A Critical Re-evaluation of "*Two residues reprogram immunity receptor kinases to signal in nitrogen-fixing symbiosis*" by Tsitsikli *et al*., *Nature* 2025; doi:10.1038/s41586-025-09696-3

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Abstract

The study by Tsitsikli *et al*. proposes that two amino-acid substitutions within a short juxtamembrane (JM) motif, termed *Symbiosis Determinant 1* (SD1), are sufficient to reprogram CERK-family immunity receptor kinases into NFR1-type symbiotic receptors capable of initiating rhizobial infection and root nodule organogenesis in *Lotus japonicus*. If valid, this would represent an unexpectedly minimal evolutionary step enabling the emergence of nitrogen-fixing symbiosis in legumes and would influence future attempts to engineer symbiotic signaling into non-leguminous crops. However, a comprehensive and figure-resolved examination of the data reveals that many of the study's conclusions exceed what the experimental evidence can robustly support. Across the main **Figures 1–4**, **Extended Data Figures 1–8 (ED1-ED8)**, and **Supplementary Figures 1–2**, the phenotypes attributed to SD1 or its engineered variants are frequently weak, low-penetrance, or inconsistent. Root-hair deformation, infection thread initiation, NIN activation, and nodule formation are often presented using highly selected images, small sample sizes, and broad phenotypic categories that obscure biological variability. Structural analyses rely on inactive kinase conformations and do not provide mechanistic insight into how SD1 residues modulate signaling specificity. Localization experiments in *Nicotiana benthamiana* and *Lotus* protoplasts suffer from overexpression artefacts, and biochemical assays do not demonstrate functional differences between engineered and native kinases. Evolutionary arguments are based on narrow sequence comparisons rather than rigorous phylogenetic or co-evolutionary analyses. Taken together, the totality of evidence does not convincingly demonstrate that two residues can reprogram immunity receptors for symbiotic signaling. This commentary provides a systematic, multidimensional critique to promote transparency, reproducibility, and constructive scientific dialogue.

1. Introduction

1.1. Background and Conceptual Premise

Nitrogen-fixing symbiosis represents one of the most intricate examples of interkingdom communication in biology. Legumes uniquely evolved the capacity to recognize rhizobial lipochitooligosaccharide signals, initiate precise epidermal reprogramming, generate infection threads, activate cortical organogenesis, and ultimately form specialized root nodules capable of hosting intracellular nitrogen-fixing bacteria[1,2]. The molecular logic enabling plants to distinguish pathogenic chitin signals from symbiotic Nod factors relies heavily on the LysM receptor kinase families, particularly CERK-type immunity receptors and NFR-type symbiotic receptors[1,2]. CERK6, CERK1, and related immune receptors recognize chitin oligomers, leading to reactive oxygen species (ROS) production, MAPK activation, transcriptional induction of defense genes, and a broader immune cascade[2,3]. In contrast, NFR1 and NFR5 perceive rhizobial Nod factors and activate a highly distinct signaling pathway encompassing calcium oscillations, CCaMK-driven decoding, NIN-mediated transcriptional reprogramming, and developmental transitions culminating in nodule formation[2,3]. Although these two receptor families share substantial structural similarity, their intracellular kinase activities, downstream interactomes, ligand affinities, and signaling outputs are profoundly different.

Central to the longstanding question in plant–microbe evolution is how legumes acquired the capacity to discriminate symbiotic from immune signals while using structurally homologous LysM domains. The prevailing model posits that symbiotic signaling emerged from gene duplications of ancestral CERK-like receptors followed by subfunctionalization and neofunctionalization across the extracellular, JM, and intracellular regions. This classical view assumes that changes in ligand-binding ectodomains and gradual rewiring of kinase signaling surfaces shaped the evolutionary divergence of Nod factor receptors. In this context, the study by Tsitsikli *et al*.[4] makes a striking and provocative assertion: *that only two amino-acid substitutions in a short JM motif, SD1, are sufficient to convert the intracellular signaling output of a CERK-type immunity kinase into that of an NFR1-type symbiotic kinase*. Such a claim challenges the foundational understanding of receptor evolution, suggesting that the functional gulf between immunity and symbiosis may be bridged far more simply than previously assumed.

The extraordinary biological implications of this claim demand rigorous scrutiny. If receptor reprogramming can indeed be achieved through minimal mutational steps, one might infer that the evolutionary emergence of root nodule symbiosis could have proceeded rapidly and with relatively few genetic innovations. Moreover, such

a finding would reshape strategies for engineering nitrogen-fixing capabilities into cereals, implying that a small set of rationally chosen amino-acid edits could suffice to redirect endogenous immunity receptors toward symbiotic signaling pathways. These conceptual stakes underscore the need for a careful, systematic, and critical examination of the evidence provided by Tsitsikli *et al.*[4], including data from main **Figures 1 through 4**, **SD1 through 8**, and **S1** and **S2**.

1.2. Central Claims of Tsitsikli *et al.*

The study advances four major assertions that frame the logic of its experimental narrative. First, the authors claim to have identified a 23-residue JM region in NFR1, termed SD1, that uniquely governs the receptor's capacity to activate symbiotic developmental and infection responses. Second, through domain swapping and chimeric constructs, they propose that SD1 is both necessary and sufficient to convert CERK6 from a chitin-triggered immune kinase into an NFR1-like symbiotic kinase. Third, they argue that installing just two NFR1-type residues into CERK6 reconstitutes nearly full symbiotic signaling, including infection thread formation and nodule organogenesis. Fourth, they extend this logic to monocots by engineering SD1 into barley RLK4, asserting that this manipulation endows RLK4 with functional symbiotic activity in *L. japonicus* roots. Collectively, these claims imply that intracellular signaling specificity in LysM receptors hinges predominantly on the SD1 motif and that the divergence of CERK6 and NFR1 signaling outputs can be mechanistically collapsed to a surprisingly small mutational difference.

These assertions rely on evidence spanning cellular imaging, genetic complementation, structural overlays, biochemical assays, and evolutionary comparisons. However, the strength of the conclusions depends heavily on the quantitative robustness, representativeness, and interpretational clarity of the data. Across the figures, the phenotypes attributed to SD1 and its engineered variants often show substantial variability, low penetrance, and marginal rescue levels. The structural argument relies on inactive conformations lacking mechanistic explanatory power. Localization experiments assume equivalence between transient overexpression and native receptor trafficking. And the evolutionary argument rests on narrow alignments that do not reflect the broader diversity of LysM receptor kinases. Given these limitations, a comprehensive commentary must evaluate whether the data convincingly support the sweeping scope of the proposed mechanistic reprogramming.

1.3. Purpose and Scope of This Commentary

The purpose of this commentary is to provide a rigorous, multidimensional assessment of the scientific validity of the claims made by Tsitsikli *et al.*[4], grounded in a detailed figure-by-figure analysis of all primary, extended, and supplementary

data. The critique integrates perspectives from receptor structural biology, plant–microbe signaling, evolutionary genomics, developmental physiology, and biochemical mechanism. Central to this analysis is an examination of whether the experiments presented adequately justify the conclusion that two amino-acid substitutions are sufficient to convert an immunity kinase into a symbiosis kinase. Beyond evaluating data quality, we analyze conceptual coherence, methodological transparency, statistical robustness, and the logical inferences drawn from phenotypes. This commentary does not aim to refute the possibility that small mutational changes could influence signaling specificity; rather, it aims to assess whether the evidence provided in this study is strong, consistent, and reproducible enough to support the transformative biological conclusions that the authors advance.

2. Conceptual Evaluation of the Central Hypothesis

2.1. Biological Plausibility of Minimal Mutational Reprogramming

The central premise of Tsitsikli *et al.*[4]—that two amino-acid substitutions within a short JM motif can reprogram a CERK-type immunity receptor kinase into an NFR1-like symbiosis receptor—is conceptually striking and carries deep implications for receptor evolution, signaling fidelity, and the engineering of nitrogen-fixing capabilities in non-legumes. However, the plausibility of such a minimalistic mechanism must be examined within the broader context of plant receptor biology, where signaling outputs arise not from discrete sequence motifs acting in isolation but through the concerted interplay of extracellular domains, transmembrane helices, JM regions, kinase activation loops, protein–protein interaction interfaces, receptor dynamics, and downstream cellular scaffolds. The transition from immunity to symbiosis is not merely a change in ligand perception; it involves an entire rewiring of downstream signaling logic, including calcium oscillations, CCaMK decoding, transcription factor cascades, and organogenic reprogramming. Receptor kinases are entry points into these complex networks, but they rarely determine signaling specificity solely through one small region of the intracellular domain.

Evolutionarily, the emergence of legume–rhizobial symbiosis required multiple gene duplications, functional specializations, co-evolution with rhizobial Nod factor synthases, and recruitment of nodulation signaling modules that do not exist in non-legumes. Key components such as NIN, CYCLOPS, CCaMK, ENOD genes, and cortical developmental regulators form a multi-tiered machinery that integrates ligand recognition with morphogenetic processes. Mutations in any one component often produce incomplete or aberrant phenotypes, demonstrating the distributed nature of signaling control. Against this backdrop, the suggestion that the massive

functional divergence between CERK6 and NFR1 can be collapsed to two amino acids within SD1 challenges fundamental principles of receptor evolution and systems-level signaling integration.

Furthermore, the intrinsic modularity of LysM receptor kinases does not automatically imply that their outputs can be reprogrammed by minute changes. While extracellular LysM domains do determine ligand affinities, the intracellular kinase domains are responsible for complex substrate recognition patterns, phosphosite selection, and interactions with co-receptors and scaffolds. Studies in Arabidopsis, rice, and Medicago have shown that kinase activation depends on a distributed set of structural elements involving the αC helix, activation loop, HRD motif, DFG motif, and the conserved regulatory spine. Mutations in these areas typically produce mild or partial effects, emphasizing the robustness of kinase architecture. The idea that a surface-exposed JM loop can override all these regulatory layers to redirect signaling output is therefore biologically implausible unless accompanied by extensive downstream rewiring, which Tsitsikli *et al*.[4] do not address.

Another conceptual concern involves the assumption that symbiotic and immune signaling differ primarily at the receptor level. In reality, immune receptors feed into canonical defense pathways, whereas symbiosis receptors activate a highly specialized suite of signaling components that are absent or nonfunctional in non-legumes. This implies that even if CERK6 could theoretically phosphorylate the same substrates as NFR1 after SD1 introduction, the downstream signaling machinery remains fundamentally incompatible. For example, CERK6-based chimeras cannot induce calcium spiking unless correctly integrated with SYMRK, NUP133, and nuclear pore complex regulators, none of which are addressed in the study. Thus, receptor reprogramming alone cannot account for the multiphasic logic of symbiosis signaling.

A further layer of complexity is the ligand-triggered receptor complex. NFR1 does not act alone but forms heteromeric complexes with NFR5, a pseudokinase with critical scaffolding functions. CERK6, in contrast, acts alongside CERK1 and LYK-family partners with distinct binding affinities and stoichiometric requirements. Engineering SD1 into CERK6 does not alter the identity or interaction landscape of these co-receptors. Without demonstrating that CERK6 chimeras physically interact with NFR5 in the manner required for symbiotic signaling, the assertion that SD1 reprograms receptor output remains speculative. The study presents no biochemical co-immunoprecipitation, FRET–FLIM, or phosphoproteomics to show changes in complex formation or substrate specificity. Instead, it relies on

morphological phenotypes that are inherently variable and prone to overinterpretation.

The physiological plausibility of the proposed mechanism also warrants scrutiny. Symbiotic signaling requires coordinated activation of both epidermal infection and cortical organogenesis. In nature, root hair deformation and microcolony formation precede infection-thread penetration, while cortical cell divisions produce a nodule primordium instructed by NIN and other transcriptional regulators. These steps require robust, high-fidelity signaling. However, the engineered receptors in Tsitsikli *et al*. show extremely low penetrance, often producing one or two nodules per plant or none at all. Such weak phenotypes raise the possibility of artefactual activation due to overexpression rather than genuine signaling reprogramming. Nodule-like structures observed in engineered constructs may represent pseudonodules, typically caused by cytokinin dysregulation or partial activation of nodulation pathways unrelated to proper NFR1-like signaling.

In summary, the biological plausibility of minimal mutational reprogramming of CERK6 into an NFR1-like receptor is low when evaluated within the broader landscape of receptor evolution, kinase biochemistry, signaling network complexity, and physiological requirements for symbiosis. While small motifs can modulate aspects of receptor function, the sweeping conclusion drawn in the study—that two residues suffice to redirect immunity to symbiosis—is incongruent with current understanding and therefore demands exceptionally strong and multidimensional empirical support. As the subsequent sections show, the evidence presented falls significantly short of meeting this threshold.

2.2. Structural, Evolutionary, and Signaling Constraints

Any mechanistic explanation for the conversion of an immunity receptor kinase into a symbiotic receptor must account for the distributed nature of functional determinants across the receptor's architecture. LysM receptor kinases share high structural similarity, but minor differences in surface charge distribution, substrate specificity pockets, activation loop flexibility, and C-terminal docking sites contribute collectively to signaling specificity. Structural studies in receptor kinases reveal that small motifs can act as regulatory switches only when embedded within a larger conformational framework that includes multiple cooperative surfaces. The SD1 region identified by Tsitsikli *et al*.[4] is located in a loop followed by the αB helix, a region known for conformational flexibility and frequent disorder in active kinase states. Because the authors solved structures of CERK6 and LYK3 only in inactive, DFG-mutated conformations, they capture the kinase in a crystallographically convenient but functionally irrelevant state. The SD1 residues, which appear surface

exposed in static structures, may reposition dynamically during activation, making static inference unreliable.

Evolutionary constraints further challenge the study's minimalistic interpretation. CERK6, NFR1, RLK4, and LYK3 belong to divergent clades that have undergone extensive gene duplication and neofunctionalization. Comparative genomic analyses show that symbiosis-related LysM receptors emerged through duplication followed by stepwise accumulation of changes in both ectodomains and kinase domains, with co-evolution of accessory signaling components. The narrow sequence alignment presented in the study lacks adequate taxon sampling and does not reconstruct the evolutionary history of SD1 or its surrounding regions. Without robust phylogenetic context, it is impossible to conclude that SD1 mutations were decisive evolutionary steps rather than downstream refinements.

In signaling terms, NFR1's ability to activate the nuclear calcium oscillator requires specific phosphorylation of protein partners distinct from those engaged by CERK6 in immunity. CERK6 operates through MAPKs and reactive oxygen species machinery, whereas NFR1 activates SYMRK, the nuclear calcium spiking apparatus, and NIN transcriptional pathways. These downstream branches are deeply divergent and depend on appropriate recruitment of interacting proteins. The study does not demonstrate that CERK6 chimeras interact with NFR5 or downstream symbiotic signaling modules, nor does it test whether the engineered receptors engage MAPK signaling differently. The absence of biochemical interaction data or phosphoproteomic profiling means that the assertion of signaling pathway reprogramming rests solely on weak morphological evidence.

Taken together, structural plasticity, evolutionary divergence, and the complexity of downstream pathway activation impose strong constraints on the plausibility of SD1-mediated receptor reprogramming. The minimalistic explanation proposed by Tsitsikli *et al*.[4] oversimplifies these constraints and attributes causality to a motif whose functional role has not been rigorously validated through biochemical, genetic, structural, or evolutionary evidence.

3. Assessment of Experimental Design

3.1. Design Logic of Chimeric Receptors and the Structural Continuity Problem

The experimental framework of Tsitsikli *et al*.[4] depends almost entirely on the construction and functional evaluation of a large family of domain-swapped LysM receptors. These include combinations of NFR1 and CERK6 ectodomains, transmembrane helices, JM motifs, and kinase lobes, along with additional constructs involving LYK3 and RLK4. The authors position these chimeras as

mechanistic probes to identify regions that determine signaling specificity. However, the interpretability of such constructs is inherently limited by the structural and functional discontinuities introduced through domain recombination. Receptor kinases consist of tightly integrated modules whose conformational relationships are essential to their function. The ectodomain must undergo ligand-induced rearrangements that are transmitted through the transmembrane helix and JM region to the intracellular kinase. Disruption of these linkages through artificial domain boundaries can easily produce loss-of-function artefacts or nonphysiological gain-of-function phenotypes misinterpreted as evidence for signaling specificity.

In this study, many constructs combine modules from receptors that diverged significantly during evolution, making it unlikely that the exact conformational dynamics required for native signaling are preserved. Even small perturbations in linker geometry or domain orientation may produce receptors that fold inefficiently, bind ligands abnormally, fail to dimerize with appropriate partners, or become hyperactive through destabilized autoinhibition. The authors attempt to mitigate these concerns by stating that all chimeras localize to the plasma membrane. Yet plasma membrane localization is not equivalent to correct folding or functional competence. Receptors that misfold yet escape the endoplasmic reticulum can still reach the plasma membrane, particularly in overexpression contexts, but they will not adopt conformations that allow bona fide activation or interaction with native co-receptors such as NFR5. Without biochemical validation, it remains impossible to know whether chimeric constructs behave as intended or whether their phenotypes arise from unpredictable structural distortions.

Another major concern is promoter usage. Although the constructs are described as being driven by native promoters, the study provides no measurements of receptor protein abundance across constructs. Even when native promoters are used, transgene insertion sites and copy numbers vary among hairy root transformation events. This variation creates substantial expression heterogeneity. A construct that appears to "*rescue*" nodulation in one or two roots may simply be overexpressed relative to endogenous receptors, driving ligand-independent activation or saturating downstream pathways without reflecting native receptor function. Because small numbers of positive events are interpreted as evidence of functional rescue, the absence of quantitative receptor abundance data severely limits the credibility of the conclusions regarding SD1 or two residue substitutions.

Finally, the nanobody-mediated synthetic receptor dimerization strategy, used as supplementary support for SD1 function, introduces additional complications. Forced association between CERK6 variants and NFR5 bypasses natural receptor affinities, stoichiometric requirements, and conformational constraints. In such a

setting, the appearance of occasional nodules cannot be taken as evidence that SD1 confers physiological compatibility. Synthetic dimerization is known to cause ligand-independent activation of receptor kinases, and any signaling events arising in this context must therefore be interpreted as artefacts rather than mechanistic demonstrations.

3.2. Vulnerabilities Inherent to the Assay Systems Used to Measure Receptor Function

The central functional assays in this study rely on root-hair deformation, infection thread formation, NIN promoter activation, microcolony organization, and nodule initiation. These phenotypes are classical readouts of symbiotic signaling but must be interpreted cautiously. Root-hair deformation is a highly sensitive phenotype that can be triggered by Nod factors independently of correct receptor signaling. It can also arise from stress responses, subtle perturbations in cell wall mechanics, or hormonal imbalances. Without calcium spiking assays or downstream transcriptional profiling, root hair deformation alone cannot be considered definitive evidence of symbiotic pathway activation.

Infection thread initiation is a more specific indicator but is still vulnerable to misinterpretation. Superficial penetration events, swirls of bacteria in curled hairs, or aborted threads do not necessarily reflect proper activation of the NFR1–NFR5–SYMRK–CCaMK pathway. The authors frequently infer receptor functionality from rare or incomplete infection threads in engineered constructs, but these ambiguous early events do not validate downstream developmental reprogramming. Moreover, infection thread morphology is not quantified in detail. Thread length, penetration depth, branching frequency, and alignment with cortical cell files are all essential metrics absent from the study.

NIN promoter activation is another widely used symbiotic marker but must be supported by time-resolved patterns and cortical expression imaging to confirm full pathway activation. The study occasionally shows cortical NIN activation in SD1-modified constructs, yet many of these signals appear faint, spatially diffuse, or limited to isolated cells. NIN expression can be induced by aberrant cytokinin signaling, making it an unreliable indicator when displayed without corresponding infection thread progression or nodule histology.

Nodule initiation is presented as a final, decisive readout. However, the study rarely distinguishes between true infected nodules and pseudonodules. Pseudonodules can arise from cytokinin accumulation, misregulated cell division, or partial pathway activation upstream of infection. In images provided for CERK6- and RLK4-derived constructs, nodules often lack the uniformly fluorescent infection zone characteristic of functional rhizobial colonization. The absence of consistent

longitudinal and cross-sectional histology prevents the conclusion that the nodules formed by engineered receptors represent genuine symbiotic structures. Finally, ROS assays used to assess immunity signaling are insufficiently calibrated. ROS production depends strongly on receptor abundance, plant age, and tissue preparation. Without normalization to expression levels, the apparent retention or loss of immune signaling in modified receptors cannot be meaningfully interpreted.

3.3. Statistical, Replication, and Validation Limitations Affecting Interpretive Strength

Across the study, phenotypic conclusions rest heavily on small sample sizes and high variability. Hairy root transformation inherently produces variable expression, yet the study often considers a construct functional if only a minority of roots display the desired phenotype. In some cases, a single nodulating root is treated as evidence of rescue. Without effect-size metrics, confidence intervals, or consistent replication, such interpretations are statistically fragile. Although the authors employ nonparametric tests such as Kruskal–Wallis with post hoc comparisons, the use of these tests without correction for multiple comparisons across dozens of constructs increases the risk of type I errors.

Replication across independent experiments is also underreported. Phenotypes observed in one transformation batch may not generalize. Several infection-related assays, ROS traces, and microscopy panels appear to represent single iterations rather than reproducible trends.

The greatest limitation, however, is the near-complete absence of biochemical validation. No co-immunoprecipitation, kinase activity assays, phosphoproteomic analyses, or interaction studies are provided to show that SD1 affects kinase activation, receptor complex formation, or substrate specificity. In the absence of biochemical grounding, morphological phenotypes—especially those that are weak or sporadic—cannot be interpreted as evidence for mechanistic reprogramming of receptor specificity. In total, the design exhibits creativity but lacks the rigorous validation required to support claims that two amino acids can convert an immunity receptor into a symbiotic receptor.

4. Figure-by-Figure Critique (Figures 1–4)

4.1. Figure 1: SD1 and Early Infection Phenotypes

Figure 1 is the conceptual anchor for the study, establishing SD1 as the key motif mediating symbiotic signaling and asserting that chimeric receptors containing this JM region can restore infection and nodulation in *nfr1* mutant roots. However, a detailed analysis of the panels reveals substantial inconsistencies, selective representation, and interpretational fragility.

The infection phenotypes shown in **Figure 1** rely on root hair deformation, microcolony formation, infection thread initiation, and nodule development. These phenotypes are visually presented as clear indicators of functional NFR1-like signaling triggered by CERK6 chimeras containing SD1. Yet many images lack clear markers of robust infection. Several root hairs show mild curling without clear bacterial penetration, while microcolonies remain superficial or poorly organized. True infection threads are rare, short, or truncated. In multiple constructs, the images display local bacterial clusters at the hair tip rather than well-defined threads progressing into the epidermis and cortex. Despite this, such events are interpreted as evidence that SD1 can endow CERK6 with symbiotic signaling capacity.

The quantification of infection threads and nodules is also problematic. Some constructs show only one or two infection threads per root, often in a minority of roots. These low-frequency events could arise from stochastic overexpression or partial activation of downstream pathways unrelated to proper receptor function. The study's interpretation relies on the assumption that any degree of infection thread formation implies functional receptor reprogramming. This assumption is flawed because weak or partial infection phenotypes are common in conditions involving ligand contamination, high-level receptor expression, or perturbations of cell wall physiology.

Moreover, the statistical analyses presented in **Figure 1** do not account for high variability and small sample sizes. The distributions of nodule numbers often include many roots with zero nodules and a small tail with one or two nodules. Such distributions are difficult to interpret without effect-size estimates, repeated experiments, and appropriate corrections for multiple testing. The boxplots visually inflate the apparent magnitude of differences by showing outliers prominently, even when medians remain close to zero.

The NIN:GUS activation patterns in Figure 1 further weaken the claim of SD1 sufficiency. Many roots show patchy or diffuse staining rather than the well-organized cortical NIN induction typical of functional NFR1 activation. NIN expression can be triggered by cytokinin accumulation independently of infection, making the staining pattern an unreliable proxy for symbiotic signaling without simultaneous evidence of robust infection thread progression.

Finally, the use of nanobody-mediated receptor pairing introduces synthetic complexity that challenges the physiological meaning of the results. Forcing CERK6 variants to associate with NFR5 bypasses natural receptor–receptor compatibility constraints and does not demonstrate that SD1 can support native complex formation. Any nodulation observed in these constructs may therefore arise from

proximity-induced signaling rather than physiological reprogramming. The rare positive events in this context do not constitute strong evidence for SD1 sufficiency. Overall, **Figure 1** fails to provide convincing evidence that SD1 alone determines symbiotic signaling capacity. The phenotypes are weak, inconsistent, and confounded by experimental limitations.

4.2. Figure 2: Structural Analyses of CERK6 and LYK3

Figure 2 attempts to establish a structural rationale for SD1 function by presenting crystal structures of CERK6 and LYK3 kinase domains. The authors suggest that SD1 maps to a surface region that differs between immune and symbiotic receptors and that this difference explains signaling specificity. However, the structural data do not substantiate this mechanistic narrative.

The most fundamental limitation is that the structures were determined using DFG-inactivated mutants, which lock the kinase domains into inactive conformations. The JM region, including SD1, is known to undergo significant repositioning during kinase activation, which cannot be captured in these static structures. Therefore, the conformational presentation of SD1 in **Figure 2** does not reflect the active state that mediates substrate engagement and downstream signaling.

Moreover, the authors do not demonstrate that SD1 forms part of a functional docking surface or substrate recruitment site. The structural overlays of CERK6 and LYK3 do not reveal meaningful differences in the SD1 region beyond superficial variations in surface topology. Without biochemical evidence showing altered substrate binding or kinase activity, the structural data remain descriptive rather than mechanistic.

The evolutionary implications suggested in the figure are also tenuous. Structural comparisons between LYK3 and CERK6, which belong to different lineages, do not establish that SD1 residues are responsible for their divergent signaling outputs. The study lacks phylogenetic or co-evolutionary analyses linking SD1 divergence to functional divergence. In sum, **Figure 2** does not provide a structural basis for SD1-mediated reprogramming. It presents static, inactive structures without functional validation, leaving the central mechanistic claim unsupported.

4.3. Figure 3: Kinase Surface Modularity and Phenotype Mapping

Figure 3 expands the investigation beyond SD1, proposing that multiple surfaces across the kinase domain contribute to signaling specificity. Despite the ambitious conceptual framing, the data presented are inconsistent, noisy, and difficult to interpret.

The mapping of surfaces A through G is speculative. These surfaces were chosen by visual inspection rather than through unbiased functional or evolutionary criteria. The replacement of each surface with its counterpart from NFR1 or CERK6 yields constructs with low and highly variable phenotypic outputs. Many constructs produce no nodules or only rare nodules, yet these rare events are interpreted as evidence that the substituted surface contributes to symbiotic signaling. With such weak effects, distinguishing genuine functional contributions from background variability is practically impossible.

Additionally, the ROS assays used to evaluate retention of immune signaling are insufficiently controlled. ROS responses depend heavily on receptor expression levels, tissue preparation, and plant age. The study does not measure receptor abundance in these constructs, making it impossible to determine whether observed ROS differences reflect functional changes or expression artifacts.

The logical coherence between structural surfaces and phenotypic outcomes is poor. Surfaces predicted to be essential sometimes show no functional impact, while other surfaces yield inconsistent and marginal phenotypes. This inconsistency suggests that the domain swaps do not probe mechanistic specificity but instead introduce unpredictable perturbations to receptor architecture. Therefore, **Figure 3** fails to provide meaningful insights into the modularity of kinase signaling surfaces and contributes little to validating SD1's proposed mechanistic role.

4.4. Figure 4: Two-Residue Sufficiency and RLK4 Engineering

Figure 4 contains the study's most provocative claim: that substituting two residues in CERK6 or RLK4 suffices to convert these immunity receptors into functional symbiotic receptors. However, the evidence presented is inadequate to support such a sweeping conclusion.

The phenotypic rescue observed with CERK6(T304M/D306A) is extremely weak. Most transformed roots fail to form nodules, and those that do typically produce one or two small nodules. These rare events cannot be interpreted as robust functional reprogramming. The absence of infection thread quantification, histological validation, or bacterial load measurement further obscures the biological significance of these nodules.

The RLK4 engineering data are even weaker. RLK4 variants show faint NIN activation and rare nodulation, with nodules that lack characteristic infection zones. These structures more closely resemble pseudonodules than true symbiotic organs. Without clear cortical infection, bacteria-filled central tissue, or nitrogen-fixation markers, the claim that RLK4 can be reprogrammed into a symbiotic receptor lacks credibility. **Figure 4** overinterprets sparse and ambiguous phenotypes,

misrepresenting marginal receptor activity as evidence of mechanistic sufficiency. The data do not justify the claim that two amino acids can reprogram immunity receptors into symbiotic receptors.

5. Extended Data Figure Critique (ED1–ED8)

5.1. ED1: Epidermal Signaling, Infection Threads, and NIN Activation

ED1 is intended to provide high-resolution support for the infection phenotypes summarized in Figure 1. It includes images of root hair deformation, microcolony organization, infection thread initiation, and NIN promoter activation. However, the data presented suffer from significant variability, selective representation, and inadequate diagnostic resolution.

The root hair images show a mixture of mild curling, partial tip swelling, and loose bacterial clusters, but few examples meet the classical criteria for productive rhizobial infection. True infection threads require clear cell wall invagination, alignment with growth axes, and consistent progression into the epidermal cell body. The majority of images in **ED1** depict ambiguous structures that could represent superficial bacterial adherence rather than legitimate infection threads. In several constructs, including those claimed to depend critically on SD1, bacterial fluorescence appears scattered or externally attached, with no clear evidence of penetration.

NIN activation images also show inconsistencies. Many roots display patchy or faint GUS staining, lacking the spatial coherence expected for symbiotic induction. Canonical cortical NIN expression forms a defined longitudinal domain in roots competent for nodule primordium formation. In contrast, the staining in **ED1** often appears unpatterned, diffuse, or restricted to isolated epidermal cells. Because NIN can be induced by cytokinin or stress independently of infection, these staining patterns cannot be used as reliable surrogates for functional signaling.

ED1 therefore fails to provide convincing evidence of consistent epidermal signaling or infection thread initiation across constructs. Instead, it highlights the fragility and low confidence of the phenotypes used to support the SD1 hypothesis.

5.2. ED2: Quantification of Infection Events

ED2 attempts to quantify the number of infection events per root, distinguishing between aborted microcolonies and infection threads. While quantitative approaches are essential for validating receptor function, the methodology and presentation of **ED2** undermine the reliability of its conclusions.

The most significant limitation is the extremely small sample size. Several constructs are evaluated on fewer than ten roots, with many roots yielding no infection events at all. This makes it statistically unsound to draw functional inferences from sporadic occurrences. The inherent transformation variability in hairy root systems further exacerbates the problem, amplifying stochastic noise.

The classification of infection events into microcolonies versus infection threads is also problematic. The figure does not provide clear morphological criteria or demonstrate that the scoring was blinded. Many events included as infection threads in **ED2** appear, based on **ED1** images, to be superficial accumulations rather than legitimate penetration structures. Without time-course imaging or histological confirmation, these distinctions remain subjective.

Importantly, constructs that are categorized as "*nonfunctional*" in the main figures nonetheless show measurable infection events in **ED2**, contradicting the binary functional assignments. Conversely, constructs that supposedly function due to SD1 show only marginal improvements in infection frequency, often insufficient to confirm true pathway activation. **ED2** therefore fails to provide quantitative support for the dichotomy between SD1-containing and SD1-deficient constructs, and its inconsistencies weaken the foundational interpretation of receptor specificity.

5.3. ED3: Receptor Localization in *N. benthamiana*

ED3 presents fluorescence microscopy of CERK6-, NFR1-, and chimeric receptors transiently expressed in *N. benthamiana*. The authors use these data to argue that all receptors properly localize to the plasma membrane, and that functional differences therefore reflect signaling specificity rather than mislocalization. However, several issues compromise this interpretation.

Transient expression in *Nicotiana* frequently leads to overaccumulation, saturating the secretory pathway and driving even misfolded proteins to the plasma membrane. The images in **ED3** show bright, uniform plasma membrane fluorescence with little variation between constructs, which is typical of strong overexpression. Subtle differences in folding, trafficking, or complex formation cannot be inferred from this assay.

Colocalization with mCherry-tagged plasma membrane markers appears qualitative, with no quantification of Pearson's correlation coefficients. Minor cytoplasmic puncta or reticular structures visible in some images are not discussed, though they may represent retention in the endoplasmic reticulum or Golgi, indicating folding or trafficking defects. Because CERK6 and NFR1 differ in their native trafficking logic and co-receptor associations, the *Nicotiana* assay is not biologically informative for determining whether chimeric receptors behave properly in *Lotus*. ED3 therefore

does not substantiate the claim that functional disparities arise from differences in signaling capacity rather than localization defects.

5.4. ED4: Localization in *Lotus* protoplasts

ED4 repeats the localization analysis in *L. japonicus* protoplasts, aiming to demonstrate that receptor variants target correctly in the native cellular environment. However, the limitations of protoplast systems diminish the interpretability of the results. Protoplasts lack the polarized trafficking and cell wall interactions that define root hair plasma membrane dynamics, making them poor models for evaluating receptor positioning relevant to symbiotic signaling. Even severe folding defects can go undetected in protoplasts because such artifacts do not generate cell-wall stress responses that would otherwise occur in intact tissues.

The images again show uniform plasma membrane localization with occasional cytoplasmic fluorescence. No comparison with endogenous NFR1 distribution is provided. Additionally, receptor abundance remains unquantified, so differences in fluorescence intensity cannot be interpreted. Although **ED4** demonstrates that receptors can reach the plasma membrane when heavily expressed in protoplasts, it does not establish that they are correctly inserted, functional, or comparably expressed in hairy roots. Thus, the figure adds no meaningful evidence supporting the mechanistic claims.

5.5. ED5: Evolutionary and sequence Conservation Analyses

ED5 displays alignments highlighting variation across SD1 and adjacent regions. While the alignment is visually clear, the conceptual interpretation is weak. The taxonomic sampling appears limited, with few representatives from key clades of LysM receptor kinases. A rigorous evolutionary analysis would require extensive phylogenetic reconstruction, ancestral state inference, and co-evolution analysis with downstream signaling partners. None of these are present. The highlighted residues in SD1 appear cherry-picked, with no statistical demonstration that they experience positive selection or covary with symbiotic function. Without evolutionary context, **ED5** cannot be used to support the claim that SD1 residues determine signaling divergence.

5.6. ED6: Biochemical and Biophysical Characterization

ED6 presents size-exclusion chromatography, SDS-PAGE, and ATP-binding assays on purified CERK6 and LYK3 kinase domains. These data aim to demonstrate correct folding and biochemical competence. However, the assays fall short.

The size-exclusion chromatography traces do not confirm monodispersity or distinguish folded monomers from aggregates. SDS-PAGE purity does not imply functional integrity. Thermal shift assays are shown without replicates and lack

statistical analysis. Most critically, ATP binding is shown, but catalytic activity is not measured. The use of DFG-mutated kinases further invalidates conclusions regarding activation. Without demonstrating autophosphorylation, substrate turnover, or dynamic conformational changes, **ED6** cannot substantiate claims that SD1 affects kinase behavior.

5.7. ED7: Structural Surface Mapping and Functional Testing

ED7 presents a structural mapping of candidate functional surfaces across the kinase domain and evaluates chimeras substituting each surface. The conceptual framework is ambitious but the data do not meet the evidentiary threshold.

The designation of surfaces A–G is subjective, lacking structural or evolutionary justification. The functional assays show extremely weak and inconsistent nodulation phenotypes for most variants, making it impossible to draw conclusions about modularity. Construct performance often contradicts predictions based on structural mapping. Because the phenotypes do not correlate with the hypothesized importance of each surface, ED7 does not validate the model of distributed structural determinants.

5.8. ED8: RLK4 Mutational Analyses and Symbiotic Signaling

ED8 attempts to demonstrate that barley RLK4 can be reprogrammed into a symbiotic receptor using SD1-derived mutations. This is the boldest extrapolation of the study, yet the evidence is the weakest. ROS assays show irregular patterns that correlate more with expression variability than signaling specificity. NIN activation is faint and inconsistent. Infection images show superficial colonization with no evidence of robust cortical invasion. Nodules formed by RLK4 derivatives lack clear infection zones and resemble pseudonodules. **ED8** therefore fails to support the conclusion that RLK4 can be converted into a functional symbiotic receptor through SD1-based engineering.

6. Supplementary Figure Critique (S1–S2)

6.1. S1: Nodule Histology and Infection Zone Organization

S1 extends the phenotypic observations made in the main figures and Extended Data by providing cross-sectional and longitudinal views of nodules induced by wild-type receptors, SD1-containing chimeras, and two–residue–modified variants. Because nodulation is the physiological hallmark of successful symbiotic signaling, these histological data are critical to assessing whether the engineered receptors actually drive functional infection and organogenesis. However, a careful analysis shows that **S1** does not provide strong support for the study's claims.

The nodules produced by the engineered receptors consistently exhibit structural irregularities. In wild-type NFR1-complemented controls, infected nodules display a clear zonation pattern, including a central infected zone densely colonized by rhizobia, surrounded by peripheral uninfected tissue and vascular traces. In contrast, the nodules formed by CERK6-based chimeras and RLK4 variants frequently lack a coherent infected zone. DsRed-labelled bacteria appear patchy, sparse, or limited to small peripheral clusters rather than filling the central tissue. This pattern is characteristic of pseudonodules or abortive infection. The absence of a well-organized infection zone strongly suggests that the downstream signaling pathways responsible for proper cortical invasion and bacterial accommodation were not fully activated.

Cortical cell divisions in engineered constructs also appear disorganized. Rather than forming tightly coordinated cell files radiating from the nodule apex, cells often appear irregular in shape and orientation. This indicates perturbation rather than full developmental reprogramming. True nodule organogenesis requires coordinated action of NIN, CYCLOPS, CCaMK, NF-YA isoforms, and additional transcriptional regulators, whose proper activation cannot be inferred solely from the limited structural changes seen here.

Another limitation is the lack of quantitative histological analysis. There are no bacterial load measurements, infection zone metrics, or assessments of bacteroid morphology. Without these, the interpretation of Supplementary Figure 1 remains qualitative and subjective. The figure does not demonstrate nitrogen fixation or even sustained bacterial proliferation, both of which are essential for declaring successful symbiosis. The histology therefore undermines rather than supports the claim that two-residue substitution in CERK6 or RLK4 produces functional nodules.

6.2. S2: Additional Receptor Localization and Stability Analyses

S2 includes additional receptor localization analyses, presumably intended to strengthen the conclusion that the SD1 region or the two key residues affect signaling, not trafficking. However, the data shown do not provide clear evidence for proper receptor folding, stable membrane insertion, or comparable abundance across constructs.

The fluorescence images in **S2** show heterogeneous signal intensities across root cells, suggesting variable expression among constructs. Without quantitative comparisons, subtle trafficking defects may go undetected. Some constructs exhibit intracellular puncta consistent with partial ER retention or endosomal misrouting, but these features are not acknowledged or interpreted by the authors. A receptor that accumulates in the ER or in recycling endosomes may still reach the plasma

membrane at levels sufficient to generate detectable fluorescence but insufficient to support reliable signaling

Another interpretive gap is the absence of biochemical data confirming protein stability. Fluorescence imaging alone cannot differentiate between correctly folded receptors and partially degraded or misprocessed variants. Western blotting, protease protection assays, or mass spectrometry would have provided crucial information about receptor integrity but were not performed.

Finally, the figure does not demonstrate that chimeric receptors are present at similar levels across cell types within the same construct. Hairy roots naturally exhibit expression mosaics, and incomplete receptor expression could explain many of the weak phenotypes attributed to SD1 rather than genuine mechanistic differences. Overall, **S1** and **S2** do not reinforce the study's central claims. Instead, they reveal inconsistencies in infection structure, incomplete receptor trafficking, and a lack of quantitative validation. These limitations weaken the argument that SD1 or two-residue substitutions can mechanistically reprogram CERK6 or RLK4 into functional symbiotic receptors.

7. Structural Biology Considerations

7.1. Inactive-State Crystallography and the Problem of Mechanistic Inference

The structural component of Tsitsikli *et al*.[4] is presented as a mechanistic anchor for the SD1 hypothesis, yet the crystallographic approach used in the study reveals fundamental limitations. The kinase domains of CERK6 and LYK3 were crystallized using DFG→N mutations, which lock the catalytic loop into an artificially stabilized inactive conformation. This state differs substantially from the active conformations that govern substrate recruitment, autophosphorylation, and interaction with co-receptors. Because SD1 resides in the JM–αB region, known to undergo significant repositioning during activation, its orientation in the inactive structures is not reflective of the conformational landscape relevant to signaling. The authors treat the static structures as indicative of functional surfaces, but inactive conformations capture only one of many structural possibilities. Without active-state structures or molecular dynamics simulations, the mechanistic claims drawn from these crystallographic snapshots cannot be validated.

Inactive kinases often fold in ways that occlude regulatory residues or distort surface regions involved in substrate recognition. The SD1 region as visualized is therefore not in a physiologically meaningful configuration. The lack of structural data in an activated state means the study provides no evidence that SD1 coordinates substrate engagement, contributes to docking platforms, or undergoes

conformational shifts essential for transmitting ligand-induced changes. The conclusions drawn from the structures thus rest on an incomplete picture of receptor dynamics, undermining the mechanistic link between SD1 and signaling specificity.

7.2. Comparative Structural Analysis and Oversimplification of Kinase Divergence

The structural overlays presented for CERK6 and LYK3 are interpreted by the authors as evidence that surface-exposed differences in the SD1 region contribute to functional divergence between immunity and symbiosis. However, this comparison overlooks the fact that CERK6 and NFR1 (the true symbiotic receptor of interest) are more divergent than CERK6 and LYK3. LYK3 itself is an immune-type kinase and cannot serve as a structural stand-in for NFR1. The structural alignment between CERK6 and LYK3 therefore does not reveal differences relevant to symbiotic signaling.

Moreover, structural divergence between CERK6 and NFR1 extends far beyond SD1. Key differences exist in the activation loop, the αC helix, the catalytic HRD and DFG motifs, C-terminal extensions, and docking sites for co-receptors and substrates. These regions typically exert stronger influence on kinase specificity than superficial surface loops. The study's focus on SD1, while ignoring these broader structural determinants, results in an oversimplified narrative that does not align with established knowledge in kinase biology. The overlay does not incorporate analyses of surface electrostatics, solvent accessibility, or residue co-evolution—tools that would provide deeper insight into whether SD1 is structurally poised to influence signaling. Without such analyses, the structural comparison lacks explanatory power.

7.3. Absence of Substrate Specificity and Kinase Activity Data

Structural biology becomes mechanistically meaningful when paired with biochemical studies demonstrating how structural features affect function. The study provides no data measuring kinase activity, substrate preferences, autophosphorylation patterns, or interaction profiles for CERK6, NFR1, or their engineered variants. Without such data, structural interpretation remains speculative.

Substrate recognition pockets in kinases depend on phosphorylation hotspot organization, catalytic loop geometry, and conserved docking regions that extend well beyond the SD1 surface. The lack of biochemical validation means that the SD1 hypothesis has no connection to measurable changes in catalytic function. ATP-binding assays shown in Extended Data Figure 6 are insufficient because ATP

affinity is not predictive of kinase specificity. Likewise, thermal shift assays provide only stability information, not functional readouts.

Absent from the study are phosphoproteomic analyses, substrate-binding assays, and kinetic measurements that could reveal whether SD1 modifications alter kinase-substrate interactions or downstream phosphorylation events. Because kinase specificity arises from complex networks of residues, including those in the activation loop and C-terminal tail, two substituted residues are unlikely to dictate specificity. Without functional biochemical data, the study cannot substantiate a mechanistic role for SD1.

7.4. JM Regulatory Logic and the Possibility of Autoinhibition Disruption

In many receptor kinases, the JM region functions as a regulatory module that stabilizes inactive conformations or mediates autoinhibitory interactions with the kinase core. SD1 is located within a JM-proximal region that could influence receptor activation indirectly by modulating autoinhibitory contacts. Mutations in JM regions often destabilize autoinhibition rather than reprogram specificity. If SD1 modifications relieve autoinhibition in CERK6, this could produce weak, ligand-independent activation capable of inducing partial epidermal phenotypes or pseudonodules.

This interpretation aligns with the sporadic and low-penetrance phenotypes observed in the study. Such phenotypes are consistent with partial release from autoinhibition rather than true rewiring of kinase specificity. The study does not test this possibility and presents no evidence that SD1 restructures the autoinhibitory network. The absence of assays measuring ligand dependence, basal activation levels, or autophosphorylation status leaves open the possibility that SD1-induced phenotypes reflect dysregulated activation rather than symbiotic signaling. Without experiments measuring basal phosphorylation or ligand-dependent activation, the study cannot distinguish between autoinhibition disruption and mechanistic reprogramming. This omission severely limits the credibility of the structural interpretation.

7.5. Lack of Structural Integration with Extracellular and Co-Receptor Modules

Receptor kinases do not function as isolated kinase domains; they operate as integrated systems in which extracellular ligand-binding domains, transmembrane helices, and co-receptors coordinate conformational changes leading to intracellular activation. The study presents kinase-only structures, which lack the ectodomain context needed to evaluate how ligand binding induces activation. Because NFR1

requires NFR5 to form an active receptor complex, SD1's function cannot be assessed without structural or biochemical data showing how SD1 influences heteromerization.

Similarly, CERK6 functions with CERK1 in immunity, relying on a different co-receptor pairing mechanism. SD1 substitution cannot override these fundamental extracellular constraints. The study provides no structural models of CERK6–NFR5 or CERK6–CERK1–NFR5 assemblies, making it impossible to infer how SD1 impacts receptor complex formation.

The absence of full-length receptor structural analyses—cryo-EM, SAXS, or computational modelling—precludes any understanding of the allosteric pathways linking ligand perception to kinase activation. Without this integration, the structural component of the study remains disconnected from the biological system it seeks to explain.

In summary, **Section 7** reveals that the structural biology presented in Tsitsikli *et al*. does not support the mechanistic claims made regarding SD1. Inactive-state snapshots, overly simplified comparisons, lack of biochemical data, failure to consider autoinhibition, and absence of integrated receptor models collectively undermine the assertion that SD1 or two residues can reprogram receptor specificity.

8. Evolutionary and Comparative Genomic Context

8.1. Evolutionary Divergence among LysM Receptor Kinases

LysM receptor kinases represent one of the most ancient and functionally diversified receptor families in land plants. Their evolutionary history spans early nonvascular plants, gymnosperms, monocots, and dicots, with substantial diversification occurring long before the emergence of nitrogen-fixing symbiosis. CERK-family immunity receptors and NFR-family symbiotic receptors belong to distinct phylogenetic clades that separated early during angiosperm evolution. This means their divergence encompasses millions of years of structural, functional, and co-evolutionary refinement, involving changes far more extensive than a short JM motif such as SD1. CERK6 homologues across plant lineages are specialized for chitin-triggered immune responses, whereas NFR1 homologues evolved specifically within legume genomes after key gene duplication events that preceded symbiotic specialization.

Given this deep divergence, the premise that two amino acids can explain the functional gulf between CERK6-mediated immunity and NFR1-mediated symbiosis is inherently problematic. Receptor evolution typically proceeds through gradual accumulation of mutations that modify not only ligand binding but also downstream

signaling interactions, substrate specificities, and regulatory dynamics. No evolutionary evidence exists to suggest that the major functional differences between immunity and symbiosis pathways can be traced to a single short region. Instead, NFRs possess lineage-specific modifications across multiple domains, including distinct autophosphorylation sites, co-receptor binding modules, and extended C-terminal regions rich in regulatory motifs.

The evolutionary relationships among CERK6, NFR1, LYK3, and RLK4 therefore argue strongly against a minimal mutational switch model. Instead, they support a model in which complex co-evolution of multiple receptor regions and downstream signaling partners is required to generate the symbiotic signaling specificity upon which the legume–rhizobial partnership depends. Nothing in the genomic history of these receptors supports the notion that SD1 alone governs this specificity.

8.2. Feasibility of Minimal Mutational Pathways to Symbiosis

The hypothesis proposed by Tsitsikli *et al*.[4] implicitly assumes that the evolutionary emergence of Nod factor signaling involved only a small number of mutational steps. However, symbiosis is not merely a modified immunity pathway; it represents an entirely distinct signaling system requiring coordinated integration of multiple molecular modules absent in most plant lineages. Calcium spiking, CCaMK decoding, downstream transcriptional networks, nodule organogenesis programs, and rhizobial accommodation pathways did not evolve overnight. These components must interact with receptor kinases in ways that go far beyond the level of JM motifs.

Comparative genomic studies of legumes and non-legumes demonstrate that the capacity for nodulation required major genomic innovations and co-option events. Genes such as NIN, CYCLOPS, and multiple ENODs emerged or diversified specifically within nodulating lineages. Even within legumes, symbiotic competence evolved multiple times with complex domain rearrangements across entire receptor families. Moreover, the diversity of Nod factor structures and corresponding receptor specificities among legume species underscores the multilayered nature of ligand–receptor co-evolution.

Minimal mutational pathways would require that CERK6-like ancestral receptors possessed latent symbiotic potential awaiting only small changes to become functional. Yet CERK-family receptors are consistently dedicated to immune signaling across monocots and dicots, with no evidence of latent bifunctionality. Their kinase domains evolve under strong purifying selection to maintain immune competence. The co-receptors that partner with CERK6, including CERK1-like kinases, are also immune-adapted and do not interact with symbiotic machinery. The proposed minimal-pathway hypothesis therefore contradicts established

genomic and phylogenetic patterns and does not align with known constraints on receptor evolution.

8.3. Co-Evolution of Ligand-Binding Domains and Intracellular Signaling Regions

Symbiotic signaling cannot be understood solely in terms of intracellular kinase domains. NFR1 and NFR5 evolved extracellular LysM domains with specialized architecture enabling recognition of rhizobial Nod factors. These ectodomains co-evolved with specific ligand-binding residues tuned to detect acylation patterns, decorations, and lengths unique to their rhizobial partners. CERK6 ectodomains, in contrast, bind chitin oligomers with high affinity, a function conserved across angiosperms.

Co-evolution between extracellular ligand-binding modules and intracellular signaling domains ensures that ligand perception and downstream outputs remain aligned. If SD1 were indeed the decisive determinant of intracellular signaling specificity, one would expect a corresponding pattern of co-evolution between SD1 regions and their extracellular counterparts. However, comparative LysM-domain phylogenies do not show clustering of CERK6 and NFR1 in any way that would support shared ligand–kinase alignment. Instead, the divergence between CERK6 and NFR1 is mirrored across the entire receptor architecture, spanning the ectodomain, transmembrane helix, JM linker, kinase lobes, and C-terminal regulatory regions. This holistic divergence contradicts the idea that SD1 alone can determine signaling fate.

Furthermore, ligand-binding preferences profoundly influence receptor complex formation. NFR1 requires NFR5 to form a functional heteromer that transduces symbiotic signals, whereas CERK6 interacts with CERK1 and related co-receptors to initiate immune signaling. These partner specificities emerged through co-evolution, and such interactions cannot be reconstituted simply by editing two residues. The absence of evidence that CERK6-SD1 variants interact correctly with NFR5 further undermines the idea that co-evolution between ligand-binding and signaling regions can be bypassed by minimal genetic manipulation.

8.4. Integration of Intracellular Signaling Networks and Functional Divergence

The symbiotic and immune signaling networks into which NFR1 and CERK6 feed are fundamentally distinct in architecture, temporal dynamics, amplitude, and regulatory feedback. Immune signaling leads to rapid ROS production, MAPK activation, transcriptional induction of defense genes, and often cell death. Symbiotic signaling, by contrast, leads to nuclear calcium oscillations, CCaMK

activation, cell cycle re-entry within cortical tissues, infection thread progression, and eventual organogenesis.

These divergent outputs require different kinase-substrate relationships, distinct scaffolding proteins, and separate sets of phosphorylation targets. The substrate-binding pockets of CERK6 and NFR1 differ in crucial catalytic and regulatory residues unrelated to SD1. Without altering these regions, intracellular rewiring remains incomplete. Minimal SD1-based reprogramming cannot recreate the necessary kinase–substrate specificity that enables symbiotic signaling. This is reflected in the weak and inconsistent phenotypes observed in the engineered constructs, which often show superficial infection or pseudonodule formation but fail to achieve full symbiotic development.

Integration into downstream networks also depends on precise spatiotemporal regulation. Symbiotic signaling requires carefully orchestrated nuclear migration, cortical division activation, and transcriptional cascades that cannot be induced by partial or sporadic receptor activation. CERK6 chimeras bearing SD1 or two residue substitutions do not induce these orchestrated responses consistently, indicating that the intracellular network remains incompatible with the modified receptors.

8.5. Constraints Imposed by Multispecies Comparative Genomics

Comparative genomics across nodulating and non-nodulating lineages reveals multiple independent losses and gains of symbiotic competence. These events are not explained by small changes in a single receptor. Instead, they correlate with genomic deletions, promoter rewiring, co-option of hormone pathways, and coordinated changes across dozens of symbiosis-associated genes. For example, the absence of CCS52A, specific NF-YA paralogues, or entire components of the calcium-spiking apparatus in non-nodulating plants underscores the complexity required for symbiotic signaling.

Cross-species analyses of LysM receptor families in *Parasponia*, the only non-legume lineage that naturally evolved nodulation, show extensive receptor duplication and neofunctionalization rather than minimalistic edits. Such patterns contradict the claim that two amino acids can bridge the functional gap between immune and symbiotic receptors.

In summary, evolutionary, genomic, and comparative analyses collectively demonstrate that the emergence of symbiotic signaling required extensive, multi-layered evolutionary innovation across several gene families. The proposition that SD1 or two amino acids can recapitulate this evolutionary trajectory is inconsistent

with established principles of receptor evolution and the genomic architecture of symbiotic signaling.

9. Physiological and Systems-Level Interpretation

9.1. Linking Receptor Activation to Downstream Nodulation Signaling

A central limitation of the study by Tsitsikli *et al.*[4] lies in the gap between the phenotypic observations associated with SD1-engineered receptors and the known physiological requirements for complete symbiotic signaling. Root nodule symbiosis is a highly coordinated developmental process that begins at the epidermis and culminates in the formation of an organ specialized for intracellular bacterial accommodation and nitrogen fixation. The transition from perception of Nod factors at the plasma membrane to downstream signaling involves multiple checkpoints: nuclear calcium oscillations, CCaMK decoding, NIN transcriptional activation, cortical reprogramming, infection thread progression, and synchronized cell division. For a receptor like CERK6 to be considered reprogrammed into a symbiotic receptor, it must not only trigger early epidermal responses but also activate the full suite of downstream events in the correct spatial and temporal order.

The evidence presented by the authors does not demonstrate such a connection. The weak infection thread-like structures and faint NIN activation patterns observed in SD1-containing constructs do not meet the criteria for coordinated downstream signaling. No calcium spiking assays were conducted, and no CCaMK-dependent transcriptional markers were monitored. The lack of direct measurement of these early symbiotic signals means that the study cannot affirm that SD1-modified receptors engage the molecular apparatus that distinguishes successful symbiosis from superficial bacterial interaction. Moreover, the absence of synchronized cortical activation, as evidenced by disorganized NIN expression and irregular nodule primordia, indicates that the core signal integration checkpoint remains unmet. For these reasons, the presented phenotypes fail to demonstrate that SD1 meaningfully reconstitutes the receptor-dependent activation of the symbiotic signaling cascade.

9.2. Distinguishing Pseudonodules from Functional Symbiotic Nodules

A significant interpretational challenge arises from the study's reliance on nodule-like structures as indicators of successful symbiosis. True nodules exhibit defining physiological characteristics that cannot be inferred from surface morphology alone. These include organized central infection zones, accumulation of rhizobia-laden cells, zonation of meristematic, infection, and nitrogen-fixing regions, and specific

transcriptional signatures corresponding to nodule maturation. In contrast, pseudonodules, which arise through perturbations of cytokinin signaling, partial activation of NIN, or stress-induced cortical divisions, can visually mimic nodules yet lack the functional hallmarks of symbiotic organs.

Many nodules presented in the main and extended figures of the study fall closer to pseudonodules than true symbiotic nodules. DsRed fluorescence in engineered constructs is often peripheral, sparse, or absent in internal tissues. The cross-sectional views in the supplementary materials reveal poorly organized tissues, lacking both defined infection zones and the extensive bacterial colonization typical of mature nodules. Without quantification of bacterial load, acetylene reduction assays, oxygen permeability gradients, or vascular organization, the nodules shown cannot be assumed to reflect genuine symbiotic development. This distinction is critical, because nodules can form in the absence of functional receptor signaling. Even mutants deficient in NFR1 or in early infection can produce pseudonodules if cytokinin levels are sufficiently elevated. Thus, interpreting nodule-like structures as evidence of receptor reprogramming overstates what the data can support. The lack of rigorous anatomical and physiological validation undermines the conclusion that SD1-engineered receptors induce proper symbiotic organogenesis.

9.3. Interplay between Epidermal and Cortical Signaling

A defining feature of symbiotic signaling is the coordinated interaction between epidermal infection and cortical organogenesis. Epidermal events such as root hair curling, microcolony formation, and infection thread initiation must be synchronized with cortical responses such as NIN-dependent reprogramming, pericycle activation, and division of cortical cells to form a nodule primordium. This synchrony is regulated by signaling feedback loops between epidermal cells and the cortex. When receptors fail to activate this coordination, infection threads abort, and nodules fail to develop.

In the study, many constructs exhibit epidermal phenotypes without coherent cortical responses. For example, root hair deformation is observed, but cortical NIN activation is faint, spatially diffuse, or absent. Similarly, occasional infection threads appear without corresponding primordia. These observations indicate that receptor signaling initiated in the epidermis does not propagate through the signaling cascade in a coordinated manner. The weak or inconsistent epidermal responses induced by SD1-modified receptors are therefore insufficient to drive cortical reprogramming.

Time-lapse imaging or transcriptional profiling across epidermal and cortical tissues would have been essential to confirm synchronization. Without such data, the study cannot demonstrate that SD1 mutations create receptors capable of

orchestrating the biphasic architecture of symbiotic signaling. This discordance suggests that the weak phenotypes associated with the SD1 constructs reflect partial pathway activation rather than genuine signaling reprogramming.

9.4. Limitations of Interpreting Early Epidermal Responses as Measures Of Receptor Specificity

Tsitsikli *et al*.[4] rely heavily on early epidermal responses—root hair curling, microcolony formation, and infection thread initiation—as indicators that SD1 confers symbiotic signaling potential. However, these responses are not specific to NFR1-mediated signaling. Root hair deformation can be triggered by isolated Nod factors without downstream infection. It can also be induced by cell wall perturbations, high receptor expression, or stress-induced signaling. Microcolonies of bacteria on the root hair surface likewise do not indicate successful receptor perception, as they may reflect surface adherence rather than entry into the plant tissue.

Infection thread-like structures, while more specific, must be interpreted carefully. True infection threads require a complex interplay between cell wall invagination, cytoskeletal rearrangement, and targeted secretion. Many of the structures shown in the study resemble superficial bacterial aggregations or aborted penetration attempts rather than genuine threads. Without detailed histological characterization showing cell wall architecture and penetration depth, the authors' classification remains subjective.

Furthermore, the reliance on these early responses obscures the importance of later developmental checkpoints. Even if early epidermal responses are partially activated by SD1-modified receptors, the absence of full cortical activation argues strongly against reprogramming of receptor specificity. The study does not evaluate whether SD1-modified receptors can activate SYMRK, trigger nuclear calcium oscillations, or induce robust CCaMK-dependent gene expression. These omissions weaken the interpretation that early epidermal phenotypes reflect genuine symbiotic signaling.

9.5. Confounding Influences of Overexpression and Transformation Variability

A recurring limitation throughout the study stems from the use of hairy root transformation systems, which introduce substantial variability in receptor expression levels. Overexpression can cause ligand-independent receptor aggregation, spurious activation of downstream pathways, and induction of cell wall modifications that mimic early symbiotic phenotypes. When expression levels vary widely across roots, sporadic activation in a few roots cannot be taken as evidence

of genuine receptor function. Because the authors do not quantify receptor abundance, the observed low-frequency root hair curling, microcolony formation, or nodule-like structures may simply reflect stochastic overexpression rather than SD1-mediated reprogramming.

The mosaic nature of hairy root systems also complicates interpretation. Roots expressing higher levels of receptor constructs may display artificially induced phenotypes, while others remain unresponsive. Without normalization of expression, consistency across biological replicates, or use of stable transgenic lines, the weak and inconsistent phenotypes associated with SD1-engineered receptors cannot be confidently attributed to specific amino-acid substitutions. In sum, the physiological and systems-level evidence does not support the claim that SD1 or two residues reprogram receptor specificity. The phenotypes observed are inconsistent, incomplete, and readily attributable to known artefacts of transformation and overexpression rather than to mechanistic reconstitution of symbiotic signaling.

10. Implications for Engineering Nitrogen-Fixing Traits

10.1. Limitations of Receptor-Only Engineering as A Strategy for Synthetic Symbiosis

The primary translational implication suggested by Tsitsikli *et al.*[4] is the possibility that nitrogen-fixing symbiosis might be engineered into non-leguminous plants through minimal manipulation of receptor kinases. If two amino-acid substitutions were truly sufficient to convert an immunity receptor into a symbiotic receptor, receptor - centric engineering might appear to offer a plausible roadmap toward generating nitrogen-fixing cereals. However, a deeper analysis of symbiotic signaling architecture reveals that receptor-only engineering is fundamentally insufficient. Symbiosis is a multi-tiered signaling program that involves receptor activation, nuclear calcium oscillations, decoding by CCaMK, NIN-driven transcriptional cascades, cortical developmental reprogramming, and the establishment of a unique intracellular niche for rhizobia. Even in legumes, small perturbations in any of these layers disrupt the entire symbiotic pathway. Thus, manipulating a single receptor—even if correctly folded and inserted—cannot reproduce the evolutionary complexity of symbiosis.

Furthermore, the downstream requirements for symbiosis include not only gene regulatory networks but also cellular structures such as infection threads, cytoskeletal arrangements, and nodular vasculature. These structures depend on legume-specific co-opted modules that do not exist in cereals. Therefore, even if SD1 or two modified residues produced partial receptor signaling, they would not create the developmental context required to support rhizobial entry, intracellular

accommodation, or nitrogen fixation. The weak and inconsistent phenotypes associated with CERK6- or RLK4-based constructs in *Lotus* underline this conceptual limitation. The absence of histological or biochemical evidence for functional infection reinforces the conclusion that receptor activation alone cannot confer symbiotic competence.

10.2. Challenges of Transferring Legume Symbiotic Machinery into Cereals

Attempts to engineer nitrogen-fixation into cereals must confront the reality that legumes possess a uniquely elaborated symbiotic apparatus absent from non-legumes. The calcium-spiking machinery, the CCaMK–CYCLOPS hub, the NIN master regulator, and a suite of ENODs all contribute essential functions in infection and nodule development. Cereals lack NIN orthologues and show no evidence of spontaneous cortical activation in response to Nod factors. Moreover, the cytokinin networks that mediate cortical organogenesis in legumes are tuned to symbiotic developmental modules that are absent in grasses.

Tsitsikli *et al*.[4] briefly imply that the successful reprogramming of barley RLK4 hints at a pathway toward engineering cereals. Yet the RLK4 phenotypes shown are superficial and sporadic, lacking cortical infection, coordinated nodule primordium development, or nitrogen-fixation signatures. These weak outputs underscore the deep evolutionary barriers that impede transfer of the symbiotic program into non-legumes. Even a receptor engineered to perceive Nod factors cannot overcome downstream developmental incompatibilities. Cereals lack the entire architectural foundation upon which symbiosis depends, including infection-thread guidance machinery, nodular meristem regulators, and mechanisms for intracellular bacterial accommodation. Therefore, even if SD1 substitutions could modestly modulate receptor phosphorylation, the lack of downstream components in cereals renders receptor - based engineering insufficient. Any realistic engineering strategy would require reconstitution of multi-gene modules rather than minimal mutational edits.

10.3. Risks of Overinterpreting Chimeric Receptor Phenotypes in Translational Contexts

The chimeric constructs used in the study show biologically weak and inconsistent phenotypes that cannot be translated into functional engineering principles. Occasional root hair deformation, sporadic infection-thread-like events, or rare pseudonodules should not be interpreted as evidence that receptor engineering provides a practical path to synthetic symbiosis. Many constructs reported as "*functional*" produce only a minority of roots with ambiguous phenotypes, making them unreliable templates for engineering strategies.

Overexpression artefacts further cloud translational relevance. Transgenic hairy roots used in the study represent highly artificial systems that do not reflect stable, whole-plant physiology. Engineering receptor-based systems into crops would require stable genomic integration, correct tissue-specific expression, and robust trafficking in intact organs. Weak phenotypes observed under artificial overexpression conditions cannot be extrapolated into engineering frameworks.

Another translational concern involves ligand availability. Nod factors are structurally complex molecules not produced by endogenous plant systems. Engineering Nod factor perception into cereals would require simultaneous modification or transfer of Nod factor synthesis or microbial colonization compatibility. Receptor modifications alone cannot recreate the species-specific signaling partnerships underlying symbiosis. Thus, the translational implications drawn in the study are speculative and overstated. The observed partial phenotypes do not constitute evidence for practical engineering pathways.

10.4. Lessons for Synthetic Biology and Receptor Modularity

Although the study's claims are overstated, it nevertheless highlights useful considerations for synthetic biology. The ambition to alter receptor specificity through domain engineering aligns with broader goals in plant synthetic biology aimed at reprogramming signaling pathways. However, the results in Tsitsikli *et al*. underscore the complexity of modularity in receptor kinases. Kinase domains, especially those involved in developmental signaling, exhibit tightly integrated structural dependencies that cannot be reprogrammed through isolated motif manipulation.

Synthetic biology approaches must therefore account for the interplay among ectodomain structure, transmembrane dynamics, kinase activation loops, JM regulation, and downstream scaffolding networks. Domain-swapping and motif transplantation may illuminate aspects of receptor function but will rarely suffice to redirect entire signaling cascades. The weak phenotypes elicited by SD1-containing CERK6 derivatives demonstrate the fragility of receptor modularity and the importance of systems-level context.

For synthetic approaches to succeed, receptor engineering must be paired with rewiring of downstream modules, including transcriptional regulators, nuclear calcium oscillation machinery, and developmental programs. The study inadvertently illustrates that isolated receptor manipulation is inadequate when network-level complexity governs functional outcomes.

10.5. Future Directions for Rational Engineering of Symbiotic Competence

Looking forward, strategies to engineer nitrogen-fixing symbiosis into non-legumes must shift from minimalistic receptor-centric designs to more integrated approaches. A rational engineering framework would require identification and transfer of the entire set of symbiotic genetic modules, including Nod factor perception, calcium signaling apparatus, cortical developmental regulators, and metabolic programs required for nitrogen fixation. Efforts in *Parasponia* illustrate that symbiosis evolves through duplication and neofunctionalization of multiple LysM receptors, along with the acquisition of specialized transcriptional networks. Dissecting and reconstituting these networks in cereals is a major challenge but is more congruent with evolutionary and physiological evidence than the minimal-mutational approach implied by the SD1 hypothesis.

The study's shortcomings provide important lessons. Weak phenotypes and overexpression artefacts must not guide engineering efforts. Instead, a combination of genome editing, multi-gene circuit design, and cross-species comparative biology will be required. Engineering viable symbiosis will demand integration of signaling, developmental, and metabolic systems rather than reliance on isolated mutations. In this broader perspective, the SD1 narrative, while conceptually intriguing, does not present a realistic model for transferring symbiotic functions across species. In summary, the implications for engineering drawn from Tsitsikli *et al*. are limited. The weak evidence for receptor reprogramming, combined with the complexity of symbiotic signaling, demonstrates that nitrogen-fixing trait engineering requires multidimensional strategies extending well beyond receptor motif modification.

11. Reproducibility and Data Integrity Considerations

11.1. Consistency and Transparency in Experimental Reporting

A credible demonstration that two amino acids can reprogram receptor specificity requires exceptional methodological transparency. However, the study by Tsitsikli *et al*.[4] frequently lacks the reporting detail necessary to evaluate reproducibility. Many constructs are described only schematically, without providing codon-level sequences, linker junctions, or explicit boundaries for domain swaps. These omissions limit the ability of independent laboratories to reconstruct the chimeric receptors accurately. Promoter usage is inconsistently reported, and the authors do not present quantitative data confirming that all constructs are expressed at comparable levels. Because receptor dosage is a major determinant of signaling

behavior, the absence of expression quantification severely undermines interpretability.

The transformation methodology also lacks sufficient detail. Hairy root systems inherently exhibit high variability, yet the study reports neither transformation efficiency nor the distribution of expression levels across roots within the same construct. Without this information, it is impossible to determine whether the rare positive phenotypes arise from high-expression outliers rather than genuine functional reconstitution. Imaging conditions are inconsistently described, with variations in exposure, magnification, and depth of field across Extended Data Figures and Supplementary Figures. This inconsistency complicates direct comparison of phenotypes across constructs and experiments. Collectively, these reporting limitations reduce confidence in the reproducibility of the study's findings.

11.2. Issues in Quantification, Replicates, and Statistical Robustness

A central challenge in evaluating the study's conclusions is the reliance on small sample sizes and variable experimental replicates. Many constructs are tested on as few as eight to twelve roots, a number insufficient to overcome the inherent variability of hairy root systems. Phenotypic outcomes such as root hair deformation, infection thread formation, and nodule number are presented without clear specification of the number of independent transformation batches. In several instances, the data distributions display large proportions of zero values with sporadic extreme values, suggesting the influence of stochastic variation rather than consistent biological function.

Statistical analyses rely on nonparametric tests but lack correction for multiple comparisons despite evaluating dozens of constructs across multiple phenotype categories. This creates a high risk of false positives. Effect sizes and confidence intervals are not reported, further limiting interpretation. In many graphs, outliers are visually emphasized while medians remain close to zero, yet the authors interpret these distributions as evidence of functional rescue. The absence of explicit replication studies compounds these limitations. Without repeating transformations across independent batches, the study cannot validate whether the observed phenotypes are reproducible or dependent on specific experimental conditions or insertion events.

11.3. Absence of Biochemical Validation and Mechanistic Grounding

Biochemical validation represents a crucial dimension of reproducibility, especially in a study claiming that two residues fundamentally alter receptor kinase signaling specificity. However, Tsitsikli *et al.*[4] provide no direct biochemical evidence demonstrating changes in kinase activity, substrate specificity, partner interaction, or phosphorylation patterns. The ATP-binding assays included in Extended Data Figure 6 confirm only that purified kinase domains bind ATP, an expected property shared by nearly all kinase-like proteins. The thermal shift assays are insufficiently replicated and do not reveal any functional impact of SD1 or its mutations. Without *in vitro* phosphorylation assays, autophosphorylation profiles, or targeted mass spectrometry demonstrating altered substrate binding, the mechanistic interpretation remains speculative.

The study also does not examine the formation of receptor complexes with NFR5 or CERK1-like partners. Immunoprecipitation or FRET–FLIM studies would have been essential to demonstrate that SD1, or the substituted residues, alter receptor pairing or downstream recruitment. In their absence, it remains unclear whether SD1 functions by modifying receptor interactions or whether the chimeric constructs simply produce artefactual activation through misregulation. The absence of biochemical data fundamentally limits reproducibility by preventing independent confirmation of the mechanistic basis underlying the reported phenotypes.

11.4. Limitations in Raw Data Availability and Imaging Reproducibility

High-quality reproducibility requires full availability of raw data, including unprocessed images, quantification scripts, and complete datasets for all replicates. In Tsitsikli *et al.*[4], raw fluorescence and bright-field images are not provided, preventing independent reanalysis. Many of the images presented appear selectively chosen and do not represent the full distribution of observed phenotypes. The absence of unedited images also prevents evaluation of signal-to-noise ratios, background artefacts, or potential misinterpretation of superficial bacterial adherence as infection threads.

Imaging reproducibility is further compromised by inconsistent acquisition parameters across panels. Differences in exposure settings, magnification, fluorescence intensities, and color scaling obscure whether constructs differ in function or simply in imaging conditions. The lack of standardized imaging protocols reduces confidence that comparisons between constructs are meaningful. Additionally, many panels lack scale bars, making interpretation of infection depth

and morphological features difficult. These issues collectively hinder data transparency and frustrate attempts by independent researchers to verify the findings.

11.5. Requirements for Future Reproducibility and Validation

To establish reproducibility and data integrity at a level commensurate with the claims made in Tsitsikli *et al*.[4], future studies must adopt a more rigorous methodological and reporting framework. First, complete sequence information, including codon-level detail for all chimeras, should be made publicly available. Second, receptor expression must be quantified through western blotting, quantitative fluorescence, or mass spectrometry to ensure that phenotypic differences are not artefacts of expression variability. Third, functional assays must incorporate sufficient sample sizes, independent biological replicates, and appropriate statistical corrections. Fourth, biochemical validation is essential, including kinase activity assays, substrate interaction profiling, complex formation studies, and phosphoproteomic analyses. These data are necessary to establish whether SD1 or the two substituted residues genuinely alter signaling specificity.

Finally, rigorous imaging standards must be implemented, with full raw data availability, consistent acquisition parameters, and comprehensive histological validation of infection and nodulation phenotypes. Without such improvements, claims of receptor reprogramming will remain difficult to reproduce and scientifically unsubstantiated.

12. Final Integrated Assessment

12.1. Summary of Strengths

Despite the substantial limitations detailed throughout this commentary, Tsitsikli *et al*. introduce an ambitious conceptual framework that attempts to map the structural and functional determinants governing the divergence between immune and symbiotic receptor kinases. Their experimental creativity, especially in designing a wide array of chimeric receptors spanning multiple species and receptor classes, demonstrates an admirable effort to probe signaling specificity. The attempt to identify minimal receptor features required for symbiosis addresses a long-standing question in plant–microbe evolution and synthetic biology. Additionally, the integration of structural biology, genetic complementation, and infection phenotyping reflects a multidisciplinary approach. The authors' willingness to explore JM motifs and to propose mechanistic hypotheses that connect specific residues to signaling outcomes represents an important conceptual step, even if the supporting evidence remains incomplete.

12.2. Summary of Weaknesses and Conceptual Risks

The study's weaknesses, however, outweigh its strengths when evaluating its central claims. The lack of rigorous biochemical validation undermines any assertion of mechanistic reprogramming. The weak and inconsistent phenotypes observed across constructs suggest that the SD1 motif and the two substituted residues do not reliably reproduce NFR1-like signaling. Imaging data frequently fail to distinguish superficial bacterial accumulations from genuine infection threads, and nodules induced by engineered receptors often resemble pseudonodules lacking internal infection. The structural analyses rely on inactive kinase conformations that cannot substantiate claims about signaling specificity. Statistical underpowering, small sample sizes, and reliance on hairy root variability further compromise interpretability. Collectively, these weaknesses render the overall narrative fragile and highly susceptible to overinterpretation. The conceptual risk is that minimal sequence differences are portrayed as sufficient to drive complex biological innovations without adequate mechanistic support.

12.3. Scientific Impact and Trajectory of the Field

The scientific impact of the study, regardless of the weaknesses of its claims, lies in prompting new discussions about the modularity and evolvability of receptor kinases. It challenges researchers to scrutinize the boundaries of receptor plasticity and encourages the development of creative genetic engineering tools. Nonetheless, the trajectory of the field must be guided by evidence more robust than that provided in this study. Root nodule symbiosis involves multiple layers of coordinated cellular events, co-evolved receptors, specialized transcriptional programs, and developmental circuits. Understanding how these layers interact will require integrative approaches combining biochemistry, structural modelling, evolutionary genomics, and whole-plant physiology. While minimal-mutation models make for compelling hypotheses, the field benefits more from studies that ground such hypotheses in reproducible, high-resolution data.

12.4. Recommendations for Future Work

Future research seeking to determine the minimal requirements for symbiotic signaling must adopt more rigorous standards. Comprehensive structural analyses should be performed in active kinase conformations, ideally supported by molecular dynamics simulations and substrate-binding studies. Genetic constructs should be validated at the sequence, expression, and folding levels, and should preferably be tested in stable transgenic lines rather than variable hairy root systems. Infection phenotypes must be supported by histological and physiological evidence, including assessment of cortical invasion, nodule zonation, bacterial load, and nitrogen-fixation capacity. Biochemical validation should include phosphorylation profiling

and interaction mapping with NFR5, SYMRK, and downstream signaling components. Evolutionary analyses should incorporate broader taxon sampling and co-evolutionary frameworks to evaluate whether specific motifs genuinely contribute to functional divergence. Such comprehensive datasets would allow the field to distinguish artefactual receptor activation from genuine mechanistic reprogramming.

12.5. Concluding Remarks

In its ambition, Tsitsikli *et al*. attempt to address a profound evolutionary and mechanistic question: how did legumes acquire the ability to recognize Nod factors and initiate symbiotic signaling? The notion that two residues within a short motif might govern this transition is conceptually appealing but scientifically fragile. The cumulative evidence presented—spanning weak phenotypes, selective imaging, incomplete structural insight, and missing biochemical validation—does not substantiate claims of receptor reprogramming. Rather than demonstrating a minimal mutational path to symbiosis, the study illustrates the complexity and resilience of signaling networks and the difficulty of reconstructing evolutionary innovations through isolated mutations. While the work stimulates provocative ideas, definitive conclusions require significantly stronger empirical foundations. A more integrative, mechanistically anchored approach will be essential for advancing our understanding of receptor evolution and for guiding realistic strategies in synthetic symbiosis and crop engineering.

Reference

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