Two residues reprogram immunity receptor kinases to signal in nitrogen-fixing symbiosis

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Abstract

Receptor signaling shapes cellular functions and specificity in the signaling output is central for delineating biological processes. In legume root cells, very similar and structurally conserved chitin and Nod factor receptor kinases activate immunity, or symbiosis, respectively after perception of chitinous ligands. Here we show that individual residues in the intracellular part of the Nod factor receptor NFR1 determine signal specificity and efficiency distinguishing immunity and symbiotic signaling. Functional investigation of CERK6, NFR1 and receptor variants hereof revealed a conserved motif that we term Symbiosis Determinant 1 in the juxtamembrane region of the kinase domain that is key for symbiotic signaling. We demonstrate that two residues in Symbiosis Determinant 1 are indispensable hallmarks for NFR1 receptors and are sufficient to convert *Lotus* CERK6 and barley RLK4 functioning in immunity, to initiate symbiotic signaling.

Main

Receptor kinases initiate and regulate diverse signaling events in eukaryotic cells in response to extracellular signals[1,2]. Evolution has established versatility and diversified their extracellular, ligand-binding domains while retaining the coupling to intracellular kinases to maintain the catalytic function[3-5]. Kinases share structurally similar domains but differ greatly in their determinants of specificity mediating the activation of specific downstream signaling pathways[6]. Identification or prediction of determinants of specificity has proven a challenge. In plants, Chitin Elicitor Receptor Kinases (CERK) recognize chitin molecules and initiate an immune response[7-9], and facilitate the symbiosis with arbuscular mycorrhizal fungi[10-15]. Legume plants have evolved an additional, but highly similar NFR1 receptor to perceive decorated chitin ligands, the lipochitooligosaccharides called Nod factors produced by symbiotic bacteria, to enable nitrogen-fixing symbiosis[16,17]. Studies based on receptor mutants determined that in legumes, the chitin-triggered immunity and nitrogen-fixing symbiosis are genetically and functionally separated[7,11] even though both receptors can be expressed in the same cell as illustrated by NFR1 and CERK6 transcripts detected in the same root cells[18] (Fig. 1a). This shows that in legumes the two functions are separated and suggests that the receptors themselves provide signaling specificity. Therefore, LysM receptor kinases from legumes offer an attractive test system for identifying determinants of specificity to understand how similar kinases control divergent signaling pathways.

Previous studies of CERK6 and NFR1 from *Lotus japonicus* (*Lotus*), and LYK3 (the NFR1 homolog) from *Medicago truncatula* (*Medicago*) identified that their ligand affinity and signaling ability depends on distinct regions present in their ectodomains distinguishing chitin and Nod factors[5]. These studies also found that while the ectodomains are in place to recognize the correct extracellular signal, the nature of the cellular pathways is dependent on residues present in the intracellular regions of NFR1 and CERK6 receptors. The CERK6 kinase domain can activate symbiosis only in the presence of the transmembrane-juxtamembrane (TM-JM) region from NFR1. The NFR1 kinase, on the other hand, is not able to activate immune related ROS production after chitin exposure irrespective of the origin of the TM-JM region[5]. Collectively, this indicated that root nodule symbiosis is controlled independently by specific residues or regions present in the TM-JM region or in the kinase of NFR1, while chitin immunity is controlled by those present in the CERK6 kinase. The regions in the intracellular part of NFR1 and CERK6 that control this divergent signal output are currently unknown. To discover the specificity determining regions, we performed a detailed functional investigation of chimeric NFR1-CERK6 receptors. Crystal structures informed the design of receptors, and their expression in Nod factor insensitive *nfr1* or in chitin insensitive *cerk6* loss-of-function mutant plants was ensured from native promoters. This identified key regions and residues that control signaling specificity and

determined that engineering symbiotic functionalities into chitin receptors from legume and non-legume plants is possible.

Symbiosis Determinant 1 defines the signaling specificity of NFR1

Residues present in the transmembrane and juxtamembrane regions have been identified to play distinct regulatory roles for the core kinases of single-pass receptors[19,20]. We aimed to identify residues controlling the specificity of signaling for nitrogen-fixing symbiosis present in these regions of NFR1[5]. For this, we created a series of fifteen receptor variants where the NFR1 ectodomain was coupled to the CERK6 core kinase via different configurations of the transmembrane (TM) and juxtamembrane (JM) (Fig. 1b, c, receptors 2-16) and investigated their function *in vivo.* The first results confirmed that the CERK6 kinase could initiate signaling for root nodule formation and infection only when preceded by the TM-JM of NFR1 (Fig. 1b, receptor 2), but not by the TM-JM of CERK6 (Fig. 1b, receptor 3)[5]. A region spanning 23 residues located in the C-terminal part of the JM adjacent to the kinase domain was found to be critical for the symbiotic proficiency of these receptors (Fig. 1b, c). All variants with the NFR1 version of this region (Fig. 1b, c, Fig. S1, receptors 11-16) activated the expression of the early symbiotic marker *Nin*[21] and led to the formation of infected root nodules (Fig. 1b, c). By contrast, all receptors with the corresponding version of CERK6 were unable to reconstitute symbiosis in *nfr1* irrespective of the origin of the remaining regions of the JM domain (Fig. 1b, c, receptors 3-10). We named this region of NFR1 the Symbiosis Determinant 1 (SD1). To verify the protein integrity, we expressed the receptor variants in tobacco leaves and found them to be plasma membrane localized, indicating that receptors which are symbiotically inactive, are expressed and correctly localized (Fig. S2). Next, we analyzed rhizobial infection in more detail by inspecting the root hairs of *nfr1* expressing the three receptor variants (receptors 1, 2, and 6) analyzed for nodule organogenesis. Roots expressing *NFR1* had typical root hair curling, bacteria entrapment, and long, elongated infection threads (Fig. 1d, e). Such elongated infection threads were also identified in roots expressing the symbiotically functional receptor 2 (Fig. 1b), albeit in a significantly reduced number, while none were detected for the symbiotically nonfunctional receptor 6 (Fig. 1b, d, e). Importantly, roots expressing receptors 2 and 6 responded to the presence of bacteria with root hair deformation and microcolony formation even when intracellular root hair infection was not enabled. This shows that receptor 6 recognizing the Nod factors via NFR1 ectodomain initiates a very limited response in the *nfr1* mutant, but that key events leading to symbiosis like the intracellular infection, *Nin* promoter activation and nodule organogenesis, fail to be activated. The lack of symbiotic functionality for this receptor was further confirmed in an independent experiment where synthetic coupling of receptor

6 to NFR5 using the nanobody-mediated receptor complex formation strategy[22] showed inability to induce spontaneous nodule formation (Fig. 1f). Together, our studies demonstrate that SD1 controls root nodule symbiosis ensuring activation of both organogenesis and root hair infection.

The region containing SD1 is a surface exposed motif in the kinase domain

The region containing SD1 has an amino acid composition that distinctly separates Nod factor receptors from chitin-binding counterparts and other members of this protein family in legume plants (Fig. S3). To understand the molecular details of SD1, we expressed and purified the intracellular domains of CERK6 and NFR1 (Fig. S4) for structural investigation. The purified proteins were functional and able to bind ATP (Fig. S5). We obtained well-diffracting crystals for CERK6 with an inhibitory mutation, D460N, in the DFG motif. This was not the case for NFR1 and to obtain crystals of a symbiotic kinase we turned to the NFR1 orthologue from *Medicago*, LYK3, which crystalized with the same inhibitory mutation, D459N, as CERK6 (Fig. S4). The atomic structures of CERK6 and LYK3 kinases were determined to 1.85 Å and 2.5 Å resolution, respectively. Both LYK3 and CERK6 showed a classical fold with clearly defined N- and C-lobes and hallmark structural features of active kinases (Fig. 2a, b). LYK3 and CERK6 are both crystallized in an inactive conformation closely resembling the CDK2/Src inactive kinase conformation, which is an autoinhibition state of the kinase, where the αC is in an "out"-position, while the aspartate of the DFG motif points towards the nucleotide binding side. LYK3, but not CERK6, was crystallized with a bound ATP analogue (Fig. S6A), however despite this, the two structures show an overall high similarity (RMSD=0.724 Å) (Fig. S6B). The SD1 region is clearly defined in the density of the LYK3 structure as a loop followed by the αB helix, which is a conserved feature of IRAK/Pelle-type kinases. SD1 is kept in place by specific residues that are conserved between chitin and Nod factor receptors and stabilizing the interactions to the N-lobe of the kinase (Fig. 2c). SD1 contains only six residues differing between CERK6 and NFR1, three of these are in the N-terminal loop, while the remaining three are on the αB helix. All six defining residues in SD1 are surface exposed making this motif accessible for interactions (Fig. 2d, e).

The core kinase domains are important for the two diverging signaling pathways

Using the available structural information, we aimed to identify which regions or residues in the core kinases control the specificity of the two signaling pathways. This revealed that the differing residues between CERK6 and NFR1 were dispersed across both the N- and C-lobes and were primarily surface-localized (Fig. S7), likely to facilitate protein-protein interactions. Knowing that core kinases of NFR1

and CERK6 can determine whether symbiosis or immunity is initiated, independent of the TM-JM regions, indicates that the diverging surface-localized residues contribute to signaling specificity. To identify contributing surfaces we investigated which residues of the NFR1 core kinase differing from CERK6 are able to drive symbiosis independent of SD1. We identified seven clusters (A to G) of residues with divergent residues defining potential interaction surfaces (Fig. S7, S8). These were exchanged individually from CERK6 to NFR1 in receptor 6, which was symbiotically nonfunctional (Fig. 1b, Fig. S8). None of the investigated surfaces alone was able to activate root nodule formation in *nfr1*, indicating a possible collective contribution (Fig. S8). We next tested if their combined differences might be a limiting factor for symbiosis establishment (Fig. 3c). Receptor 20, where both the αC helix (E) and the activation loop (A) of CERK6 have been exchanged to NFR1 (Fig. 3a), was found indeed able to induce nodule formation on *nfr1*, albeit with very low efficiency, when compared to functional receptor 18 containing the entire NFR1 kinase or the full-length NFR1. This provides further indications that multiple surfaces of the core NFR1 kinase contribute to symbiosis signaling. Therefore, we tested two variants where the N or C-terminal region of CERK6 was exchanged to NFR1 in receptor 6 (Fig. 3a, Fig. S8) without a negative impact on protein folding and ATP binding activities (Fig. S5). Receptor 22 which has the N-terminal region of CERK6 and C-terminal of NFR1, but not the opposite variant, receptor 21 (N-terminal region of NFR1 and C-terminal of CERK6), ensured the formation of functional nodules more efficiently than receptor 20 where only the αC helix and the activation loop of CERK6 have been exchanged to NFR1 (Fig. 3a). The symbiotically functional receptor 22 contains 45 residues diverging from CERK6 and they are scattered on several surface areas (Fig. S8). This shows that residues from the C-terminal region of NFR1 corroborate to create a larger or a particularly well-defined surface enabling symbiosis.

NFR1 core kinase lost its ability to activate immunity[5], and our findings show that residues in its C-terminal region have diverged from CERK6 to enable symbiosis (Fig. 3d). This raises the question of whether the specificity for symbiosis evolved in the core kinase at the expense of immune signaling. We investigated this possibility by exchanging the C-terminal region of the full-length CERK6 with the symbiosis-permissive region of NFR1. This receptor version expressed in the *cerk6* mutant from *Cerk6* promoter was able to induce the production of reactive oxygen species (ROS) after chitin treatment (Fig. 3c, receptor 26), indicating no negative interference from these NFR1 residues on this signaling pathway. By contrast, receptor 25 containing the N-terminal of NFR1 core kinase failed to complement the mutant phenotype (Fig. 3c) indicating that the N-terminal region of CERK6 contains determinants for immune signaling. The C-terminal region of the NFR1 core kinase acts independently of SD1 in activating symbiosis (Fig. 3a, receptor 22), and importantly, it does not interfere in immunity. Therefore, we asked if the SD1 in NFR1 may affect the immune signaling from the CERK6 core kinase.

We exchanged the corresponding region with the NFR1 variant within the full-length CERK6 (Fig. 3b, receptor 23) and found that this receptor variant 23 also induced production of ROS after chitin treatment when expressed in *cerk6* mutant. Together, these results demonstrates that neither of the two regions found here to be determinants for symbiosis have a negative impact on immunity and that the functionality from the intracellular region of CERK6 or NFR1 can be independently modulated by residues located in distinct regions.

Specific residues in Symbiosis Determinant 1 coordinate organogenesis and infection

All land plants contain CERK receptors activating immunity, but only legumes contain NFR1-type receptors activating root nodule symbiosis[23-25]. The identification of two distinct regions in NFR1 extending the molecular functions of CERK6 prompted us to investigate if this can be employed for engineering of CERKs. The C-terminus of NFR1 core kinase contains 45 residues diverging from CERK6 and our extensive analysis of receptor versions revealed a synergetic action of these variable residues (Fig. 3a). On the other hand, the SD1 contains only 6 out of 23 residues that differ from CERK6 (Fig. 4a, Fig. S3) and are thus more amenable for engineering. Therefore, we asked if all defining residues of SD1 are crucial for the symbiotic program. We examined variants of the symbiotically active receptor 2 where individual variable residues of SD1 were exchanged from NFR1 to CERK6. (Fig. 1a, 4b). All these variants retained the ability to induce nodule formation after inoculation with *M. loti*, indicating a synergetic contribution to signaling. However, closer inspection of the phenotypes revealed residue-specific variations (Fig. 4b, c, Fig. S9). Receptor 27 (M306T) ensured the formation of both infected and white nodules albeit on fewer plants (18 out of 24) and in a significantly reduced number compared to receptor 2 (29 out of 30). This indicates an impact of M306 on the symbiotic signaling efficiency (Fig. 4b, c). Receptor 28 (A308D) had the lowest symbiotic capacity. Only 15 of the 40 *nfr1* roots expressing this receptor formed infected nodules, a significantly reduced number when compared to those expressing receptor 2 (Fig. 4b, c, Fig. S9). This shows that A308 in SD1 is critical for both symbiotic processes. Nodule organogenesis and rhizobial infection are well coordinated processes in symbiosis and the phenotypes observed in receptors 27 and 28 imply that variable residues of SD1 may not only control the initiation of symbiotic signaling, but they may also coordinate organogenesis and infection. To investigate this, we inspected the transgenic roots in more detail for the expression pattern of the *Nin* promoter to have a better insight on the early signaling events. It is well-established in root nodule symbiosis that primordia arrest if the infection is impaired[26-29]. The *nfr1* roots expressing receptors 28 (A308D), 30 (Q316D) and 31 (K320T) showed a significantly larger number of nodule primordia initiated but arrested inside the roots before emergence (Fig. S9). Thus,

the large number of arrested primordia initiated by these receptors indicates that signaling from NFR1 via specific residues in SD1 is key for coordination of organogenesis and infection.

Two residues reprogram chitin receptors to signal in symbiosis

Knowing that a synergetic action of the residues from SD1 is needed we asked whether minimal engineering could be achievable for rerouting the signaling from the intracellular region of CERK6 for root nodule symbiosis. As a proof-of-concept we targeted the symbiotically nonfunctional receptor 6 where the entire intracellular region is derived from CERK6 (Fig. 1b, c). Changing individual residues in SD1 did not reconstitute symbiosis of the *nfr1* mutant (Fig. S10) but the T306M D308A substitution was sufficient to complement nodule formation and rhizobial infection (Fig. 4d, receptor 33) demonstrating that these two residues are the main drivers of specificity for symbiotic signaling. One additional change, T318K, significantly improved the efficiency of the symbiotic signaling (Fig. 4d, receptor 34). Next, we attempted to engineer RLK4, the closest receptor of NFR1 and CERK6 in barley[22]. The kinase of RLK4 is, like CERK6, able to induce immunity by producing ROS in *cerk6* upon chitin treatment (Fig. S11), but unlike CERK6 it has a very limited symbiotic signaling capacity when coupled to the ectodomain of NFR1. Two out of 17 *nfr1* roots expressing receptor 36 with the intracellular region of RLK4 produced 1 and 2 nodules, respectively (Fig. 4e). Nonetheless, the residues within the region of SD1 are conserved between CERK6 and RLK4 (Fig. 4a). Therefore, we asked if engineering this region in the cereal receptor will have a similar impact on symbiosis signaling. We created an engineered receptor 37 where the intracellular RLK4 contained SD1. This receptor, like receptor 13 (SD1 engineered in CERK6), was also able to efficiently initiate root nodule formation and rhizobial infection when expressed in the *nfr1* mutant (Fig. 4e, receptor 37). Together, these results demonstrate that SD1 provides both *Lotus* CERK6 and barley RLK4 with symbiotic signaling capacity leading to root nodule organogenesis and intracellular bacterial infection.

Discussion

Detecting specific signals present on the cell surface and responding with the right internal signaling pathway is important for all life forms. Receptor kinases play a major role in eukaryotes in relaying the correct information in the cell, and despite an impressive structural conservation[2,6,30-32], little is known about how signaling specificity is ensured in plants. Here we investigated the mechanism enabling NFR1 and CERK6 to initiate symbiosis or immune signaling pathways. NFRs evolved as homologs of CERKs and became neofunctionalized in legumes to enable root nodule organogenesis and

intracellular infection of nitrogen-fixing bacteria[17,24,33] (Fig. 3d). Thus, it is conceivable that specific residues have diverged from CERK receptors to enable root nodule symbiosis. We demonstrate here that two regions, the SD1 and the C-terminal region of NFR1, contain residues that determine symbiosis signaling. These regions can activate symbiosis independently since the necessity for SD1 can be overcome by NFR1 residues present in the C-terminal region of the kinase and vice versa. Interestingly, an intracellular conformation where the C-terminus is of NFR1-type can serve a dual function, initiating both symbiosis and immunity, depending on the type of the ectodomain. This is reminiscent to a certain extent to the functionality of intracellular region of barley RLK4 which was able, albeit with a very low efficiency, to induce root nodule formation, and to a larger extent to the reported signaling capabilities of receptors from *Parasponia andersonii* (Parasponia), the only plant forming root nodules with rhizobia outside of the legume clade[24]. Parasponia receptors LYK1, LYK3a and LYK3b can function in both symbiosis and chitin immunity[24]. These receptors lack the SD1 signature present in all NFR1-type proteins (Fig. S3) from legume species. The SD1 is a specific signature of Nod factor receptors from legumes, and we show here that it is important for initiation and coordination of root nodule organogenesis and root hair infection which in legumes runs very efficiently. It is thus conceivable that the evolution and full maintenance of SD1 in the legume clade could have contributed to the evolution of a very efficient symbiosis in Papilionoids[34].

The residues in SD1 are surface exposed (Fig. 4a), showing that this motif is accessible for interactions. Detailed inspection of CERK6 and LYK3 crystal structures revealed no evidence of intra-molecular interactions between variable residues and known regulatory regions of the core kinase that could explain a direct control over signaling. However, kinases have a dynamic structure[32,35,36] and both intra- and intermolecular interactions between protomers or with interacting partners i.e. NFR5 may occur once the compatible ligand is bound by the ectodomains. The necessity of a large surface in the C-terminal region of NFR1 kinase corroborates with a scenario whereby multiple surface-exposed residues contribute to symbiotic signaling possibly by creating more stable protein-protein interaction interfaces or for enabling dynamic interactions with multiple partners, which currently are unknown.

Minimal changes were identified here that enabled chitin receptors, CERK6 from *Lotus* and RLK4 from barley, to signal in root nodule symbiosis. Changing two residues, T304M and D306A, was sufficient for CERK6 kinase to induce formation of nodules and root hair infection threads (Fig. 4d, e). The symbiotic proficiency was improved by changing T318 K and NFR1-levels of nodulation were reached when the entire SD1 was present (Fig. 1b and Fig. 4e). Importantly, our detailed investigations revealed that engineering the intracellular regions of chitin receptors extends their functional capacities and that both symbiotic and immune functionalities can be controlled from a singular version of the intracellular domain. Together with the opportunity of minimal alterations in native

receptors, these are solid approaches in the efforts of engineering root nodule symbiosis in non-legumes[37].

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Competing interests

Aarhus University has filed a provisional patent application authored by SR, KRA, MT, BWS, CS, CK, MML, DL, SBH, KG, on using these findings for engineering LysM receptor kinases. The other authors declare no competing interests.

Author contributions:

Conceptualization: SR, KRA,

Methodology: SR, KRA, KG, CK, DL, MT, BWS, SH

Investigation: MT, BWS, CGA, MML, HR, TBL, GK, SH.

Visualization: MT, BWS, KRA, KG

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Supervision: SR, KRA, KG, CK, DL

Writing – original draft: SR

Writing – review & editing: MT, BWS, CGA, MML, KG, KRA.

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FIGURES

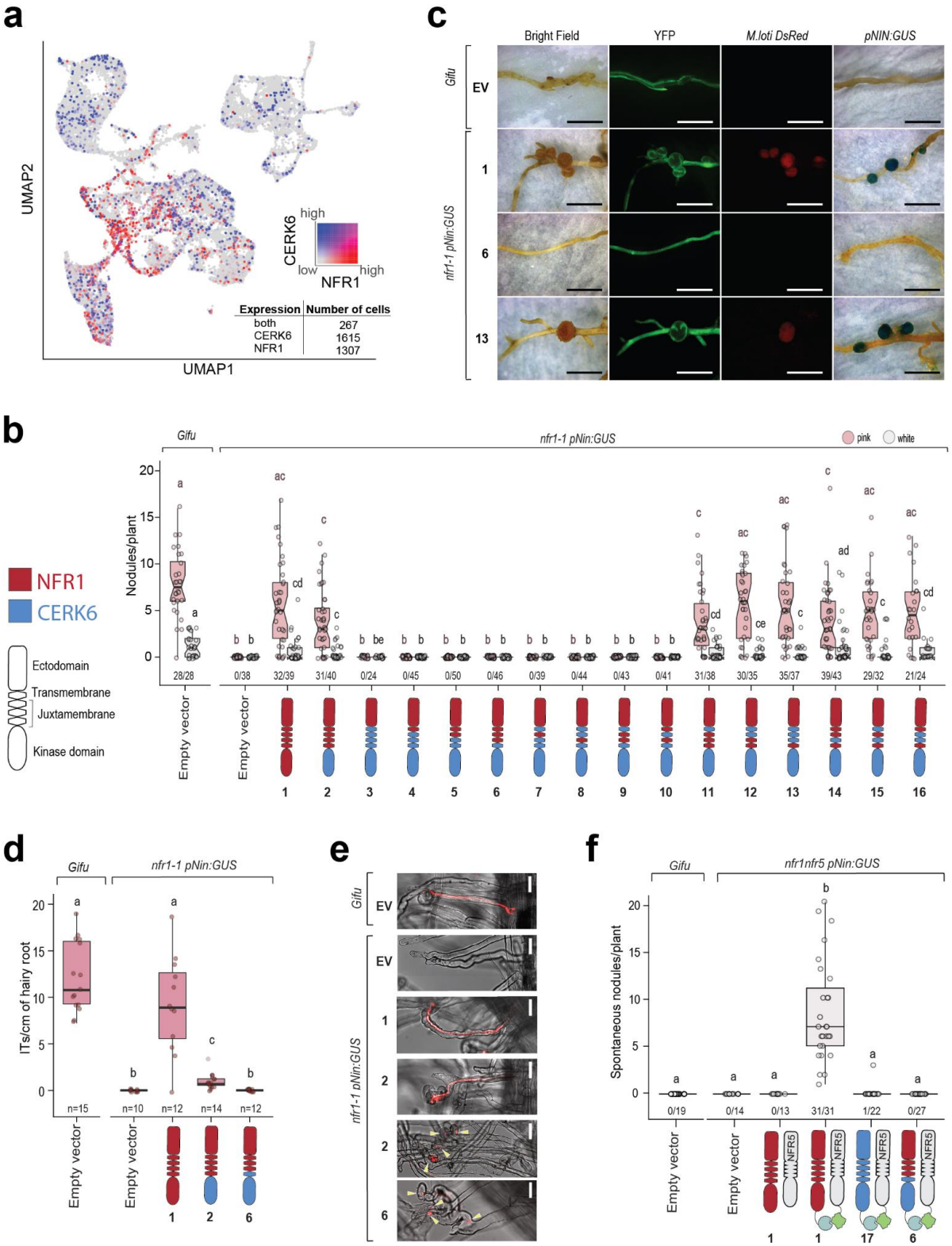
a
UMAP2
UMAP1
CERK6
high
low
NFR1
high
Expression | Number of cells
both 267
CERK6 1615
NFR1 1307
c
Bright Field
YFP
M.loti DsRed
pNIN:GUS
Gifu
EV
nfr1-1 pNin:GUS
1
6
13
b
Gifu
nfr1-1 pNin:GUS
pink
white
Nodules/plant
NFR1
CERK6
Ectodomain
Transmembrane
Juxtamembrane
Kinase domain
Empty vector
Empty vector
28/28
0/38
32/39
31/40
0/24
0/45
0/50
0/46
0/39
0/44
0/43
0/41
31/38
30/35
35/37
39/43
29/32
21/24
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
d
Gifu
nfr1-1 pNin:GUS
ITs/cm of hairy root
n=15
n=10
n=12
n=14
n=12
Empty vector
Empty vector
1 2 6
e
Gifu
EV
nfr1-1 pNin:GUS
EV
1
2
2
6
f
Gifu
nfr1nfr5 pNin:GUS
Spontaneous nodules/plant
0/19
0/14
0/13
31/31
1/22
0/27
Empty vector
Empty vector
NFR5
1 1 17 6

Figure 1. Symbiosis Determinant 1 controls symbiosis with rhizobia. a *Lotus* root cells expressing *NFR1* (red), *CERK6* (blue), or both (magenta)) 10 dpi (UMAP generated at https://lotussinglecell.shinyapps.io/lotus_japonicus_single-cell/). **b** Number of pink or white nodules formed by *Lotus* hairy roots expressing the indicated receptor variants. Nodules were counted 5 weeks post inoculation with *M. loti* R7A dsRED. Fractions under the boxplots indicate the number of nodulating plants out of total plants tested. **c** Representative photos of hairy roots carrying the indicated receptor variants. *YFP*: Yellow Fluorescent Protein (transformation marker), *M. loti DsRED* (fluorescent bacteria), *pNIN:GUS*: Bright Field photos after GUS staining. Scale bars: 5 mm. **d** Number of infection threads (ITs) formed per cm of transgenic root expressing the indicated receptor variant. ITs counted at 3 weeks post inoculation with *M. loti* MAFF. '*n'* indicates the number of plants analyzed per genotype. **e** Photos of transgenic roots expressing the indicated receptors at 3 weeks post inoculation with *M. loti* MAFF. Red shows *M. loti* MAFF bacteria. Yellow arrowheads indicate branched and scrambled root hair tips where micro-colonies with bacteria are visible. Scale bars: 30 µM. **f** Number of spontaneous nodules formed on *Lotus* hairy roots expressing the indicated constructs at 7 weeks post hairy root transformation. Fractions under the boxplots indicate the number of nodulating plants out of total plants tested. **b**, **d** and **f** lowercase letters indicate significant differences between samples. The analysis of variance was done with Kruskal-Wallis and post-hoc analysis (Dunn's test) $p < 0.05$.

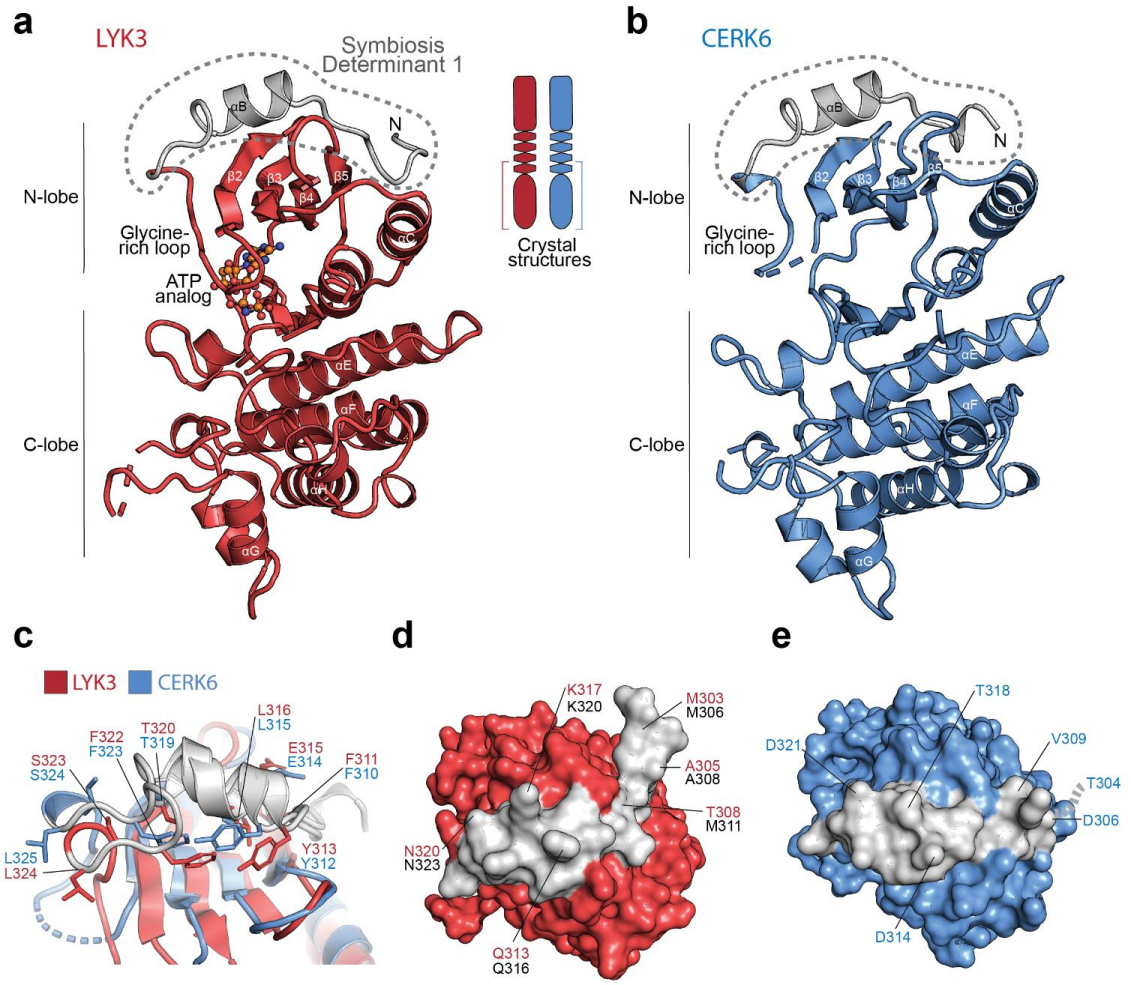

Figure 2. Crystal structures of LYK3 and CERK6 kinase domains. Structure of the LYK3 (**a**) and CERK6 (**b**) kinase domains annotated with conserved secondary structural elements (LYK3, PDB-ID: 9GFZ, CERK6 PDB-ID: 9GB9). LYK3 kinase was crystallized with AMP-PNP in the nucleotide binding site and the identified SD1 region is indicated in gray. **c** Structural overlay of the LYK3 and CERK6 showing the SD1 region. Amino acids that interact with the kinase core domain are conserved. **d-e** Zoom-in on SD1 of LYK3 and the same region in CERK6 seen from the top showing that this region is surface exposed and accessible. In (**d**) residues in red are from LYK3 and those in black are from NFR1.

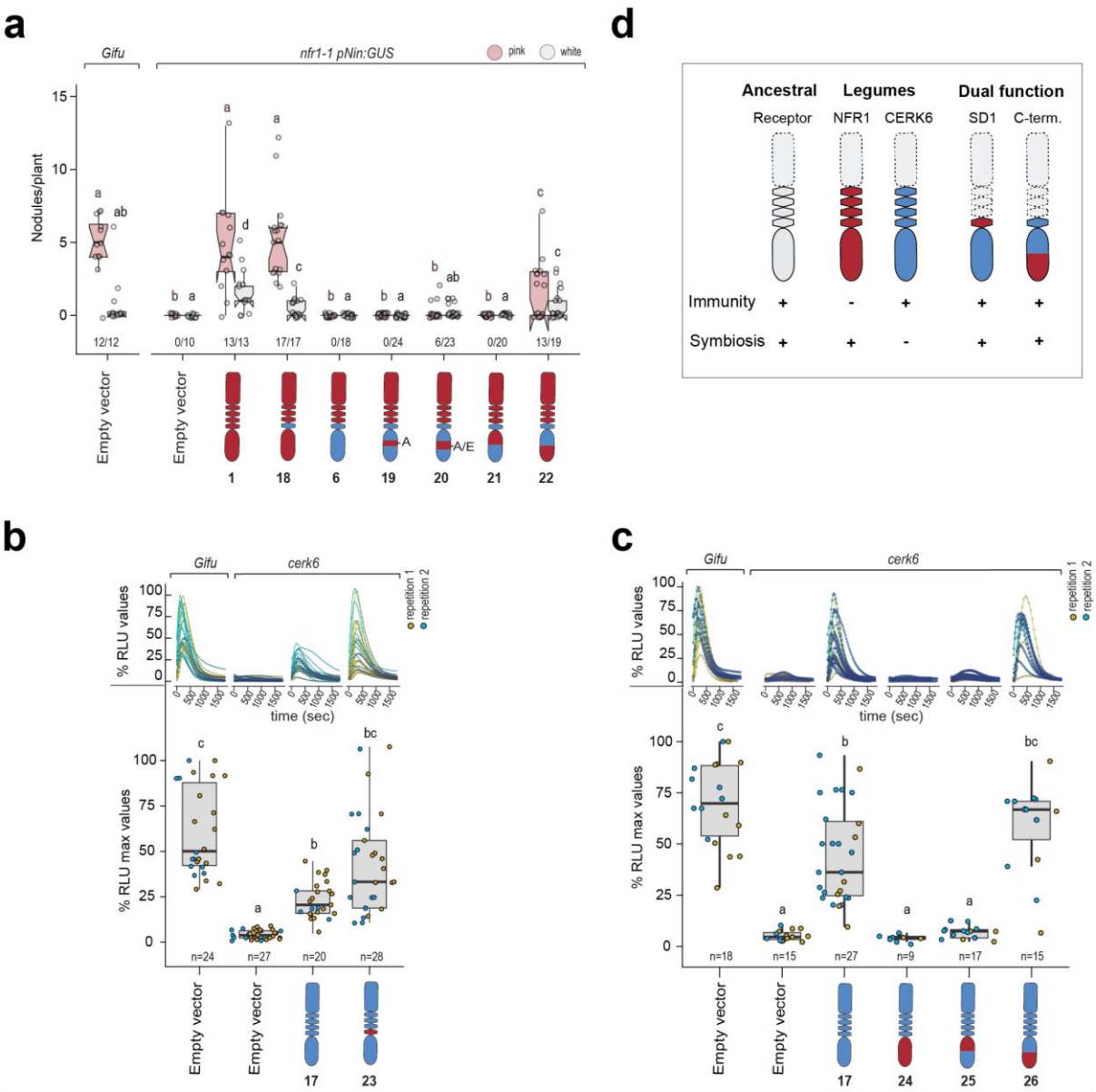

Figure 3. The N- and C-lobes contain determinants controlling independently the two signaling pathways. a The number of pink or white nodules formed on *Lotus* hairy roots expressing the indicated receptor variants. Nodules were counted 5 weeks post inoculation with *M. loti* R7A dsRED. Fractions under the boxplots indicate the number of nodulating plants out of total plants tested. Lowercase letters indicate significant differences between samples [analysis of variance (Kruskal-Wallis nd post-hoc analysis (Dunn's test) $p < 0.05$). **b-c** Reactive oxygen species (ROS) generated in *Lotus* hairy roots expressing the indicated receptor variants within a period of 30 min after treatment with 0.1 mg/ml chitin. Top: Curves show the values of RLU (Relative Luminescence Units) during the 30-min period after chitin treatment. Bottom: boxplots show the maximum value of RLU observed for each sample within the 30-min period after chitin treatment (100% is the highest value observed in Gifu WT expressing the empty vector construct). **d** Schematic representation of the observed functional capacities for the intracellular domains of the indicated LysM receptors.

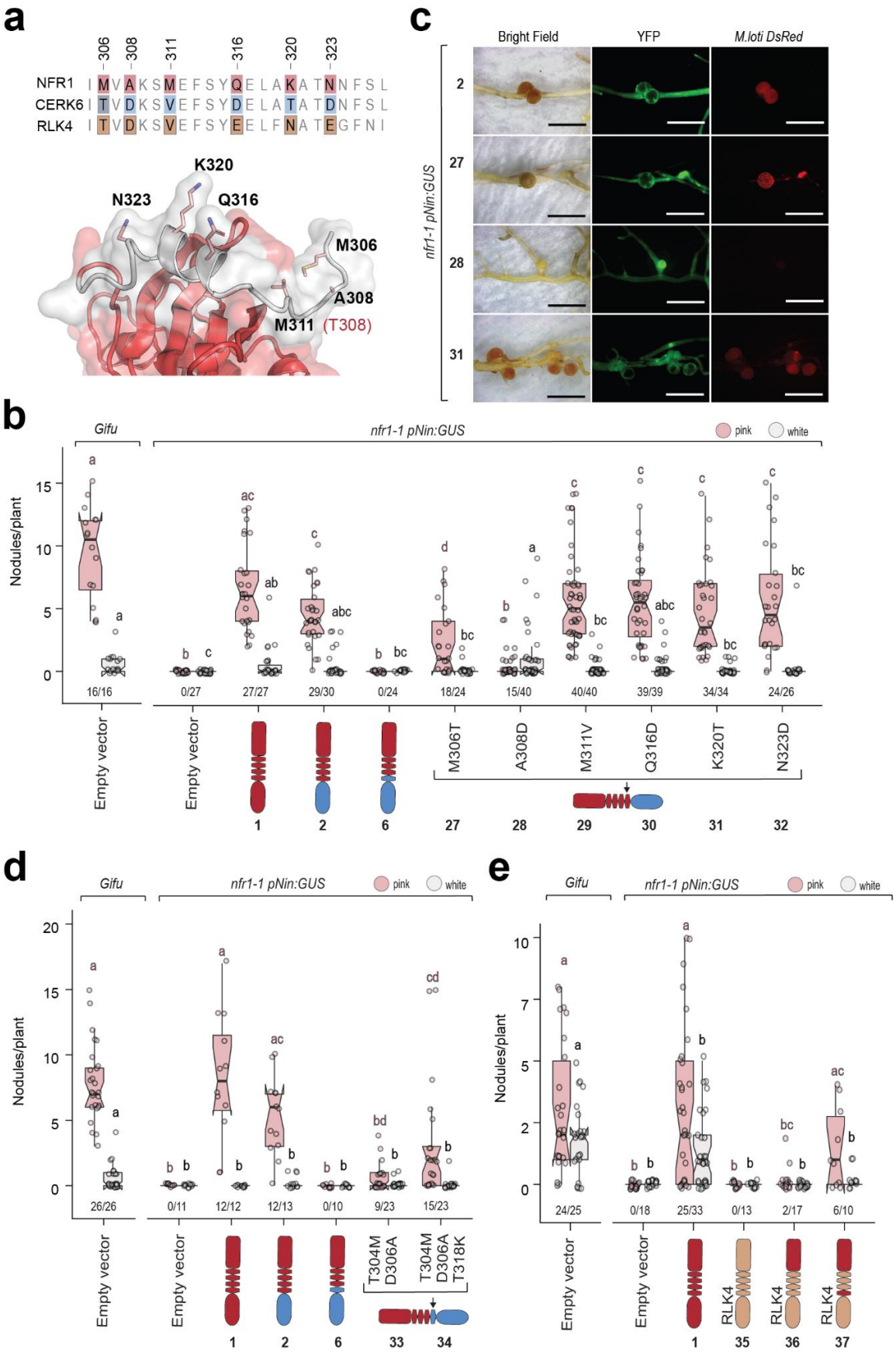
a
306
308
311
316
320
323
NFR1 I M V A K S M E F S Y Q E L A K A T N N F S L
CERK6 I T V D K S V E F S Y D E L A T A T D N F S L
RLK4 I T V D K S V E F S Y E E L F N A T E G F N I
K320
N323
Q316
M306
A308
M311 (T308)
c
Bright Field
YFP
M.loti DsRed
nfr1-1 pNin:GUS
2
27
28
31
b
Gifu
nfr1-1 pNin:GUS
pink
white
Nodules/plant
16/16
0/27
27/27
29/30
0/24
18/24
15/40
40/40
39/39
34/34
24/26
Empty vector
Empty vector
M306T
A308D
M311V
Q316D
K320T
N323D
1
2
6
27
28
29
30
31
32
d
Gifu
nfr1-1 pNin:GUS
pink
white
Nodules/plant
26/26
0/11
12/12
12/13
0/10
9/23
15/23
Empty vector
Empty vector
T304M D306A
T304M D306A T318K
1
2
6
33
34
e
Gifu
nfr1-1 pNin:GUS
pink
white
Nodules/plant
24/25
0/18
25/33
0/13
2/17
6/10
Empty vector
Empty vector
RLK4
RLK4
RLK4
1
35
36
37

Figure 4. Two residues of Symbiosis Determinant 1 are essential for root nodule symbiosis. a Amino acid alignment of Symbiosis Determinant 1 (SD1) in NFR1, CERK6 and RLK4. Numbers above the alignment indicate the amino acid positions in NFR1 protein. **c** Representative photos of hairy roots carrying the indicated receptor variants. YFP: Yellow Fluorescent Protein (transformation marker), *M. loti DsRED* (fluorescent bacteria), *pNIN:GUS*: Bright Field photos after GUS staining. Scale bars: 5 mm. **b**, **d**, and **e** Number of pink or white nodules formed on *Lotus* hairy roots expressing the indicated receptor variants. Nodules were counted 5 weeks post inoculation with *M. loti* R7A dsRED. Fractions under the boxplots indicate the number of nodulating plants out of total plants tested. Lowercase letters indicate significant differences between samples [analysis of variance (Kruskal-Wallis and post-hoc analysis (Dunn's test) $p < 0.05$).