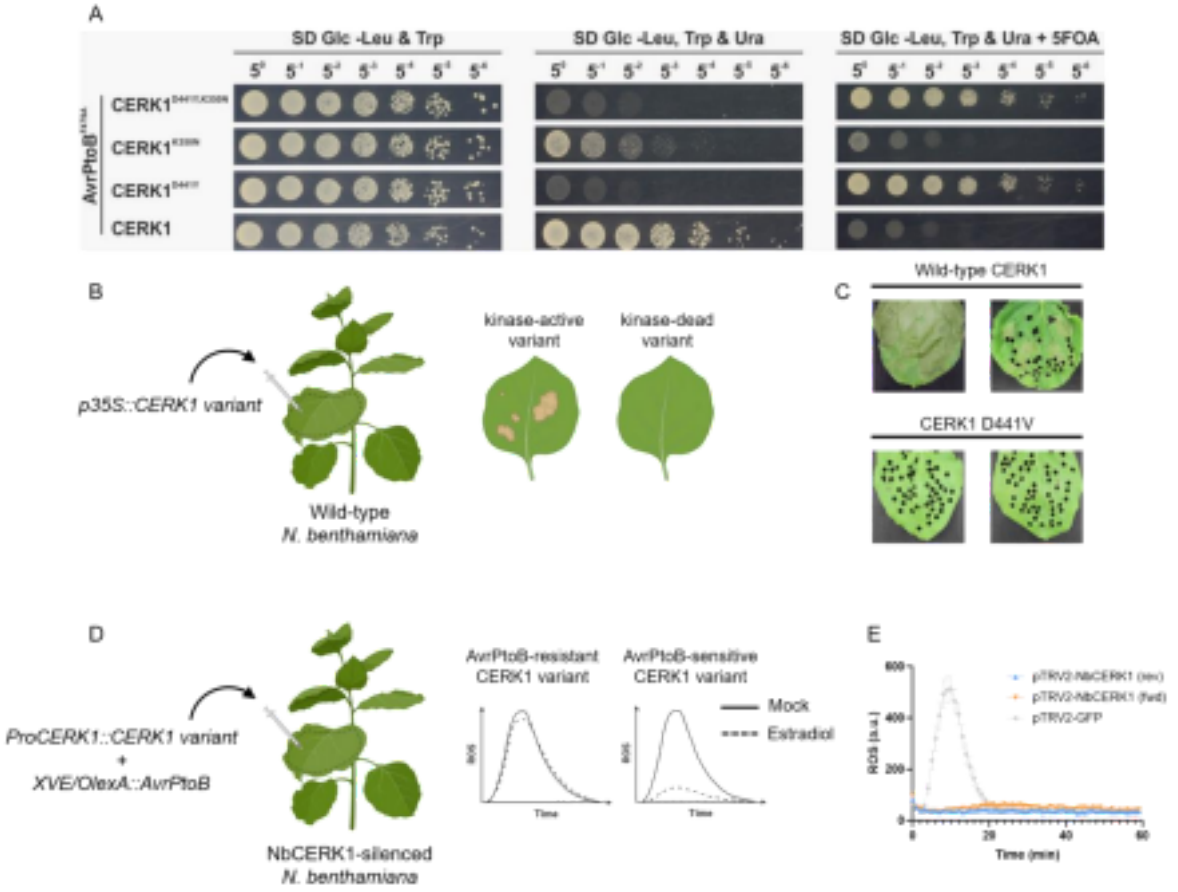

Figure 4. Design of *in planta* screening assays for kinase-active and *bona fide* AvrPtoB resistant CERK1 mutants. (A) Kinase-dead CERK1 mutants lost interaction with AvrPtoB suggesting CERK1's kinase activity and capability to interact with AvrPtoB are closely correlated. (B) The assay used to screen for CERK1 variants that retain kinase activity, which is based on overexpression of CERK1 variants and observation of cell death phenotype. (C) Expression of wild-type CERK1 and not the D441V kinase-dead mutant caused cell death in *N. benthamiana*. (D) The assay used to screen for AvrPtoB-resistant CERK1 variants *in planta*. (E) Successful silencing of native CERK1 of *N. benthamiana*.

Development of a reverse split ubiquitin-based membrane protein yeast two-hybrid system using E2A-BleoR anti-truncation system

Apart from the conventional yeast two-hybrid, diverse genetic methods have been developed to test protein-protein interactions (Stynen et al., 2012). For example, the split ubiquitin-based membrane protein yeast two-hybrid (MYTH) system, which is designed to study the interaction between membrane proteins (Iyer et al., 2005).

Except for the conventional Y2H, no reverse system was successfully developed for other genetic PPI assays, with there being only a failed attempt (Sahin, 2019). In terms of truncation elimination, some strategies are limited to the conventional Y2H (Kim et al., 2007; Vincent et al., 2020), some can potentially be used for other genetic PPI assays like MYTH but with the risk of impairing the interaction detection. Therefore, we strived to develop a reverse MYTH system using E2A-BleoR to eliminate truncation mutations.

To enable the reverse selection, a *Ura3* reporter gene not found in common MYTH strains is needed Therefore we repurposed strain FRY1537 from Ottoz et al., 2014, which carries a *Ura3* gene driven by a synthetic promoter, as a reporter strain for our rMYTH system. A fluorescent reporter strain FRY70+FRP795 (similar to FRY482 from Ottoz et al., 2014) was also constructed to precisely quantify the interaction on a single-cell level using flow cytometry (Fig. 5A).

As a proof-of-concept, we used two well-studied single-pass membrane proteins in the plant immune systems, BAK1 and BIR2, to test our rMYTH system and intracellular domains (Fig. 5A). BAK1 and BIR2 have been reported to interact with each other through both extracellular and intracellular domains(Ma et al., 2017; Halter et al., 2014). As the residues on the interaction surfaces were previously identified, we generated mutations on some of these residues to disrupt either extracellular or intracellular interaction or both of them (Fig. 5B). This resulted in BAK1-BIR2 pairs with different levels of interaction strength, which were quantified by the fluorescence readout (Fig. 5C bar plot). As shown in the plate assay results (Fig. 5B), there was a positive correlation between the interaction strength and the cell growth on the plate without uracil, while the 5-FOA plate showed an opposite trend. These results suggest the success of repurposing FRY1537 as a reporter strain for rMYTH.

We then tested whether E2A-BleoR can be also applied in rMYTH. E2A-BleoR and $(GS)_3$-BleoR were fused after Cub-LexA-VP16 for the bait construct and after Nub for the prey construct, respectively. The fusions of E2A-BleoR after Nub and Cub-LexA-VP16 have no effect and only a mildly negative effect on BAK1-BIR2 interaction, respectively (Fig. 4D). An increase in the interaction strength implies that $(GS)_3$-BleoR can cause false positive results, perhaps because of the homodimerization of BleoR (Dumas et al., 1994).

The rMYTH system can be potentially used to evolve BAK1, the co-receptor of many pattern recognition receptors in plants (Yasuda et al., 2017), to become resistant to plasma membrane-localised *P. syringae* effector AvrPto. Only MYTH, but not the conventional Y2H system is capable of detecting AvrPto-BAK1 interaction in yeast (Halter et al., 2014).

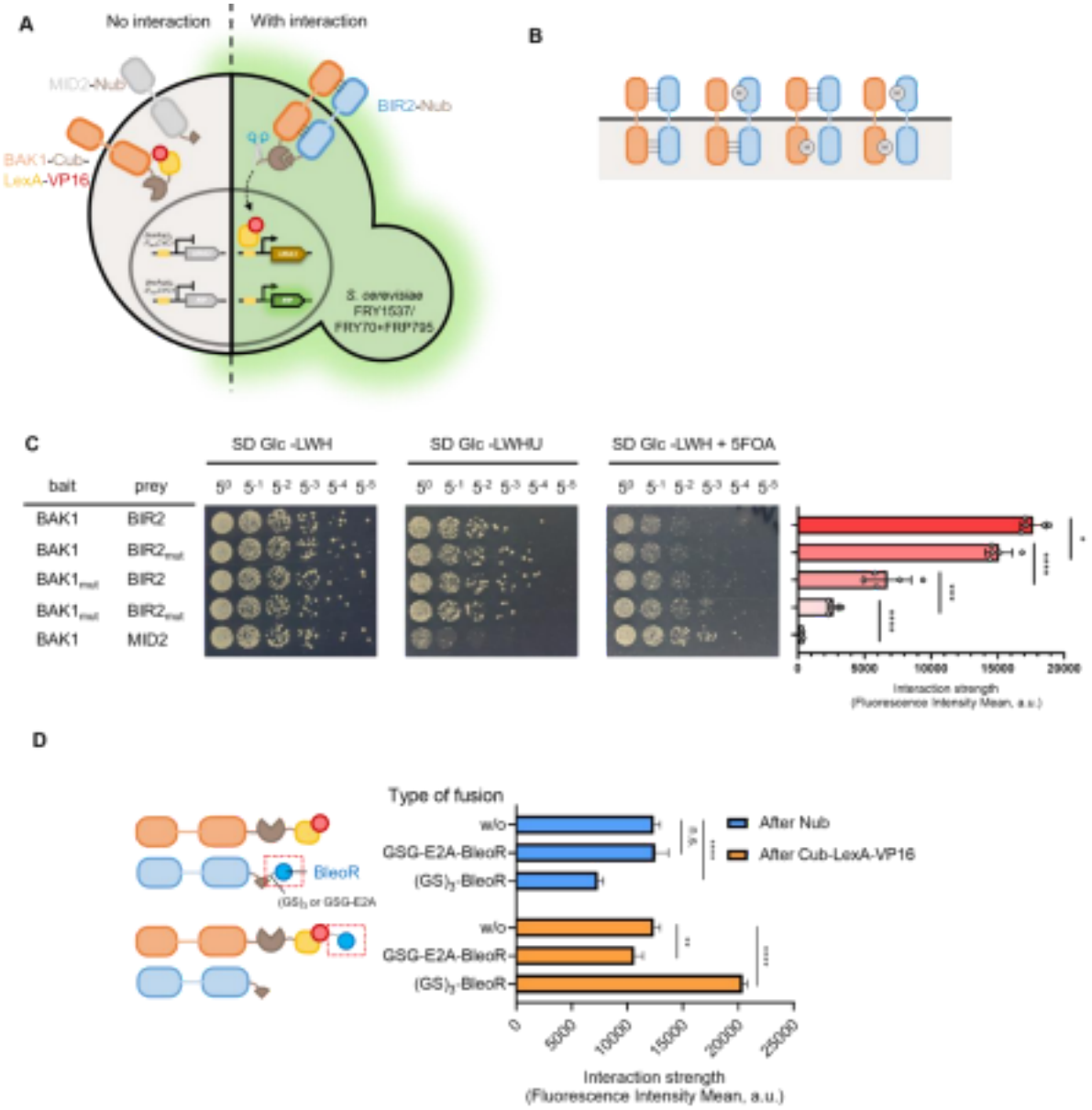

Figure 5. Development of a reverse split ubiquitin-based membrane protein yeast two hybrid (rMYTH) system with E2A-BleoR truncation elimination strategy. (A) Schematic diagram of the rMYTH design used for selection of loss-of-interaction mutations on the plate with 0.5 mg/mL 5-FOA. (B) BAK1 and BIR2 with interaction-disrupting mutations are used to test the rMYTH system. (C) E2A-BleoR can be fused after either Nub or Cub LexA-VP16 without or only mildly affecting BAK1-BIR2 interaction

Differences between sample means in the flow-cytometry data were assessed using one-way ANOVA with Dunnett's post-hoc test; <0.01 (*), <0.0001(****)

Discussion

Compared to existing rY2H systems, our TRUST-rY2H is more robust as seen through the advantages covered in Table 1 and its compatibility with the MYTH system. Although carefully designed (removing the start codon of BleoR, out-of-frame empty vector), the system was still leaky to truncation mutations (Table 2). Perhaps this is due to the leaky expression of BleoR, which might be caused by multiple methionine codons at the C-terminus of CERK1 and upstream sequences with IRES property, or non-canonical

translation initiation (Monteuuis et al., 2019). To overcome this, we plan to introduce D25A mutation to BleoR to compromise its activity by ~ 50% (Dumas et al., 1994), which has been proven useful to counteract the leaky expression (Tominaga et al., 2021).

Surprisingly, among the ten sequenced colonies from the rY2H without anti-truncation selection, only two carried truncation mutations. This truncation rate (20%) is far lower than reported (>97%) (Barr et al., 2004, Gray et al., 2007). One possibility is that some of the hits are false positives. This can be because of mutations disrupting DBD, promoter, and *Ura3* gene, which can also lead to a 5-FOA-resistant phenotype. Therefore, we need to confirm these mutations by performing a round of forward Y2H. Another possibility is that CERK1-
AvrPtoB is sensitive to mutations, i.e., among all the potential interaction-disruption mutations, there are many more non-truncation mutations than truncation mutations. The result could also be due to stochastic colony picking and requires more colonies to achieve statistical robustness.

We also experimented with a mutation discovery method entirely based on machine learning (Bryant et al., 2022; Homma et al., 2023). With the predicted models, we were able to pinpoint the binding interface and design point mutations that prohibit binding interactions, which were experimentally validated. Based on the experimental results, five out of the seven predicted mutations successfully disrupted the CERK1-AvrPtoB interaction.

To ensure the long term functionality of mutant CERK1 and to guarantee that it does not negatively impact fitness, stable expression lines should be constructed by complementing *A. thaliana cerk1* mutant or *fec* (*fls2 efr cerk1*) mutant with obtained CERK1 variants. Multiple immune outputs should be evaluated, for example, activation of mitogen-activated protein kinases (MAPKs) (Yamada et al., 2016), expression of the immune marker genes, and callose deposition. (Gimenez-Ibanez et al., 2009). Ultimately, disease resistance should be quantified by inoculating *Pseudomonas syringae* pv. tomato DC3000 D36E strain carrying AvrPtoB to CERK1 variants complementation lines and measuring pathogen growth.

Our strategy can be used to engineer not only the resistance (R) genes, but also the susceptibility (S) genes, which are exploited by pathogens. Since S genes are often involved in physiological processes of plants, complete knock-out or disruption of a S gene sometimes leads to fitness cost (Koseoglou et al., 2022). Therefore, using rY2H technology to design gene variants resistant to effectors meanwhile retaining its physiological functions can be another potential application scenario of our strategy.

Materials and methods

Yeast two-hybrid

For the Y2H plate assay 4-5 medium-sized yeast colonies were picked and thoroughly resuspended in 50 µL of Milli-Q H_2O. The obtained yeast suspensions were adjusted to an OD_{600} value of 0.2, serially diluted by a factor of 5, and plated on the appropriate selection medium. The base medium was SD glucose with -Leu & Trp as

control, -Leu, Trp & Ura to select for interactions, -Leu, Trp & 5-Fluoroorotic acid (γ = 1 mg mL^{-1}) to select for non
interactions. Zeocin (γ = 1 mg mL^{-1}) was added to the plates to select against truncated and out-of-frame mutations. The MYTH system is based on the same principle, using media that are additionally -His.

Flow cytometry

For each group, 6 colonies are picked, resuspendedended in 1.5 mL yeast SD medium and cultured overnight in 96-deep well plates (Corning®) sealed with 3M™ Micropore™ Surgical Tape. For measurement using flow cytometry, the overnight cultures are diluted 5- fold using PBS (Phosphate-buffered saline) buffer and loaded in the flow cytometer. The measurement of yeast fluorescence using flow cytometry followed the protocol described in Ottoz et al., 2016. The data analysis was performed using FlowJo software, using a one-way ANOVA with Dunnett's posthoc test to assess differences between sample fluorescence means.

Agrobacterium-mediated transient expression in *Nicotiana benthamiana*

N. benthamiana was co-infiltrated with agrobacteria containing a plasmid coding for AvrPtoB, and a plasmid containing P19. 100 ng of plasmid was added to electrocompetent agrobacteria GV3101, which were electroporated at 1.4 kV. 2 h of recovery in 500 ml LB medium was carried out at 28 C. The bacteria were then plated and grown overnight in selective medium. The following day, a liquid culture was started using 5-6 colonies from the plate. The next morning, the bacteria were centrifuged and resuspended in infiltration buffer. The OD600 of the cultures was adjusted to 0.5. P19 culture was added to each trial culture at a ratio of 1:5, with a final OD600 of 0.5. The mixed cultures were then incubated for 1-3 hours at room temperature while shaking. *N. benthamiana* leaves were then infiltrated with the culture mixture using a syringe. The plant was left at room temperature overnight before the leaves were brushed with estradiol (100 mM in DMSO, diluted with water to 50 mM) using cotton swabs to induce expression of AvrPtoB. Negative controls were incubated in 10-50 mM DMSO to mimic experiment groups.

ROS burst assay

Leaf discs were harvested from *Nicotiana benthamiana* using a 4-mm diameter biopsy punch. To decrease the background signal, leaf disks were incubated overnight on 100 µL of distilled water in white 96-well-plates (Greiner Bio-One) wrapped with aluminum foil. Chitin stock solution (10 mg/mL) was diluted for 100 times in to the ROS assay solution (100 µM L0-
12 (FUJIFILM), 20 µg mL−1 horseradish peroxidase (HRP)). Prior to ROS measurement, the water was removed and replaced with ROS assay solution with or without the addition of elicitors. Immediately following the addition of the assay solution, light emission was measured from the plate using Tecan SPARK microplate reader.

Binding complex prediction with AlphaFold2

The CERK1-AvrPtoB binding complex was first predicted using AlfaFold-Multimer V2.3.1 using default parameters with cloned protein sequences as input. Based on the obtained prediction, intrinsically disordered regions in CERK1 and AvrPtoB which don't participate in the binding complex were removed from the input sequences. A second round of predictions was performed using ColabFold V1.5.1 with the new input sequences, recycling number set at 15, and using templates. The predicted models were evaluated by the model
predicted TM scores using the following formula:
$$0.8 \cdot \text{ipTM} + 0.2 \cdot \text{pTM}$$

Analysis of binding interface

The binding interface was determined in PyMol by predicting all possible interactions within 3 Å. The seven most interactive CERK1 residues in the binding interface were selected for site-directed mutagenesis. The selection specifically avoided the residues close to the potential ATP-binding as well as the substrate-binding domain to preserve the CERK1 kinase function. Additionally, glycine and proline were avoided to reduce potentially negative effects on protein stability and folding.

Discovery of structure-preserved mutations

The final mutations were discovered with the help of a protein message-passing neural network (ProteinMPNN). Which takes the most confidence predicted CERK1 structure as input and enables the sequence redesign based on the given structure. The redesigned amino acid sequence is expected to refold into the given backbone. In our work, the redesign only happened at the selected residue positions. The output returns the conditional probabilities of all 20 amino acids at given positions. The higher conditional probability has an amino acid at a given position, it is more favourable in the defined structure. Considering both chemical properties and conditional probabilities, we have chosen the rational mutations, which could prohibit interactions with AvrptoB and preserve the initial CERK1 structure.

Error-prone PCR

GoTaq polymerase was used according to the corresponding protocol. Mutation rates were calculated using the Thermofisher fidelity calculator. In order to have a single point mutation predicted across the CERK1 sequence, regular conditions sufficed. The annealing temperatures were adjusted to the primers and the thermocycler was run for 35 cycles. The PCR product was purified as normal.

Virus-induced gene silencing (VIGS)

Electrocompetent agrobacteria were transformed as described above with either

pTRV1 or pTRV2-CERK1. The bacteria were then recovered and cultured overnight as described above. The following day, the cultures were adjusted to an OD600 of 2.0 and pTRV1 and pTRV2-CERK1 cultures were mixed in a 1:1 ratio. The mixture was then incubated for 1-
3 h at room temperature while shaking. The true leaves of 1-week-old *N. benthamiana* were then infiltrated with the culture mixture using a syringe. The infiltrated plants were grown for 3- 4 weeks at (greenhouse conditions). A ROS burst assay can be performed to validate the gene silencing.

Acknowledgement

We would like to thank Prof. Cyril Zipfel for scientific supervision, as well as for providing the lab space and reagents. We extend our deepest gratitudes to the University of Zurich and ETH for sponsoring the project, as well as to Jena Biosciences, Zymo Research, and Microsynth for providing free services and reagents. Moreover, we appreciate the help of Lucie Reijman with fundraising. We would like to thank Keran Zhai, Yoonyoung Lee, and Sera Choi for their support with experiments. We thank Prof. Jörg Stelling for providing plasmids FRP70, FRP1642, and yeast strains FRY795, FRY1537. We thank Prof. María-Angeles Navas and Prof. Olivier Vincent for providing yeast strain OVY216 and plasmid pACT2-PTAP. We would like to thank Alessia Cambria for contributing to the experiments, as well as Joseph Iwasyk and Flavio Rump for helping to set up the project.

References

American Phytopathological Society, Plant Health to Combat World Hunger. (2023).

P. M. M. Martins, M. V. Merfa, M. A. Takita, and A. A. De Souza, Persistence in phytopathogenic bacteria: Do we know enough?. Frontiers in Microbiology, vol. 9, no. MAY. Frontiers Media S.A., (2018).
DOI: https://10.3389/fmicb.2018.01099

S. Savary et al., Crop health and its global impacts on the components of food security. Food Secur, vol. 9, no.2, pp. 311–327, (2017).
DOI: https://10.1007/s12571-017-0659-1

V. M. Pathak et al., Current status of pesticide effects on environment, human health and its eco-friendly management as bioremediation: A comprehensive review. Frontiers in Microbiology, vol. 13. Frontiers Media S.A., (2022).
DOI: https://10.3389/fmicb.2022.962619

C. Zipfel, Plant pattern-recognition receptors. vol. 35, no. 7. Elsevier Ltd, (2014).
DOI: https://10.1016/j.it.2014.05.004

F. Boutrot and C. Zipfel, Function, Discovery, and Exploitation of Plant Pattern Recognition Receptors for Broad-Spectrum Disease Resistance. (2017).
DOI: https://10.1146/annurev-phyto-080614

A. P. Macho and C. Zipfel, Targeting of plant pattern recognition receptor-triggered immunity

by bacterial type-III secretion system effectors. Current Opinion in Microbiology, vol. 23. Elsevier Ltd, pp. 14–22, Feb. 01, (2015).
DOI: https://10.1016/j.mib.2014.10.009

J. Mansfield et al., Top 10 plant pathogenic bacteria in molecular plant pathology. Molecular Plant Pathology, vol. 13, no. 6. pp. 614–629, Aug. (2012).
DOI: https://10.1111/j.1364-3703.2012.00804.x

J. A. Oguiza and A. C. Asensio, “The VirPphA/AvrPtoB family of type III effectors in Pseudomonas syringae,” Research in Microbiology, vol. 156, no. 3. Elsevier Masson SAS, pp. 298–303, (2005).
DOI: https://10.1016/j.resmic.2004.10.017

F. Xiao, P. Giavalisco, and G. B. Martin, Pseudomonas syringae type III effector AvrPtoB is phosphorylated in plant cells on serine 258, promoting its virulence activity. Journal of Biological Chemistry, vol. 282, no. 42, pp. 30737–30744, (2007).
DOI: https://10.1074/jbc.M705565200
S. Gimenez-Ibanez, D. R. Hann, V. Ntoukakis, E. Petutschnig, V. Lipka, and J. P. Rathjen, AvrPtoB Targets the LysM Receptor Kinase CERK1 to Promote Bacterial Virulence on Plants. Current Biology, vol. 19, no. 5, pp. 423–429, (2009).
DOI: https://10.1016/j.cub.2009.01.054

C. Yang, E. Wang, and J. Liu, CERK1, more than a co-receptor in plant–microbe interactions. New Phytologist, vol. 234, no. 5. John Wiley and Sons Inc, pp. 1606–1613, Jun. 01, 2022.
DOI: https://10.1111/nph.18074

S. Fields and O.-K. Song, A novel genetic system to detect protein-protein interactions. (1989). DOI: https://10.1038/340245a0

C. A. Leanna and M. Hannink, The reverse two-hybrid system: a genetic scheme for selection against specific protein/protein interactions, (1996).
DOI: https://10.1093/nar/24.17.3341

M. Vidal, R. K. Brachmannt, A. Fatraey1, E. D. Harlow, and J. D. Boeket, Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein and DNA-protein interactions (URA3/negative selection/5-fluoroorotic acid). (1996).
DOI: https://10.1093/nar/24.17.3341

P. N. Gray, K. J. Busser, and T. G. Chappell, A novel approach for generating full-length, high coverage allele libraries for the analysis of protein interactions. Molecular and Cellular Proteomics, vol. 6, no. 3, pp. 514–526, (2007).
DOI: https://10.1074/mcp.T600023-MCP200

J. Jumper et al., Highly accurate protein structure prediction with AlphaFold. Nature, vol. 596, no. 7873, pp. 583–589, (2021).
DOI: https://10.1038/s41586-021-03819-2

R. Evans et al., Protein complex prediction with AlphaFold-Multimer. (2022).
DOI: https://10.1101/2021.10.04.463034

R. Yin, B. Y. Feng, A. Varshney, and B. G. Pierce, Benchmarking AlphaFold for protein complex modeling reveals accuracy determinants. Protein Science, vol. 31, no. 8, Aug. 2022.
DOI: https://10.1002/pro.4379

M. Mirdita, K. Schütze, Y. Moriwaki, L. Heo, S. Ovchinnikov, and M. Steinegger, ColabFold: making protein folding accessible to all. Nat Methods, vol. 19, no. 6, pp. 679–682, (2022).
DOI: https://10.1038/s41592-022-01488-1

B. Kobe and B. E. Kemp, Principles of Kinase Regulation, vol. 1–3. Elsevier, (2003).
DOI: https://10.1016/B978-012124546-7/50450-2

J. Dauparas et al., Robust deep learning-based protein sequence design using ProteinMPNN. [Online]. (2022).
DOI: https://10.1126/science.add2187
B. Stynen, H. Tournu, J. Tavernier, and P. Van Dijck, Diversity in Genetic In Vivo Methods for Protein-Protein Interaction Studies: from the Yeast Two-Hybrid System to the Mammalian Split-Luciferase System. Microbiology and Molecular Biology Reviews, vol. 76, no. 2, pp. 331– 382, (2012).
DOI: https://10.1128/mmbr.05021-11

K. Iyer et al., Utilizing the Split-Ubiquitin Membrane Yeast Two-Hybrid System to Identify Protein-Protein Interactions of Integral Membrane Proteins. Science's STKE, vol. 2005, no. 275, (2005).
DOI: https://10.1126/STKE.2752005PL3

O. Vincent, A. Gutierrez-Nogués, A. Trejo-Herrero, and M. A. Navas, A novel reverse two hybrid method for the identification of missense mutations that disrupt protein–protein binding. Scientific Reports 2020 10:1, vol. 10, no. 1, pp. 1–11, (2020).
DOI: https://10.1038/s41598-020-77992-1.

J. Y. Kim, O. G. Park, J. W. Lee, and Y. C. Lee, One- plus two-hybrid system, a novel yeast genetic selection for specific missense mutations disrupting protein/protein interactions. Molecular and Cellular Proteomics, vol. 6, no. 10, pp. 1727–1740, (2007). DOI: https://10.1074/mcp.M700079-MCP200

T. Halter et al., The Leucine-Rich Repeat Receptor Kinase BIR2 Is a Negative Regulator of BAK1 in Plant Immunity. Current Biology, vol. 24, no. 2, pp. 134–143, (2014). DOI: https://10.1016/J.CUB.2013.11.047

T. Stellberger, R. Häuser, A. Baiker, V. R. Pothineni, J. Haas, and P. Uetz, Improving the yeast two-hybrid system with permutated fusions proteins: the Varicella Zoster Virus interactome. Proteome Science. (2010).
DOI: https://10.1186/1477-5956-8-8

G. Monteuuis et al., Non-canonical translation initiation in yeast generates a cryptic pool of mitochondrial proteins. Nucleic Acids Res, vol. 47, no. 11, pp. 5777–5791, (2019). DOI: https://10.1093/NAR/GKZ301

P. Dumas, M. Bergdoll, C. Cagnon, and J. M. Masson, Crystal structure and site-directed mutagenesis of a bleomycin resistance protein and their significance for drug sequestering. EMBO J, vol. 13, no. 11, pp. 2483–2492, (1994).
DOI: https://10.1002/J.1460-2075.1994.TB06535.X

M. Tominaga, K. Nozaki, D. Umeno, J. Ishii, and A. Kondo, Robust and flexible platform for directed evolution of yeast genetic switches. Nature Communications 2021 12:1, vol. 12, no. 1, pp. 1–13, (2021).
DOI: https://10.1038/s41467-021-22134-y

R. K. Barr, R. M. Hopkins, P. M. Watt, and M. A. Bogoyevitch, Reverse Two-hybrid Screening Identifies Residues of JNK Required for Interaction with the Kinase Interaction Motif of JNK interacting Protein-1. Journal of Biological Chemistry, vol. 279, no. 41, pp. 43178–43189, (2004).
DOi: https://10.1074/JBC.M405900200

P. Bryant, G. Pozzati, and A. Elofsson, Improved prediction of protein-protein interactions using AlphaFold2. Nature Communications 2022 13:1, vol. 13, no. 1, pp. 1–11, (2022). DOI: https://10.1038/s41467-022-28865-w

F. Homma, J. Huang, and R. A. L. van der Hoorn, AlphaFold-Multimer predicts cross-kingdom interactions at the plant-pathogen interface. Nature Communications 2023 14:1, vol. 14, no. 1, pp. 1–13, (2023).
DOI: https://10.1038/s41467-023-41721-9

K. Yamada et al., The Arabidopsis CERK1-associated kinase PBL27 connects chitin perception to MAPK activation. EMBO J, vol. 35, no. 22, pp. 2468–2483, (2016). DOI: https://10.15252/EMBJ.201694248

L. Shan et al., Bacterial Effectors Target the Common Signaling Partner BAK1 to Disrupt Multiple MAMP Receptor-Signaling Complexes and Impede Plant Immunity. Cell Host Microbe, vol. 4, no. 1, pp. 17–27, (2008).
DOI: https://10.1016/J.CHOM.2008.05.017

W. Cheng et al., Structural Analysis of Pseudomonas syringae AvrPtoB Bound to Host BAK1 Reveals Two Similar Kinase-Interacting Domains in a Type III Effector. Cell Host Microbe, vol. 10, no. 6, pp. 616–626, (2011).
DOI: https://10.1016/J.CHOM.2011.10.013

M. Suzuki, T. Watanabe, I. Yoshida, H. Kaku, and N. Shibuya, Autophosphorylation site Y428 is essential for the in vivo activation of CERK1. Plant Signal Behav, vol. 13, no. 2, (2018)
DOI: https://10.1080/15592324.2018.1435228

Q. Zhou et al., The juxtamembrane domains of Arabidopsis CERK1, BAK1, and FLS2 play a conserved role in chitin-induced signaling. J Integr Plant Biol, vol. 62, no. 5, pp. 556–562, (2020).
DOI: https://10.1111/JIPB.12847

C. Ma et al., Structural basis for BIR1-mediated negative regulation of plant immunity. Cell Research 2017 27:12, vol. 27, no. 12, pp. 1521–1524, (2017).
DOI: https://10.1038/cr.2017.123
T. Halter et al., The leucine-rich repeat receptor kinase BIR2 is a negative regulator of BAK1 in plant immunity. Current Biology, vol. 24, no. 2, pp. 134–143, (2014)
DOI: https://10.1016/j.cub.2013.11.047

S. Yasuda, K. Okada, and Y. Saijo, A look at plant immunity through the window of the multitasking coreceptor BAK1. Curr Opin Plant Biol, vol. 38, pp. 10–18, (2017). DOI: https://10.1016/J.PBI.2017.04.007

T. Halter et al., The leucine-rich repeat receptor kinase BIR2 is a negative regulator of BAK1 in plant immunity. Current Biology, vol. 24, no. 2, pp. 134–143, (2014). DOI: https://10.1016/j.cub.2013.11.047
E. Koseoglou, J. M. van der Wolf, R. G. F. Visser, and Y. Bai, Susceptibility reversed: modified plant susceptibility genes for resistance to bacteria. Trends Plant Sci, vol. 27, no. 1, pp. 69– 79, (2022).
DOI: https://10.1016/J.TPLANTS.2021.07.018

M. Shabab, M. J. Kulkarni, and M. I. Khan, Study of papain-cystatin interaction by intensity fading MALDI-TOF-MS. Protein J, vol. 27, no. 1, pp. 7–12, (2008).
DOI: https://10.1007/S10930-007-9102-3

J. G. Bishop, D. R. Ripoll, S. Bashir, C. M. B. Damasceno, J. D. Seeds, and J. K. C. Rose, Selection on Glycine β-1,3-Endoglucanase Genes Differentially Inhibited by a Phytophthora Glucanase Inhibitor Protein. Genetics, vol. 169, no. 2, p. 1009, (2005). DOI: https://10.1534/genetics.103.025098

A. Pietraszewska-Bogiel et al., Interaction of Medicago truncatula Lysin Motif Receptor-Like Kinases, NFP and LYK3, Produced in Nicotiana benthamiana Induces Defence-Like Responses. PLoS One, vol. 8, no. 6, p. e65055, (2013).
DOI: https://10.1371/JOURNAL.PONE.0065055

Leanna, C. A. & Hannink, M. The reverse two-hybrid system: a genetic scheme for selection against specific protein/protein interactions. Nucleic Acids Res. 24, 3341–3347 (1996). DOI: https://doi.org/10.1093/nar/24.17.3341

Vidal, M., Braun, P., Chen, E., Boeke, J. D. & Harlow, E. Genetic characterization of a mammalian protein–protein interaction domain by using a yeast reverse two-hybrid system. Proc. Natl. Acad. Sci. USA 93, 10321–10326 (1996).
DOI: https://doi.org/10.1073/pnas.93.19.10321

One-plus Two-Hybrid system: One- plus Two-hybrid System, a Novel Yeast Genetic Selection for Specific Missense Mutations Disrupting Protein/Protein Interactions Kim et al. - Molecular & Cellular Proteomics (2007).
DOI: https://doi.org/10.1074/mcp.M700079-MCP200