A Pair of Receptor Kinases Trigger Cellular Death in *Nicotiana benthamiana*

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Introduction

Cysteine-Rich Receptor-Kinases are Upregulated in SSI

Solanum sisymbriifolium (SSI), is a distant relative of potato and is resistant to several plant-parasitic nematodes, including *Globodera pallida*, which poses a significant threat to the potato industry. Commercially available potato lines lack an effective resistance against *G. pallida*. SSI, which allows the nematodes to hatch but not reproduce, is being evaluated as a potential source of novel nematode resistance.

Transcriptome comparisons of uninfected and infected SSI revealed 277 genes that dramatically changed expression during nematode infection. Of these, 143 genes were only upregulated by *G. pallida*, and not by treatments with salicylic acid, jasmonic acid, or wounding (Wixom, *et al.* 2020). Of these 143 genes, two are cysteine-rich receptor-like-kinases (CRK). CRKs are a class of receptor-like-kinases shown to function in defense against biotic and abiotic stresses in plants. The defining feature of these proteins is a pair of DUF26 domains, a motif that contains 3 cysteines arranged in a C-[aa_8]-C-[aa_2]-C pattern (Fig1).

These two proteins, provisionally named "*Robin*" and "*Blue-Jay*" were upregulated 4.9 and 6.5 times higher after 3 days in infected SSI. While the proteins are only 42.3% homologous, the homology throughout the two DUF26 motifs increases to 57.7%. When expressed in *Nicotiana benthamiana*, both genes cause cell death, characteristic of the hypersensitive response.

Hypothesis

The observed phenotype is partially due to several conserved amino acids in the DUF26 motifs. Mutagenesis on these amino acids will reveal if they are necessary for the cell death function.

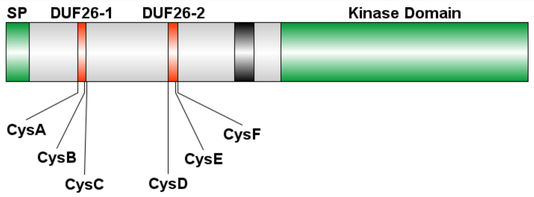

Fig 1: Domain Structure of a Cysteine-Rich Receptor-like-Kinase Protein. The shared structures are a single peptide (SP), followed by two cysteine-containing DUF26 domains in the extracellular portion of the protein, a transmembrane domain, and an intracellular kinase domain.

Methods

- *Robin* and *Blue-Jay* were cloned under a 35S promoter and transformed into the *Agrobacterium tumefaciens* vector Agl-1.
- Protein structures were visualized using ColabFold and ChimeraX
- For noninvasive monitoring, 35S:RUBY was used (Addgene plasmid # 160908 ; http://n2t.net/addgene:160908 ; RRID:Addgene_160908)
- A possible potato homolog of *Robin* "*StRobin*" was identified using BLAST and cloned from *Desirée* potato cDNA and transformed into Agl-1.
- Transient expression was performed by infiltration of *Nicotiana benthamiana* plants and results were imaged at 6 days post-infection.
- Mutagensis was performed using Agilent's QuikChange Lightning Multi Site-Directed Mutagenesis Kit. Mutations were made as follows and summarized in Table 1:
  - *Robin* C(90, 99, 102, 203, 212, 215)→S
  - *Blue-Jay* C84→ S and K373→N

Results

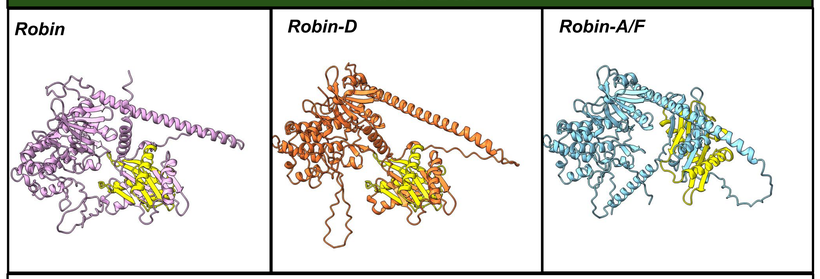

Fig 2: Predicted Structures of Mutants from ColabFold, visualized in ChimeraX. The 26 amino acids that contain both DUF26 motifs are highlighted in yellow.

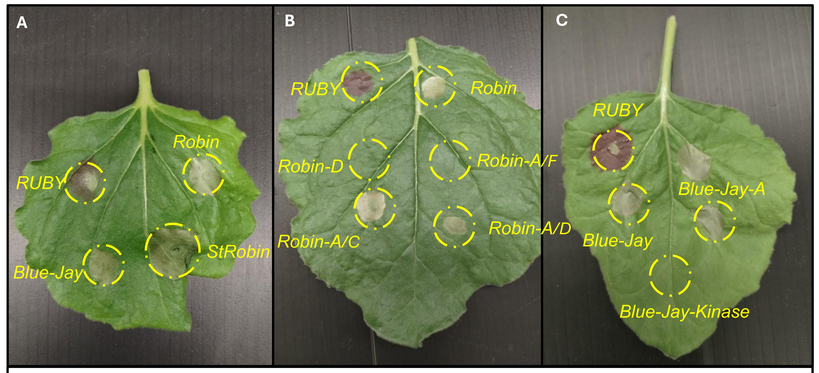

Fig 3: Transient Expression of Mutants in *Nicotiana benthamiana.* Leaves were infiltrated with *A. tumefaciens* with an OD of 0.90 to 1.0 and imaged after 6 days post infection. To show the responses were gene specific, 35S::RUBY was infected in the top left corner of each leaf. Each infiltration was repeated 3 times. (A) *Robin*, *Blue-Jay*, and *StRobin*, all cause cell death. (B) Expression of the *Robin* mutants. *Robin-D* and *Robin-A/F* all do not cause cell death, while *Robin -A/C* and *Robin -A/D* do. (C) Expression of the *Blue-Jay* mutants. *Blue-Jay-A* causes cell death, while *Blue-Jay-Kinase* does not.

Gene	Mutation	Phenotype
Robin-A	C90→S	No Death
Robin-A/C	C(90, 99, 102)→S	Death
Robin-A/D	C(90, 99, 102, 203)→S	Death
Robin-D	C212→S	No Death
Robin-A/F	C(90, 99, 102, 203, 212, 215)→S	No Death
Blue-Jay-A	C84→ S	Death
Blue-Jay-Kinase	K373→N	No Death

Table 1: Summary of mutants created, and their phenotype expressed in *Nicotiana benthamiana*.

Acknowledgments

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Conclusions

Cell Death in *N. benthamiana*

The cell death seen in *N. benthamiana* indicates that both genes are executing a hypersensitive-like response, a component of the plant's antipathogen defense. We identified a potential potato homolog of *Robin* (87.7% homology), *StRobin*, that produced an identical phenotype. We hypothesized that this phenotype would be in part due to the DUF26 motifs, due to the low levels of homology in the non-DUF26 portion *Robin* and *Blue-Jay*.

Potato Has a Functional Homolog of *Robin,* but not *Blue-Jay*

Since *StRobin* produced the same phenotype as its homolog, native potato CRK's may also be functioning in pathogen defense. Currently, we are working on determining how *StRobin* is expressed in potatoes during infection with the root knot nematode, *Meloidogyne hapla*.

We have not been able to identify a close homolog of *Blue-Jay* in the potato databases.

Mutagenesis Confirms Cysteine Residues Are Needed for Cell Death

Mutagenesis of all six cysteines in *Robin* stopped cell death, confirming the hypothesis that these residues are necessary for the function. Further mutagenesis produced conflicting results. Mutagenesis of the three cysteines in DUF26-1 allowed cell death to continue, however, mutating only the first cysteine in DUF26-1 stopped cell death. Similarly, a mutation of 4 residues across both DUF26 motifs allowed cell death to continue, while a single mutation in DUF26-2 stopped cell death. Expression levels of the proteins need to be measured in future experiments to verify that the conflicting results are not due to differences in CRK protein accumulation.

Blue-Jay is still undergoing mutagenesis. As expected, the mutagenesis of the critical lysine residue in the kinase domain stopped cell death. Mutagenesis on the first cysteine in the DUF26-1 motif, did not stop cell death. This is unlike how *Robin* responded to the same mutation, which may be indicative of differential functions between the two proteins.

Future Directions

Several questions remain unanswered by this preliminary research, including the question as to why *S. sisymbriifolium* has two CRKs upregulated by *G. pallida*. It is possible that *Robin* and *Blue-Jay* are either functionally identical or recognize different substrates.

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