

Original Article

In-silico analysis of Rcr3 polymorphism in Solanaceous species and its influence on tomato Cf-2 mediated recognition of the *Fulvia fulvum* Avr2 effector

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Article Info

Abstract



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Understanding how pathogen recognition has evolved in plants is essential for uncovering the mechanisms of host resistance and enhancing crop resilience. In this study, we examined how polymorphisms influence the evolutionary dynamics of Avr2 effector recognition from *Fulvia fulvum* across selected Rcr3 proteins in Solanaceae species. Rcr3, a co-receptor protein central to pathogen recognition, displays notable sequence variation that may affect its interaction with Avr2. Amino acid sequences of Rcr3 from ten Solanaceous plant species were obtained from public databases and categorized into compatible or incompatible groups according to their hypersensitive response. Structural modeling and comparative analyses revealed significant polymorphism in the Rcr3 binding sites, with 73 amino acid substitutions in the compatible group and 33 in the incompatible group, relative to the reference species *Solanum lycopersicum*. Structural assessments indicated that amino acids within the binding grooves of Rcr3 variants exhibit considerable diversity, particularly within incompatible species, where tighter binding interactions (less than 2Å) were observed. In contrast, compatible species displayed more variable, longer-range interactions (up to 6Å), suggesting a correlation between binding site polymorphism and recognition capability. These findings provide critical insights into the molecular basis of pathogen resistance in Solanaceae, highlighting the evolutionary pressures shaping Rcr3 diversity and its functional implications for plant immunity.

Keywords: Protein-protein interaction, Decoy model, Selection pressure, Avr2-binding protein.

1. Introduction

Proteins are essential macromolecules that perform a wide range of functions in all living organisms. They are broadly categorized by function into structural, defensive, signaling, enzymatic, and storage proteins [1]. In the context of plant–fungal interactions, proteins play a pivotal role in mediating key biological processes such as inter-organismal communication, defense signaling, nutrient exchange, and host colonization [2–7]. Throughout their co-evolution, plants have developed sophisticated immune mechanisms to recognize and resist fungal pathogens, while fungi have concurrently evolved strategies to evade or suppress host defenses [3]. This ongoing molecular arms race exerts strong selective pressure on protein–protein interactions, particularly at interface regions, often resulting in adaptive changes. Such adaptations may include amino acid substitutions at interaction surfaces, leading to altered protein structures and binding affinities, thereby influencing the functional dynamics and evolutionary trajectories of both host and pathogen proteins [8].

Fulvia fulvum, previously known as *Cladosporium fulvum*, is a biotrophic fungal pathogen that exclusively infects tomato (*Solanum lycopersicum*), its only known host. It secretes various molecules essential for colonization and evasion of host immune responses [9]. These molecules

are small, low-molecular-weight proteins secreted into the apoplast, commonly referred to as effectors. In response to such pathogen-derived signals, many members of the Solanaceae family have evolved both passive and active defense strategies to detect and counteract microbial invasion [10–13]. The tomato–*F. fulvum* interaction is widely regarded as a model system for studying recognition specificity and the evolutionary dynamics of plant disease resistance genes [9]. The effectors secreted into the apoplast act as virulence factors; however, when these molecules are recognized by corresponding host receptors encoded by resistance (R) genes, they are classified as avirulence (Avr) factors, triggering defense responses [11, 14].

Many plant species possess specific resistance genes that influence disease outcome by detecting corresponding pathogen Avr factors via direct or indirect mechanisms [15]. Within the guard–decoy model, R proteins recognize pathogen effectors indirectly by monitoring modifications to either the effector's native host target (the guardee) or a structurally similar plant-derived decoy protein that mimics the target and binds the Avr protein [14].

F. fulvum harbors a suite of effector genes, including well-characterized avirulence (Avr) genes—*Avr2*, *Avr4*, *Avr4E*, *Avr5*, and *Avr9*—as well as extracellular protein (Ecp) genes such as *Ecp1*, *Ecp2*, *Ecp2-1*, *Ecp3*, *Ecp4*,

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Ecp5, and *Ecp6* [16]. Recognition of these effectors in tomato is mediated by *Cf* resistance genes, which encode receptor-like proteins that initiate a hypersensitive response (HR) upon effector detection. Within the Solanaceae family, the *Cf-2* gene—introgressed from wild tomato species into cultivated varieties—encodes a leucine-rich repeat receptor-like protein (LRR-RLP) that plays a key role in effector perception and defense activation [9, 10, 17].

The *Cf-2* resistance protein recognizes the *Avr2* effector through the involvement of a co-receptor *Rcr3*, which encodes a papain-like cysteine protease (PLCP) [18]. According to the guard-decoy model, a tripartite complex is formed involving *Cf-2* (resistance protein), *Rcr3* (acting as a decoy), and *Avr2* (a protease inhibitor) [17, 19]. *Avr2* inhibits the enzymatic activity of *Rcr3*, a secreted cysteine protease in the apoplast. This inhibition is sensed by *Cf-2*, which triggers a hypersensitive response (HR) and confers resistance to *C. fulvum* [20]. Kourelis and colleagues (2020) utilized transient expression assays in *Nicotiana benthamiana* to investigate the *Cf-2/Rcr3/Avr2* interaction via agroinfiltration. Their study explored how amino acid polymorphisms in both *Rcr3* and *Avr2* influence recognition and HR signaling, highlighting the evolutionary dynamics of this defense system across Solanaceous species [17]. It is proposed that conformational changes in *Rcr3* induced by *Avr2* binding are key to its recognition by *Cf-2*, ultimately initiating immune signaling and localized cell death. These molecular adaptations have led to diverse recognition specificities among different members of the Solanaceae family. In this study, we examined both compatible and incompatible interactions within Solanaceae species by analyzing the polymorphism of *Rcr3* sequences across diverse taxa. We further evaluated the structural implications of these variations on the three-dimensional conformation of the *Rcr3* protein, particularly at the interface with *Avr2* (active site), in both responsive and non-responsive plant accessions.

2. Materials and Methods

2.1. Sequence retrieval and structural modelling

To assess the evolutionary impacts of *Rcr3* on *Avr2* recognition, amino acid sequences of *Rcr3* from ten Solanaceae species were obtained from UniProt and NCBI RefSeq proteomes. These species were categorized into compatible and incompatible groups based on their hypersensitivity response to *Avr2*. The *Rcr3* accession number from compatible group included *Capsicum chinense* (accession PHT97811), *Nicotiana sylvestris* (accession XP-009775922), *Nicotiana tabacum* (accession XP-016437926), and *Solanum melongena*, while the incompatible group comprised *Solanum pimpinellifolium* (accession AAM19207), *Solanum demissum* (accessions AHA62773 and AHA62774), *Solanum lycopersicoides* (accession AFP73348), and *Solanum verrucosum* (accession XP-049370299). *Rcr3* sequences were aligned using MAFFT, and their three-dimensional structures were modeled using the Swiss-Model server.

2.2. Structural analysis and polymorphism assessment

The structural polymorphism of *Rcr3* variants was analyzed using PyMOL. The *Rcr3* interface, including the catalytic and binding sites, was examined for each species to identify polymorphic regions. Comparative structural analysis was performed to assess amino acid variations

in the binding site, focusing on residues near the groove interface. Variability among compatible and incompatible species was further quantified by mapping these differences onto the *Rcr3* structures from *S. lycopersicum* as a reference.

2.3. In Silico diversity assessment

The diversity of *Rcr3* amino acids was examined separately for compatible and incompatible groups. In the compatible group, sequence comparisons revealed 73 amino acid differences across the *Rcr3* variants, with a focus on changes within the binding site. Amino acid variations were specifically noted in the binding sites relative to *S. lycopersicum*.

2.4. Protein-protein interaction analysis

To investigate the impact of *Rcr3* polymorphisms on *Avr2* binding, we employed H-DOCK and CLUSPRO for docking studies. The resulting models were visualized and analyzed using PyMOL to identify optimal *Rcr3-Avr2* interaction patterns. The binding interfaces of *Rcr3* from both compatible and incompatible groups were examined, revealing distinct interaction distances.

2.5. Data visualization and structural comparison

Variability in amino acid positions within the *Rcr3* structures was visually inspected to identify conserved and polymorphic regions. Figures representing the amino acid substitutions in the binding sites and the overall polymorphism distribution were generated to highlight the structural variations within and between the two groups.

3. Results

3.1. Divergence of *Rcr3* and its impact on *Avr2* recognition and HR induction

The evolutionary divergence of *Rcr3* was initially assessed by co-expression of *Avr2* of *C. fulvum*, tomato *Cf-2*, and *Rcr3* from ten species from the Solanaceae family [21]. The interaction of these species was categorized as either compatible or incompatible based on the presence or absence of a hypersensitive response. Using their accession numbers, the amino acid sequences of *Rcr3* from these species were acquired from UniProt and NCBI RefSeq proteome databases. The *Rcr3* sequence from the compatible group encompasses *C. chinense* (accession PHT97811), *N. sylvestris* (accession XP-009775922), *N. tabacum* (accession XP-016437926), and *S. melongena*. Conversely, and from the incompatible group include *S. pimpinellifolium* (accession AAM19207), *S. demissum* (AHA62773, AHA62774), *S. lycopersicoides* a and b (AFP73348), and *S. verrucosum* (accession XP-049370299). To facilitate our bioinformatic study, we designed the three-dimensional structures of *Rcr3* for all accessions, derived from Swiss-Model, and subsequently analyzed their 3D model. The active site of proteins comprises two distinct regions: the catalytic site and the binding site. Alignment using MAFFT showed that the conserved regions are present in other parts of *Rcr3* rather than interface. The polymorphism of *Rcr3* structures was analyzed utilizing the software PyMOL, through which we conducted a comparative structural analysis of all *Rcr3* variants, categorizing them into two groups and comparing them with *Solanum lycopersicum* to assess variations in abundance. We also acquired the 3D structure of *Avr2* to investigate the effect

of mutations on the interaction between Rcr3 and Avr2 in both compatible and incompatible groups.

3.2. Diversity assessment of Rcr3 in the compatible group

The amino acid diversity of Rcr3 within the compatible group was assessed by comparing it to the amino acids present in the wild Rcr3 of *Solanum lycopersicum*. In total, 73 amino acids difference across Rcr3 of compatible members was observed. Commonly, amino acids located around the groove interface demonstrated fewer variations than those situated far from the groove. Comparing variations in the binding site of Rcr3, *C. Chinese*, specifically exhibited one amino acid change, *S. melongena* displayed four amino acid changes, *N. sylvestris* had six amino acid changes, and *N. tabacum* exhibited three amino acid changes in the binding site (Fig. 1). Further, the polymorphism of amino acids in Rcr3 was analyzed among members in the compatible group. In total, 54 amino acid variations were found among compatible members, while nine of these variants were located in their binding sites. Comparing *C. chinese* and *S. melongena*, 33 amino acids were different, nine of which, including H24N, Y67M, R149A, N189T, R160Q, and Y73W, were located in the binding site (Fig. 2), while *N. sylvestris* and *N. tabacum* presented five amino acid variations including Y23Q, L26H, Y158D, Q159R, and K69N in their binding sites (Fig. 3).

3.3. In silico assessment of Rcr3 diversity in the incompatible group

We investigated the diversity of Rcr3 within the incompatible group in comparison to Rcr3 of *S. lycopersicum*. In this group, a total of 33 amino acids of Rcr3 exhibited polymorphism relative to the wild-type species, of which six amino acids are situated in the binding site. In *S. pimpinellifolium*, the amino acid changes L73F and Q160R of Rcr3 were found. In *S. demissum*, the polymorphic amino acids Y24H and T189S of Rcr3 were observed. In *S. lyco-*

persicoides, the N24H and Q27R variations of Rcr3 and in *S. verrucosum*, amino acid changes Y24H, A188T, and T189S have occurred in their binding sites (Fig. 4). We further examined the diversity of Rcr3 among the members of the incompatible group. The amino acids of Rcr3 displayed 19 distinct variations, with five located in the binding site. *S. pimpinellifolium* and *S. demissum* exhibited differences at positions H24Y, S189T, Q160R, and L73F (Fig. 5). In the case of *S. verrucosum* and *S. lyco-*

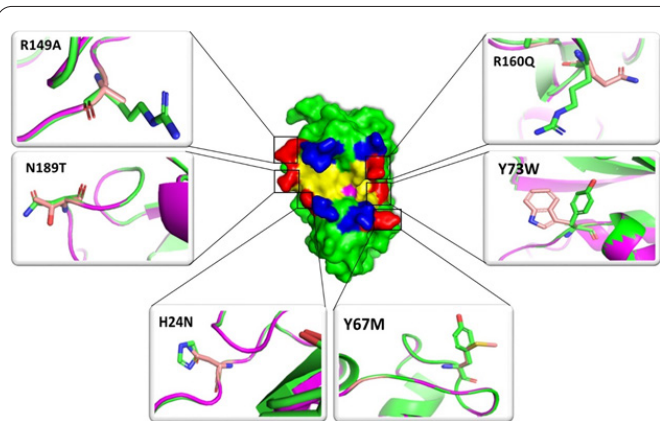


Fig. 2. Rcr3 variation in *C. chinense* and *S. melongena*. Amino acid changes in both species are observed at positions 24, 67, 73, 149, 160, and 189. In *C. chinense*, His24 is replaced by Asn in *S. melongena*, Tyr67 by Met, Tyr73 by Trp, Arg149 by Ala, Arg160 by Gln, and Asn189 by Thr.

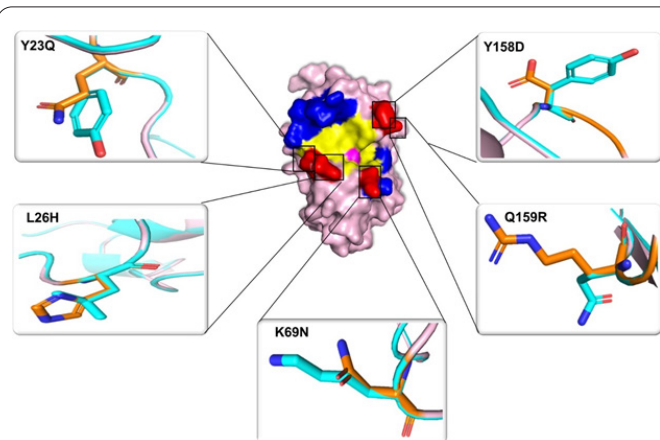


Fig. 3. Rcr3 variation in *N. sylvestris* and *N. tabacum*. Variations are noted at positions 23, 26, 69, 158, and 159. In *N. sylvestris*, Tyr23 is substituted by Gln in *N. tabacum*, Leu26 by His, Lys69 by Asn, Tyr158 by Asp, and Gln159 by Arg.

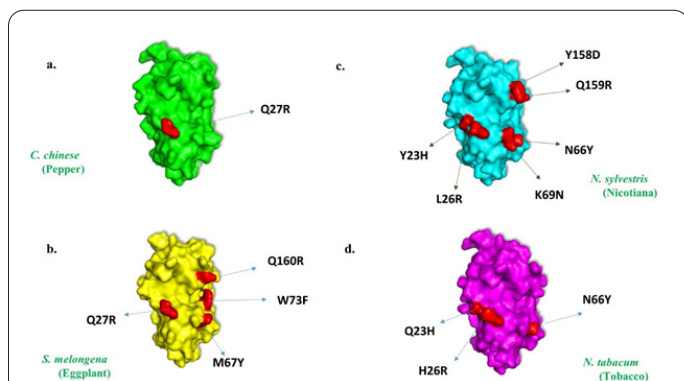


Fig. 1. Rcr3 diversity in nonresponsive Solanaceous species vs. *S. lycopersicum*. Amino acid variations in the Rcr3 binding site are highlighted in red, with a root mean square deviation (RMSD) of 0.247, indicating diversity in compatible group members compared to *S. lycopersicum*. **a.** In *Capsicum chinense*, glutamine (Gln) at position 27 is replaced by arginine (Arg). **b.** In *Solanum melongena*, Gln27 is substituted by Arg, methionine (Met) 67 by tyrosine (Tyr), tryptophan (Trp) 73 by phenylalanine (Phe), and Gln160 by Arg. **c.** In *Nicotiana sylvestris*, Tyr23 is replaced by histidine (His), leucine (Leu)26 by Arg, lysine (Lys)69 by asparagine (Asn), asparagine (Asn) 66 by Tyr, Gln159 by Arg, and Tyr158 by aspartic acid (Asp). **d.** In *Nicotiana tabacum*, Gln23 is replaced by His, His26 by Arg, and Asn66 by Tyr.

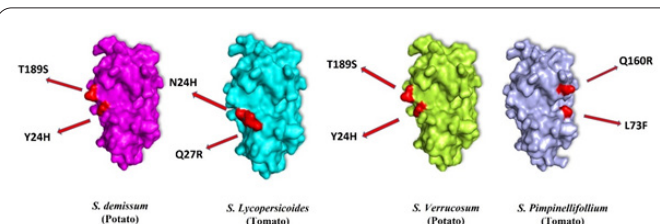
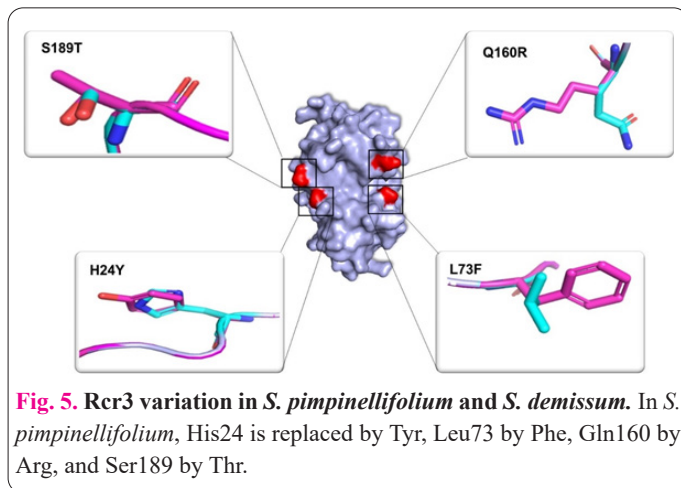


Fig. 4. Rcr3 diversity in incompatible group vs. *S. lycopersicum*. Amino acid changes in this group have an RMSD of 0.007. In *S. demissum*, Tyr24 is replaced by His and Thr189 by serine (Ser). In *S. lycopersicoides*, Asn24 changes to His and Gln27 to Arg. In *S. verrucosum*, Tyr24 to His and Thr189 to Ser. In *S. pimpinellifolium*, Leu73 is replaced by Phe and Gln160 by Arg.



persicoides, polymorphisms were identified at positions Y24N, R27Q, and T189S within the binding site (Fig. 6).

3.4. Variability of Rcr3 amino acids among the selected solanaceous species

We evaluated the polymorphism of amino acids in the Rcr3 across these two compatible and incompatible groups. Generally, variability assessment of amino acids among all members in the compatible and incompatible groups showed more variation around the groove than far amino acids. Amino acids located at the back side of Rcr3 are mainly conserved, but more variations have occurred in binding site (Fig. 7).

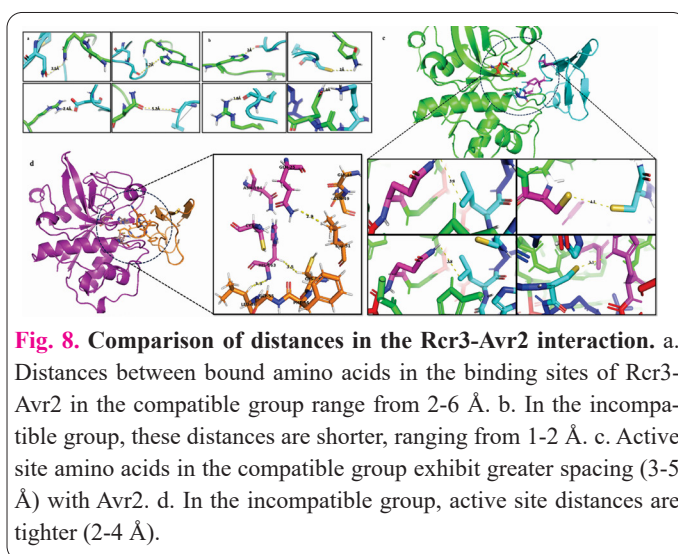
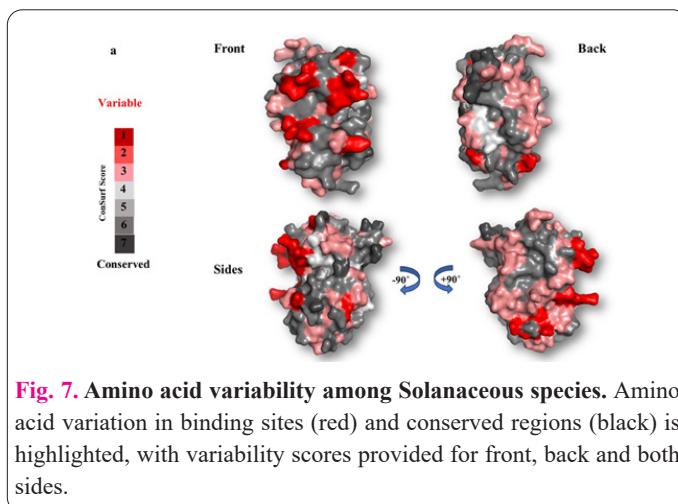
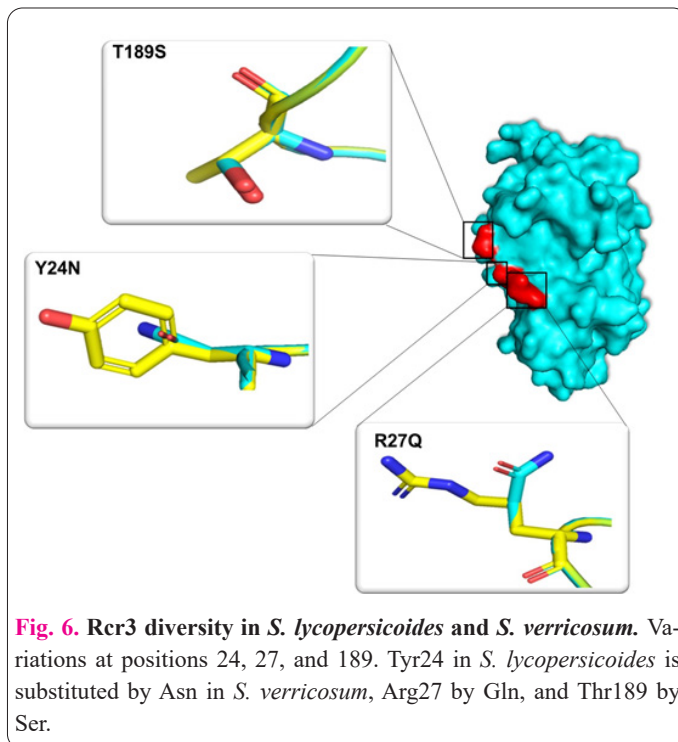
3.5. Effect of polymorphism on the interaction of Rcr3-Avr2

In our investigation, we analyzed the diversity of Rcr3 across various species within the Solanaceae family, examining the implications of amino acid polymorphism in Rcr3 on its interaction with Avr2. We derived an acceptable model for Avr2 using Swiss Model. We employed H-DOCK and CLUSPORO to generate models reflecting the diverse interactions between Rcr3 and Avr2. Subsequently, we utilized the Pymol software to identify the optimal model while assessing the effects of polymorphism on the interaction within two distinct groups. The amino acids located in the binding site of Rcr3 established clear connection with the amino acids of Avr2; within the compatible group, these interactions exhibited more distances (2-6 Å), whereas the incompatible interactions presented closer proximities, being less than 2 Å (Fig. 8).

4. Discussion

The co-evolutionary dynamics between *F. fulvum* and tomato are characterized by the deployment of small effector proteins like Avr2, which are critical for fungal virulence. These effectors typically are low-molecular-weight proteins that circumvent plant immunity by targeting host defense mechanisms [20]. In Solanaceous plants, the Cf-2 resistance gene encodes a receptor that, in complex with the protease Rcr3, detects Avr2, thereby triggering a robust hypersensitive response (HR) that limits pathogen spread [17]. According to the guard-decoy model, the Cf-2/Rcr3 complex functions as a molecular sensor, detecting conformational changes in Rcr3 induced by Avr2 binding, ultimately activating immune signaling pathways [22].

The evolutionary divergence of Rcr3 across Solanaceae species has profound implications for the recognition



of the fungal effector Avr2 and the subsequent induction of the hypersensitive response (HR). Mutational analyses indicate that amino acid substitutions within Rcr3 significantly influence its interaction with Avr2, affecting both structural conformation and binding affinity. These muta-

tions can alter the physicochemical properties of Rcr3, including charge, hydrophobicity, and steric interactions at the binding interface. Importantly, such polymorphisms are more pronounced in Rcr3 alleles from wild *Solanum* species, which display greater sequence variability compared to domesticated *S. lycopersicum*. Co-expression assays with tomato Cf-2 and Avr2 [21] categorized species based on their ability to mount an HR, highlighting the adaptive divergence of Rcr3 in response to selective pressures from pathogen effectors. Our analysis, integrating sequence alignment, structural modeling, and interaction modeling, provides a detailed understanding of how amino acid polymorphisms shape Rcr3 function in both compatible and incompatible plant-pathogen interactions. Our sequence alignment and 3D modeling of Rcr3 revealed that amino acid variability is unevenly distributed. The binding interface, crucial for Avr2 recognition, exhibited significant polymorphisms in both groups, whereas regions distant from the interface remained largely conserved. This pattern suggests that the structural core of Rcr3 is under stabilizing selection, whereas residues directly involved in effector recognition are subject to diversifying selection, likely to modulate immune specificity. In the compatible group, a total of 73 amino acid differences were observed relative to *S. lycopersicum*, with 9 changes located in the binding site. Specific substitutions, such as H24N in *C. chinense* and R160Q in *S. melongena*, potentially reduce binding affinity to Avr2 by altering local electrostatics or steric complementarity. Comparisons among members of the compatible group (e.g., *C. chinense* vs. *S. melongena*) revealed up to 33 variations, indicating substantial intraspecific polymorphism that may contribute to a weakened or absent HR. Conversely, the incompatible group exhibited fewer total variations (33 amino acids), with six substitutions in the binding site, suggesting that these species have retained critical residues that maintain tight Rcr3-Avr2 interactions necessary for HR induction. For instance, conserved residues like Tyr24, Gln27, and Thr189 maintain close contact with Avr2, consistent with the stronger interaction observed in this group.

Structural superimposition and RMSD analysis revealed notable differences between groups. The compatible group exhibited higher RMSD values (~0.247), reflecting greater structural divergence, whereas the incompatible group showed minimal RMSD (~0.007), indicating high structural conservation. This reinforces the hypothesis that Rcr3 in incompatible species is evolutionarily constrained to preserve functional interactions with Avr2.

Docking simulations using H-DOCK and CLUSPRO provided mechanistic insight into how polymorphisms influence recognition. In the compatible group, distances between Rcr3 binding site residues and Avr2 ranged from 2–6 Å, suggesting weakened interactions that may fail to trigger HR. By contrast, in the incompatible group, distances were consistently shorter (1–2 Å), indicative of tighter binding and a higher likelihood of eliciting a defense response. These results demonstrate a clear structural basis for functional divergence.

The observed patterns of Rcr3 polymorphism highlight the dynamic co-evolution between Solanaceae hosts and *C. fulvum*. Species in the compatible group appear to have accumulated substitutions that reduce recognition efficiency, potentially as a trade-off between immune sensitivity and autoimmunity. In contrast, species in the incompatible

group preserve key residues in the binding interface, ensuring robust HR induction upon Avr2 detection. This dichotomy underscores the role of selective pressures in shaping immune receptor specificity, balancing pathogen defense with the metabolic costs of constitutive immune readiness. Furthermore, our findings indicate that not all amino acid changes are equally impactful. Variations at the groove interface directly influence effector binding, whereas distant substitutions have minimal structural consequences. This suggests that targeted engineering of Rcr3 residues at the interface could potentially restore or modulate Avr2 recognition, providing a route for crop improvement through rational design of immune receptors.

Overall, this study illustrates the tight link between Rcr3 sequence divergence, structural variation, and functional outcomes in plant-pathogen interactions. Compatible species display higher polymorphism at critical interface residues, resulting in reduced Avr2 recognition and suppressed HR, whereas incompatible species maintain conserved binding site residues that facilitate tight interactions and robust defense activation. These findings contribute to a deeper understanding of molecular co-evolution in plant immunity and may inform strategies for engineering broad-spectrum disease resistance in Solanaceae crops.

Conflict of interest

There is no potential conflict of interest.

Author contribution

MK-J and FD conceived the project. FD performed the experiment, designed the models, evaluated all analyses and wrote the manuscript. MK-J critically reviewed and edited the manuscript. All authors read and approved the final manuscript for publication.

Availability of data and material

Authors declare that they embedded all data in the manuscript.

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