A Critical Commentary on "*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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1. Introduction

The study by Tsitsikli *et al*.[1] presents the bold claim that two amino-acid changes in a juxtamembrane motif ("Symbiosis Determinant 1", SD1) can reprogram chitin-responsive CERK6-type immunity receptors into NFR1-type symbiotic receptors capable of initiating nodule organogenesis and intracellular rhizobial infection in *Lotus japonicus*. The mechanistic implications are significant: the work suggests a minimal mutational path by which legumes diversified LysM receptor kinases into immune versus symbiotic modules, and further implies that engineering these determinants could extend nitrogen-fixing capabilities to non-legume crops.

Despite its conceptual significance, the study exhibits substantial weaknesses in experimental logic, figure design, receptor nomenclature consistency, statistical rigor, and over-interpretation of subtle phenotypic outputs. Some central claims rely on low-penetrance phenotypes, confounded chimeric constructs, or indirect inferences rather than direct biochemical evidence of altered signalling.

Below, we provide a figure-by-figure critique, including panel-specific and methodological concerns.

2. Figure-by-Figure Critique

Figure 1 — Symbiosis Determinant 1 controls symbiosis with rhizobia

Figure 1 is presented as the anchor for the central discovery: the identification of a 23-residue juxtamembrane motif (SD1) required for symbiotic output. However, several conceptual and technical issues undermine the strength of the claim.

Figure 1a — UMAP of NFR1 and CERK6 expression in root cells

The UMAP visualization shows relative expression of NFR1 (red) and CERK6 (blue) in single-cell datasets. Several concerns emerge:

1. **Interpretation without statistical quantification.**
   The authors infer co-expression and infer "potential for pathway separation." Yet no metrics (co-expression index, Pearson correlation, cluster enrichment) are provided.
2. **UMAP instability.**
   UMAP is a non-deterministic manifold projection. Without reporting parameters or replicates, it is unclear whether the observed clustering is robust or an artefact.
3. **No direct relevance to SD1.**
   While visually appealing, this panel contributes little mechanistic insight and seems placed mainly to motivate receptor expression context.
4. **Absence of CERK6 protein-level verification.**
   mRNA co-expression does not confirm that both receptors are co-present at the PM, or in the same root hair at infection onset.

Thus, Figure 1a provides circumstantial, not causal, support.

Figure 1b — Nodule formation by NFR1–CERK6 chimeras

This panel is central, displaying the effects of 16 receptor configurations on nodule numbers. However:

1. Statistical weakness

- Many boxplots show extremely low biological penetrance (e.g., only 2–4 nodulating plants out of >30).
- The authors treat the mere presence of one nodule as evidence of "functionality."
- Kruskal–Wallis tests are performed, but multiple testing corrections are not applied, despite >16 constructs.

2. Chimera design is confounded

The chimeras couple differing numbers of residues from TM, JM, and kinase domains. However:

- Many constructs differ in more than one region, making attribution of phenotype shift to the SD1 motif ambiguous.
- Some receptors (e.g., 6) combine CERK6 kinase with partial NFR1 JM but still fail; authors interpret this as evidence of SD1's necessity, but alternative explanations exist (misfolding, wrong linker geometry, altered membrane insertion).

3. Expression variation not controlled

Although Supplementary Figures S2–S3 supposedly verify membrane localization, no data on receptor abundance or promoter strength heterogeneity is shown here.

4. Phenotypic scoring may be biased

“Pink” versus “white” nodules are counted together in multiple panels. Pink nodules indicate successful infection and nitrogen fixation; white nodules often represent aborted infections. Combining them artificially inflates “functional” phenotype.

Thus, the evidence that SD1 is “necessary” for symbiosis is not conclusively demonstrated.

Figure 1c — Representative transgenic root images

This panel attempts to visually confirm phenotypes, but:

- Images are low magnification; infection thread details are barely visible.
- No quantification accompanies morphological differences.
- Constructs 6 and 13 appear to differ dramatically, yet expression levels are not shown to be comparable.

More importantly, infection thread existence is shown qualitatively, with no enumeration of thread lengths, branching frequency, or penetration depth.

Figure 1d — Infection thread counts

This panel shows infection thread numbers per cm of root.

Problems:

1. **Considering infection thread count as proxy for signalling efficiency is simplistic.** Infection thread initiation and persistence depend on multiple downstream genes (*NIN*, *CYCLOPS&, *RINRK1*), none of which are controlled for.
2. **Thread scoring is done at 3 weeks**, long after typical infection thread formation windows (usually 3–7 days). At this time, many threads collapse or mature into cortical infection threads. This makes counts unreliable.
3. **Infection thread morphology is not quantified**, although the authors mention “branched and scrambled” root hairs.

Figure 1e — Root hair deformation and microcolony formation

Microscopy images show root hair curling and microcolonies.

Critique:

- The images lack scale consistency and appear selectively chosen.

- Root hair deformation can occur even with non-functional chitin receptor overexpression, due to ligand hypersensitivity.
- No negative controls (empty vectors with Nod factors, etc.) are shown at equivalent magnification.

Figure 1f — Nanobody-mediated receptor reconstitution

A clever idea, but:

Problem 1: Over-interpretation

The presence of a few spontaneous nodules in receptor 13 (SD1-containing CERK6 chimera) is taken as "reconstitution of signalling," but spontaneous nodulation is an inherently noisy phenotype influenced by cytokinin hypersensitivity, not solely LysM receptor activity.

Problem 2: Missing biochemistry

The authors do not demonstrate:

- Dimer formation between NFR5 and CERK6-derived constructs
- Signal phosphorylation events downstream of the engineered receptors

Thus, the claim that SD1 is "sufficient" is overstated.

Figure 2 — Crystal structures of LYK3 and CERK6 kinase domains

Figure 2 is used to justify a structural basis for SD1 functionality, but the conclusions are weaker than presented.

Figure 2a–b — Structures of LYK3 and CERK6

The authors crystallized:

- LYK3 kinase from *Medicago truncatula* (2.5 Å)
- CERK6 kinase from *Lotus japonicus* (1.85 Å)

Both structures include a DFG-inhibitory mutation (D460N or D459N) required for crystallization. This mutation forces a kinase-inactive conformation.

Critical issues:

1. **Inactive conformations do not represent signalling states**

The SD1 motif's orientation under active conditions cannot be inferred reliably, because:

- Juxtamembrane regions undergo major conformational rearrangements upon activation.

- Crystal structures capture a static “off” conformation.

2. **Absence of NFR1 kinase structure**

The authors use LYK3 as a surrogate because NFR1 would not crystallize.
This undermines the claim that the structure reveals NFR1-specific interactions.

3. **Low relevance of SD1 mapping**

Mapping SD1 differences onto CERK6 structure is speculative:

- The region is poorly resolved in CERK6 (T304 not visible).
- No interacting partners are shown.
- No structural perturbation is demonstrated between CERK6 and LYK3.

Thus, Figure 2 is mostly descriptive, not mechanistically explanatory.

Figure 2c — Structural overlay of LYK3 and CERK6

While aesthetically convincing, this overlay does not show meaningful SD1 divergence.
Only surface exposure is shown, not functional interfaces.

Figure 2d–e — Zoom on SD1

These panels illustrate surface exposure, but:

- No biochemical validation (mutagenesis combined with in vitro signalling assays)
- No evidence of SD1 binding partners

The structural analysis does not identify why SD1 mutations alter signalling. Therefore, the structural claims in Figure 2 are overinterpreted.

Figure 3 — N- and C-lobes of the kinases contain determinants for signalling specificity

Figure 3 attempts to broaden the scope beyond SD1 by suggesting additional kinase-surface determinants control immunity vs symbiosis.

Figure 3a — Nodule formation with various kinase swaps

This is one of the weakest parts:

1. **Extremely low nodule numbers**

Many constructs produce 0–3 nodules per plant, yet these are used to map functional surfaces. Such weak phenotypes cannot robustly define mechanistic regions.

2. **Redundancy is not rigorously tested**

The authors interpret that multiple surfaces act “collectively,” but they never test:

- Additive combinations
- Epistatic interactions
- Compensatory mutations

3. **Variable expression not controlled**

As earlier, no protein abundance quantification.

Figure 3b–c — ROS assays in CERK6 mutants

These panels claim that adding symbiotic residues to CERK6 does not impair immunity.

Critical concerns:

1. Missing replicates

ROS traces are shown but replicate number is unclear.

2. ROS readouts are extremely sensitive to promoter activity

Overexpression of receptors often induces ligand-independent ROS; this is not accounted for.

3. Only early immunity is measured

No MAPK activation, no transcriptional reporter assays. Thus, Figure 3 lacks signalling depth and employs overly simplistic readouts.

Figure 3d — Schematic model

A conceptual summary, but:

- The model is reductive and does not incorporate cooperativity between NFR1 and NFR5.
- It ignores downstream signalling hubs (SYMRK, CYCLOPS, CCaMK) that determine pathway bifurcation.

Figure 4 — Two residues of SD1 are essential for root nodule symbiosis

This figure makes the most provocative claim: two amino acids (T304M and D306A in CERK6) are sufficient to convert an immunity receptor into a symbiosis receptor. However, the evidence is problematic.

Figure 4a — Sequence alignment of SD1

The alignment highlights six variable residues across NFR1, CERK6, and RLK4.

Issues:

1. **Residues chosen appear cherry-picked**
   The authors do not define clear criteria for residue selection.

2. **No evolutionary analysis**

   - Are these residues under positive selection?
   - Are they conserved among all NFR1 orthologs?
   - Are analogous positions crucial in other LysM-RLK subclades?

Figure 4b — Single-residue substitutions weaken symbiosis

Single mutations reduce nodulation, but:

- The authors do not show whether the mutated receptors reach the plasma membrane normally.
- The reduction in nodulation may reflect dosage issues or destabilization, not signalling defects.

Figure 4c — Representative root images

Same issues as earlier:

- Only qualitative data
- Images chosen selectively
- Nodules not quantified by cross-section or bacterial load

Figure 4d — The "Two-residue rescue" experiment

This panel is central, showing that **T304M + D306A** in CERK6 restores nodulation. However:

1. The phenotype is weak

- Many plants still fail to nodulate (e.g., 12/12 → 0/10 versus 15/29 modest recovery).
- The "rescued" plants often produce only 1–2 nodules, far below wild-type levels.

2. Receptor abundance is not measured

A CERK6(T304M/D306A) receptor may accumulate at abnormally high levels, artificially promoting signalling.

3. No infection thread quantification

The constructs are judged solely by nodule count, ignoring early signalling defects.

4. No biochemical evidence of signalling

No phosphorylation assays, no NIN induction kinetics, no calcium spiking measurements. Thus, the bold claim is not sufficiently supported.

Figure 4e — RLK4 engineering

The claim that barley RLK4, a monocot receptor, can be engineered to support symbiosis is highly speculative.

- RLK4(T304M/D306A) induces only 1–2 nodules in a minority of plants.
- This is not evidence of a functional symbiotic program; legumes can form pseudo-nodules under hormonal perturbation.
- The ROS phenotype is abolished, but the downstream pathway (infection vs organogenesis) is not dissected.

Overall, Figure 4 does not convincingly demonstrate that two residues “reprogram” a receptor; instead, they modulate a weak, partially functional chimeric signalling environment.

3. Critical Re-evaluation of the Extended Data and Supplementary Figures

The Extended Data (ED) and Supplementary Figures are intended to provide mechanistic reinforcement beyond the main Figures 1–4. However, upon close inspection, many of these ED and Supplementary data sets reveal substantial weaknesses in experimental design, data quantification, construct validation, and interpretation. In several cases, the ED figures contradict claims made in the main text, or provide evidence far weaker than asserted.

Extended Data Figure 1 — Root hair and epidermal infection phenotypes

This figure presents root hair deformation, infection thread initiation, and NIN promoter activity across constructs 1–16.

1. Lack of uniform imaging conditions

The panels show inconsistent lighting, scale bars, and magnification. Some images are visibly oversaturated (especially YFP channels), while others appear faint. This variability makes comparison unreliable.

2. Highly selective imaging

Across constructs 1–16, only a single representative root is shown. Given the extreme variability of infection thread initiation, especially in *nfr1* backgrounds, single images cannot support claims of "functional rescue."

3. Misleading comparison to empty vector controls

In several constructs (e.g., 5, 6, 9, 10), weak root hair deformation occurs but no infection threads. The authors interpret this as "partial signalling," but similar deformation can arise from:

- mild stress
- transient Nod factor contamination
- overexpression artefacts

Without time-course quantification, root hair curling is not a reliable phenotype.

4. NIN:GUS activity inconsistently reported

Some constructs show faint GUS staining that may stem from background activity rather than receptor-driven signaling. No negative staining controls (e.g., *nin* mutants) are presented.

Conclusion: ED1 does not convincingly establish infection phenotypes for the majority of constructs and relies heavily on anecdotal images.

Extended Data Figure 2 — Quantification of infection events

ED2 attempts to quantify infection events (organised into "infection threads" vs. "arrested microcolonies") for constructs 1, 2, 6, and 18.

1. The boxplots are extremely noisy

Even "functional" constructs (1 and 2) exhibit wide variance. Construct 2 shows roots with zero events alongside roots with many events.

2. Small sample size

Most constructs involve n = 8–14 roots — too low for the high biological noise of infection assays.

3. Categorization is ambiguous

The categories "arrested" vs. "infection threads" often overlap morphologically. The figure does not indicate:

- scoring criteria

- depth of penetration
- presence of bacteria beyond the epidermis

4. Discrepancy with main Figure 1

Construct 6, described in the main text as “nonfunctional,” appears to produce several infection events here, contradicting the supposed necessity of SD1.

Conclusion: ED2 quantification is insufficiently robust and undermines the binary functional/nonfunctional claims.

Extended Data Figure 3 — Transient expression and localization in *Nicotiana benthamiana*

This figure addresses receptor localization using receptor-YFP and AtPIP2-mCherry.

1. Overexpression in tobacco does not model endogenous localization

Transient overexpression saturates the secretory pathway, producing artefactual PM localization even for misfolded or nonfunctional receptors.

2. No quantitative colocalization analysis

Colocalization appears visual, not analytical. Ideally, Pearson’s R or Manders coefficients should be computed.

3. Absence of ER retention markers

Many constructs may misfold, yet ER retention (a failure mode consistent with functional deficiencies) is not tested.

4. Plasma membrane enrichment appears identical across constructs

This undermines claims that “nonfunctional” constructs fail due to mislocalization. Paradoxically, even constructs 6, 9, and RLK4 variants show strong PM localization.

Conclusion: ED3 does not support claims about receptor localization affecting function.

Extended Data Figure 4 — Localization in *Lotus* protoplasts

This figure attempts to validate localization in the endogenous plant system.

Major weaknesses:

1. Protoplasts lack polarized membrane trafficking

Localization in protoplasts does not accurately represent root hair PM targeting, which depends on:

- cytoskeletal trafficking
- lipid microdomains
- cell wall anchoring

2. No side-by-side comparison of wild-type NFR1

Without a WT comparator, the relative localization of chimeric receptors cannot be evaluated.

3. Overexpression again obscures functionality

High expression yields PM signal regardless of correct folding.

4. Unclear construct stability

The figure does not show whether receptors accumulate at different levels. Unequal fluorescence intensity can mask degradation or misprocessing.

Conclusion: ED4 largely repeats the limitations of ED3 without resolving localization concerns.

Extended Data Figure 5 — Structural sequence alignment and homology analysis

This ED figure shows SD1 alignment across various LysM receptors.

1. Alignment appears curated rather than comprehensive

Several well-characterized NFR and CERK homologues are omitted. No phylogenetic tree accompanies the alignment to validate clustering.

2. Conservation metric lacks methodology

The “high–low conservation” heatmap does not specify:

- the scoring scheme
- evolutionary model
- gap penalties

3. Residue choice appears post-hoc

The residues highlighted as “determinants” are chosen based on known function, not unbiased analysis.

4. Lack of structural validation

No modelling shows how divergent residues alter surface properties or interact with NFR5.

Conclusion: ED5 provides only correlative evolutionary data and cannot substantiate the functional role of SD1.

Extended Data Figure 6 — Purification and ATP-binding assays of kinase domains

This figure includes size-exclusion chromatography (SEC), SDS-PAGE, and thermal shift assays.

1. Incomplete folding assessment

SEC curves show elution peaks, but:

- no evidence of monodispersity
- no dynamic light scattering (DLS)
- no circular dichroism (CD) spectra

Thus, the proteins may not be properly folded.

2. Thermal shift assays lack replicates

Only single curves are shown. No indication of Tm for each condition ± ATP/Mg.

3. ATP binding does not equal catalytic activity

The authors show binding but not autophosphorylation or substrate phosphorylation.

4. Missing control for kinase-dead DFG mutants

Since NFR1/LYK3 were crystallized as DFG mutants, ATP binding could be artefactual.

Conclusion: ED6 fails to provide biochemical evidence that SD1 mutations alter kinase behaviour.

Extended Data Figure 7 — Surface mapping of divergent regions (A–G) and functional rescue

This figure attempts to rationalize which kinase regions contribute to symbiosis.

1. Region selection appears arbitrary

Surfaces A–G are visually mapped but lack quantitative criteria.

2. Structural mapping does not correspond to functional rescue

For example:

- Region E (αC helix) shows minimal impact in isolation.

- Receptor 21 (N-terminal CERK6, C-terminal NFR1) fails despite predicted functional relevance.

3. Nodule counts remain low

Receptor 20 and 22 produce few nodules, often only 1–3 per plant, questioning whether these represent true rescue.

4. Infection thread phenotypes inconsistent with nodule counts

In several cases, infection thread abundance does not correlate with nodule production, suggesting unrelated or partial signalling.

Conclusion: ED7 does not convincingly support modular contributions of kinase surfaces and raises concerns about interpretational bias.

Extended Data Figure 8 — RLK4 engineering and symbiotic phenotypes

ED8 is intended to demonstrate that SD1 engineering enables barley RLK4 to function in legumes.

1. ROS data (panel a) are inconclusive

RLK4 and its variants show wide variance in ROS responses. However:

- ROS amplitude depends on expression level
- RLK4's intrinsic signalling in *Lotus* is unknown
- No internal control (e.g., CERK6 in same experiment) is shown

2. NIN activation (panel b–c) inconsistent

Construct 37 activates cortical NIN in 7/15 roots, but:

- NIN activation may not always lead to nodules
- Cortical NIN may reflect cytokinin hypersignalling, not symbiosis

3. Infection events are poorly characterized

Images show only superficial staining; cortical penetration is unclear.

4. Nodules in panel d appear pseudonodules

The nodules formed by RLK4 constructs:

- are small
- show limited red fluorescence from *M. loti*
- lack the morphology typical of functional nodules

Conclusion: ED8 does not convincingly show that RLK4 can be converted into a bona fide NFR1 analog.

4. Supplementary Figures (from ESM PDF)

Supplementary Figure 1 — GUS staining and nodule sectioning

This figure shows internal structure of nodules formed by chimeric receptors.

Critical issues:

1. Several nodules lack clear infected zones (zone II), indicating incomplete infection.
2. Vascular patterns appear disorganized, suggesting pseudonodule identity.
3. No longitudinal sections are provided to verify infection thread continuity.
4. The authors do not provide comparison with WT NFR1 nodules.

Conclusion: S1 reveals developmental irregularities inconsistent with fully functional symbiosis.

Supplementary Figure 2 — Additional localization and expression validation

This figure attempts to support PM localization of engineered receptors.

Problems:

1. Fluorescence intensity varies widely, again indicating inconsistent expression.
2. No western blots confirm equal expression levels across constructs.
3. PM localization does not imply correct receptor activation or complex formation with NFR5.
4. Cytoplasmic and vesicular signals are visible in several constructs, suggesting trafficking defects.

Conclusion: S2 does not provide robust evidence that engineered receptors are properly processed.

Overall Assessment for Extended Data and Supplementary Figures

The Extended Data and Supplementary Figures were intended to reinforce the manuscript's central claims that a small juxtamembrane motif (SD1) and two amino acids can reprogram CERK-type immunity receptors into symbiotic receptors. However, a careful evaluation shows that most Extended Data figures:

- lack quantitative rigor

- show inconsistent phenotypes
- rely on small sample sizes
- present unconvincing or anecdotal images
- fail to control for expression levels
- do not provide biochemical evidence
- sometimes contradict the main figures

The Supplementary Figures further demonstrate structural abnormalities, inconsistent infection patterns, and possible pseudonodule formation in engineered constructs.

Overall, the Extended Data do not substantiate transformative claims about minimal evolutionary mutational steps enabling nitrogen-fixing symbiosis. Instead, they reveal a fragile, partial, and highly variable signalling response that likely reflects overexpression artefacts, weak receptor compatibility, and developmental misregulation rather than true pathway reprogramming.

5. Overarching Methodological Concerns

1. Lack of biochemical mechanistic validation

The authors make molecular claims but never show:

- Autophosphorylation patterns
- Partner binding assays (e.g., NFR5 interactions)
- Kinase activity differences between variants
- Downstream signalling flux (MAPKs, calcium signatures)

2. Inadequate genetic controls

The study would be stronger with:

- Complementation in *nfr1/nfr5* double mutants
- Reconstitution in heterologous species
- Dominant-negative analyses

3. Statistical analysis is underpowered

- Few roots per construct
- Low nodulation penetrance
- No correction for multiple comparisons
- High biological variability unaccounted for

4. No computational modelling

The structural claims about SD1 would benefit from:

- MD simulations
- Surface electrostatics
- Docking analysis with known interactors

6. Conclusions

Tsitsikli *et al*. present intriguing experiments that attempt to pinpoint minimal determinants of symbiotic vs immune signalling in legume LysM receptor kinases. The discovery of the SD1 motif is conceptually interesting; however, the evidence provided does not justify the paper's strongest claims, particularly:

- That SD1 is necessary and sufficient for symbiotic signalling
- That two residues can convert immunity receptors to symbiosis receptors
- That CERK6 or RLK4 can be rationally engineered for nitrogen-fixing symbiosis

The phenotypes demonstrated are weak, inconsistent, and poorly quantified, and mechanistic insights remain speculative. The structural data, while elegantly presented, do not resolve how SD1 controls receptor output.

Nonetheless, the study raises important hypotheses that may drive future work on rational engineering of NFR-type receptors. A more rigorous suite of biochemical, genetic, evolutionary, and structural analyses will be required to convincingly demonstrate minimal mutational steps enabling symbiosis evolution.

Reference

1 Tsitsikli, M. *et al.* Two residues reprogram immunity receptors for nitrogen-fixing symbiosis. *Nature* (2025). https://doi.org/10.1038/s41586-025-09696-3