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ORIGINAL ARTICLE

Role of a receptor-like kinase K1 in pea *Rhizobium* symbiosis development

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Abstract

Main conclusion **The LysM receptor-like kinase K1 is involved in regulation of pea-rhizobial symbiosis development.**

The ability of the crop legume *Pisum sativum* L. to perceive the Nod factor rhizobial signals may depend on several receptors that differ in ligand structure specificity. Identification of pea mutants defective in two types of LysM receptor-like kinases (LysM-RLKs), SYM10 and SYM37, featuring different phenotypic manifestations and impaired at various stages of symbiosis development, corresponds well to this assumption. There is evidence that one of the receptor proteins involved in symbiosis initiation, SYM10, has an inactive kinase domain. This implies the presence of an additional component in the receptor complex, together with SYM10, that remains unknown. Here, we describe a new LysM-RLK, K1, which may serve as an additional component of the receptor complex in pea. To verify the function of K1 in symbiosis, several *P. sativum* non-nodulating mutants in the *k1* gene were identified using the TILLING approach. Phenotyping revealed the blocking of symbiosis development at an appropriately early stage, strongly suggesting the importance of LysM-RLK K1 for symbiosis initiation. Moreover, the analysis of pea mutants with weaker phenotypes provides evidence for the additional role of K1 in infection thread distribution in the cortex and rhizobia penetration. The interaction between K1 and SYM10 was detected using transient leaf expression in *Nicotiana benthamiana* and in the yeast two-hybrid system. Since the possibility of SYM10/SYM37 complex formation was also shown, we tested whether the SYM37 and K1 receptors are functionally interchangeable using a complementation test. The interaction between K1 and other receptors is discussed.

Keywords Heterologous expression · Legume-*Rhizobium* symbiosis · LysM receptor-like kinases · Mutants · Nod factor perception · Pea · *Pisum sativum* L.

Abbreviations

ECD	Extracellular domain
GUS	Beta-glucuronidase
HR	Hypersensitivity reaction
IT	Infection thread
RLK	Receptor-like kinase
Y2H	Yeast two-hybrid system

Introduction

The establishment of symbiosis between legume plants and rhizobial bacteria is based on signal exchange between partners, which finally leads to the development of new organs on the plant roots, the nitrogen-fixing nodules. Bacterial signals Nod factors are lipochitooligosaccharide

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molecules composed of a few residues of N-acetylglucosamine ($n = 3–6$), connected by β-1,4-glycosidic bonds; they carry the acyl chain at the non-reducing end of the molecule and exhibit various substitutions on the backbone (Lerouge et al. 1990; Spaink et al. 1991; Mergaert et al. 1997; Perret et al. 2000). Nod factors induce the plant's responses in picomolar-to-nanomolar concentrations, and their structural characteristics determine the host specificity of the symbiosis (van Brussel et al. 1982; Spaink et al. 1991; Dénarié et al. 1996; Schultze and Kondorosi 1998; Cullimore et al. 2001; Riely et al. 2004). These data indicate that specific receptors to the Nod factors are present in plants, and play a pivotal role in the perception of these extracellular signals (Dénarié and Cullimore 1993; Heidstra and Bisseling 1996).

A number of genes encoding receptor-like kinases (RLKs) with LysM motifs in extracellular domains (ECDs) have been described in model legumes *Medicago truncatula* Gaertn and *Lotus japonicus* (Regel) K. Larsen (Arrighi et al. 2006; Zhang et al. 2007; Lohmann et al. 2010; Kelly et al. 2017). Analysis of nodulation-deficient mutants in model (*M. truncatula, L. japonicus*) and crop legumes (*Pisum sativum* L., *Glycine max*) allowed for the identification of a set of candidates for the role of putative receptors to Nod factors from LysM-RLK family (Ben Amor et al. 2003; Madsen et al. 2003; Limpens et al. 2003; Radutoiu et al. 2003, 2007; Arrighi et al. 2006; Smit et al. 2007; Zhukov et al. 2008; Indrasumunar et al. 2010). In the model legume *L. japonicus*, which forms a determinate type of nodules, it was found that two LysM-RLKs, *Lj*NFR5 and *Lj*NFR1, were both essential for all symbiotic responses (Madsen et al. 2003; Radutoiu et al. 2003, 2007) and involved in Nod factor binding (Broghammer et al. 2012). An *L. japonicus* double mutant in genes *nfr5/nfr1* is characterized by a complete blockage of plant responses to rhizobial inoculation, as well as to the application of Nod factors (Radutoiu et al. 2007). Due to its structural features, the *Lj*NFR5 kinase domain is not capable of autophosphorylation, while *Lj*NFR1 contains a functional kinase domain involved in intra- and intermolecular phosphorylation (Madsen et al. 2011). This indicates that *Lj*NFR5 and *Lj*NFR1 receptors in *L. japonicus* may form a heteromeric complex. Indeed, only the transfer of both RLKs (*Lj*NFR5 and *Lj*NFR1) into *M. truncatula* plants, which differ in specificity from *L. japonicus*, conferred them capacity to respond to *M. loti* inoculation, leading to symbiosis development (Radutoiu et al. 2007). Subsequently, the possibility of forming a complex was demonstrated for the *Lj*NFR5 and *Lj*NFR1 RLKs by bimolecular fluorescence complementation, as well as by modified FRET analysis, which resulted in hypersensitivity reaction (HR) development (Madsen et al. 2011).

Homologues of the *LjNFR5* and *LjNFR1* genes have been found in other legume species *M. truncatula* (*MtNFP* and *MtLYK3*) and *P. sativum* L. (*PsSym10* and *PsSym37*), forming indeterminate type of nodules (Ben Amor et al. 2003; Limpens et al. 2003; Madsen et al. 2003; Arrighi et al. 2006; Smit et al. 2007; Zhukov et al. 2008). However, the phenotypic characteristics of the mutants defective in these genes indicate that the perception of Nod factors may be organized in a more complex way in these legumes. The *Mtnfp*/*Pssym10* mutants were found to be completely deficient for nodulation as the *Ljnfr5* mutant (Duc and Messager 1989; Kneen et al. 1994; Sagan et al. 1994; Schneider et al. 2002; Ben Amor et al. 2003; Madsen et al. 2003). Though analyses performed on the *Mtlyk3/Pssym37* mutants showed that the plants were able to initiate the first responses upon rhizobial inoculation in contrast to *Ljnfr1*, and they were impaired in infection thread (IT) initiation (Engvild 1987; Postma et al. 1988; Wais et al. 2000; Catoira et al. 2001; Smit et al. 2007; Borisov et al. 2007; Zhukov et al. 2008).

A hypothetical model involving two types of receptors that recognize Nod factors during symbiosis establishment was previously proposed for *Medicago* plants based on the analysis of the influence of bacterial *nodF*$^-$, *nodL*$^-$, and *nodFL*$^-$ mutant strains producing Nod factors with altered substitutions (Ardourel et al. 1994; Wais et al. 2000; Oldroyd et al. 2001; Cullimore and Dénarié 2003). *S. meliloti nodL*$^-$ and *nodFE*$^-$ mutants making non-*O*-acetylated Nod factors or Nod factors with modified acyl substituents at the non-reducing end had a reduced capacity to initiate ITs in root hairs, but elicited root-hair deformations and cortical cell divisions (Ardourel et al. 1994). Double *nodFL*$^-$ mutant showed an additive effect with complete blocking of ITs initiation. Therefore, a receptor with low stringency for substitutions at the Nod factor non-reducing end was suggested to be involved in the control of the early stages of nodulation, such as root-hair deformations and initial cortical cell division, while a receptor with higher structural requirements is involved in the infection initiation and development (Ardourel et al. 1994).

A similar proposal about the functioning of two types of receptors was suggested for pea *P. sativum* L. based on the analysis of Nod factor-dependent responses (Walker and Downie 2000). *R. leguminosarum* bv. *viciae* produces a mixture of Nod factors with either a C18:4 acyl chain or a C18:1 acyl chain at the non-reducing end. *nodL* and *nodFE* genes are required to produce Nod factors with O-acetyl group and specific acyl chain at the non-reducing end (Spaink et al. 1991). A *nodE* mutant produced only Nod factors containing a C18:1 and showed a reduced nodulation of pea (Economou et al. 1994). This effect can be compensated by bacterial protein NodO, probably amplifying the signal from Nod factor in plant cells. A *nodE nodO* double mutant has initiated aberrant infection (initial entry), but was almost unable to stimulate the formation of ITs (Walker and Downie 2000). Hence, unlike *Medicago*, the initial entry of rhizobia in pea requires minimal host-specific substitutions on the

Nod factors, while IT growth in the root hairs and their distribution in cortical root cells require additional specificity involving the Nod factors' acyl chain structure. In addition, initiation of the cortical cell division and pre-ITs formation in pea can strictly dependent on the Nod factors' specific acyl chain C18:4 and O-acetyl group (Spaink et al. 1991; van Spronsen et al. 1994).

The presence of mutants *Mtnfp*/*Pssym10* and *Mtlyk3*/*Pssym37* defective in two types of LysM-RLKs with different phenotypic manifestations corresponds well with assumption about two-receptor system. At the same time, the structural features of the *Mt*NFP and *Ps*SYM10 kinase domains suggest that they can form complexes with other LysM-RLKs (Madsen et al. 2003; Arrighi et al. 2006). Although the possibility of functional redundancy could not be excluded, *Mt*LYK3 or *Ps*SYM37 is unlikely to be co-receptors at symbiosis initiation, as the initial stages of symbiosis are not impaired in the mutants defective in these genes (Catoira et al. 2001; Smit et al. 2007; Zhukov et al. 2008). Consequently, in *Medicago* and pea, an additional and not yet identified co-receptor may comprise part of the complex with *Mt*NFP or *Ps*SYM10. In accordance with this suggestion, swap experiments between various LysM motifs of *Mt*NFP or *Ps*SYM10 were indicative that an additional component exists in complex with the *Mt*NFP receptor, which is required for the activation of the early symbiotic responses (Bensmihen et al. 2011). These swap experiments also showed that *Mt*LYK3 is apparently not the additional component that triggers the earliest symbiotic responses together with *Mt*NFP receptor protein, although the functional redundancy could not also be excluded in this case (Bensmihen et al. 2011). While taking this into consideration, the hypothesis that there are two receptors that recognize Nod factors should be revised. Rather, it must be stated that a few receptor complexes that consist of not one, but several proteins are involved in legumes with the indeterminate type of nodules. Therefore, searching for additional receptors that are responsive to Nod factors is of great interest. Indeed, the identification of new receptor proteins such as *Mt*LYK4 in *M. truncatula*, as well as the recently found *Mt*LYR3, supports the assumption that Nod factor receptors are organized in a more complicated manner in such legumes (Limpens et al. 2003; Fliegmann et al. 2013).

In pea *P. sativum* L., there are two more possible candidates for the role of additional receptors, as shown using molecular genetic approaches (Lie 1984; Geurts et al. 1997; Zhukov et al. 2008). On one hand, the existence of the *Sym2* gene, which controls infection development dependent on the Nod factor's structure, suggests that it may encode another additional Nod factor receptor (Lie 1984; Geurts et al. 1997). On the other hand, screening of a cDNA library using *LjNFR1* as a probe resulted in the identification of the additional gene *K1*, which encodes an LysM-RLK with a high degree of similarity to *Sym37* (Zhukov et al. 2008). This allows us to consider the pea as an attractive model to find and characterize additional Nod factor receptors. Based on the assumption that several receptor complexes are involved in the recognition and binding of the rhizobial Nod factors in pea, we investigated the possible role of K1 in pea-rhizobial symbiosis. A few mutants in the *k1* gene were isolated using the TILLING approach. Their phenotyping revealed the blocking of symbiosis development at the early stages. Using various approaches, we also investigated the capacity of K1 to form in vivo complexes with both RLKs (SYM10 and SYM37).

Materials and methods

Bacterial strains and inoculation

The *Rhizobium leguminosarum* biovar *viciae* strain RCAM 1026 (WDCM 966) was cultivated at 28 °C on TY (Orosz et al. 1973) or YEB (Krall et al. 2002) agar medium supplemented with 0.5 mg/ml of streptomycin. Triparental mating mediated by pRK2013 was performed to introduce the vector pJP2neo-*gusA* Amp^R (Prell 2003) into *R. leguminosarum* bv. *viciae* strain. To prepare plant inoculum, fresh liquid cultures were grown in B^- medium (Van Brussel et al. 1977). The optical density at 600 nm (OD_{600}) of the suspension was adjusted to 0.1–1.0 depending on the experiment type.

Plant material and growth conditions

Pisum sativum L. cv. Finale or cv. Cameor and Rondo seeds (ARRIAM collection, Saint-Petersburg, Russia) were surface sterilized with sulphuric acid for 5 min, washed three times with water, transferred on 1% water agar plates, and germinated at room temperature in the dark. After germination (for 4–5 days), plants were transferred into pots with vermiculite saturated with Jensen medium (van Brussel et al. 1982), grown in a growth chamber at 21 °C at 16 h light/8 h dark cycles, 60% humidity. Pea seedlings were inoculated with 1 ml of *R. leguminosarum* bv. *viciae* RCAM 1026 suspension prepared as described above (OD_{600} = 0.5–1.0) per plant. For the temporal expression analysis during nodulation, pea roots (segments of main roots corresponding to the susceptible zone for rhizobial infection without lateral roots) or segments of main roots with nodules were harvested at 1–9 days after inoculation (dai), while the nodules were collected at 14–28 days.

N. benthamiana seeds were surface sterilized with 10% hypochlorite for 10 min, washed 5 times with water, and left for imbibition on plate with sterile filter paper at 4 °C. All seeds were germinated in a big plastic box for 7 days and then transferred into individual pots with soil. Plants

were grown at 25 °C with 18 h light/6 h dark cycles, 60% humidity.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from plant tissues using the PureZOL RNA isolation reagent (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions followed by DNAseI (Thermo Fisher Scientific, Massachusetts, USA) treatment to remove traces of genomic DNA. Complementary DNA was prepared from 1 to 2.5 µg of RNA with the RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific) using oligo(dT) primers (Sileks www.sileks.com) according to the manufacturer's protocol. The quantitative reverse transcription PCR (qRT-PCR) analysis was performed using the CFX96 Real-Time System and iQ SYBR Green Super Mix (Bio-Rad Laboratories). All primer pairs (Supplementary Table S1) were designed using Vector NTI program and produced by Evrogen company (www.evrogen.com). PCR amplification specificity was verified using a dissociation curve (55–95 °C). mRNA levels were normalized against *Ubiquitin* and *Actin,* and values were calculated as ratios relative to non-inoculated root expression levels. The data of two–three independent biological experiments were analyzed. Statistical analysis was conducted by Student's test ($P < 0.05$) to assess the differences between variants.

Generation of constructs for plant and yeast transformation

To generate the pBIN19 vectors for plant transformation, carrying the genes of interest, the coding sequences of *Sym37*, *Sym10,* and *Sym19* genes without stop-codons have been amplified using cDNA as a matrix (total RNA was isolated from 21 dai pea nodules of cv. Finale) with corresponding primers (Supplementary Table S1). Amplification was done using Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific). The amplified products were restricted with NheI/EcoRI and subcloned in the pMON vector under 35S promoter in the frame with the sequences encoding YFP, RFP, or 3xFLAG and nopaline synthase terminator (Tnos). For *Sym37* (cv. Finale) with internal EcoR1 site, the large fragment (1080 bp) was first cloned followed by small EcoR1/EcoR1 fragment (786 bp) cloning. The inserts were verified by sequencing. At the next step, the cassette composed of the 35S promoter; gene of interest fused with YFP, RFP, or 3xFLAG and *Tnos* was excised from pMON using HindIII/SmaI and cloned in the pBIN19.

To clone the full-length *K1* gene (without stop-codon), two pairs of primers for amplification of two overlapping fragments on the matrix of cDNA have been used. The first fragment (1–650 bp) containing the internal AccI site was subcloned into the pMON vector using NheI/EcoR1 sites. The second fragment (418-1860 bp) was later inserted into this vector using AccI/EcoRI restriction enzymes. The full-size *K1* gene insert was verified by sequencing. Finally, the cassette with full-size *K1* gene fused with *YFP* or *RFP* under 35S promoter was transferred into pBIN19 in the same way as described for other genes.

To generate pB7WG2D plasmids, the *K1*, *Sym37,* and *Sym10* full-length sequences with stop-codons (cv. Finale, Cameor or Rondo) were amplified using cDNA (total RNA was isolated from 2 dai roots and 14 dai nodules) and inserted into pENTRY/D-TOPO or pDONR221 vectors (Thermo Fisher Scientific), respectively, and finally into the pB7WG2D using the LR clonase enzyme (Thermo Fisher Scientific). The construct in pDONR221 vector with *K1* gene, carrying mutation as in *k1-2* line, was obtained using site-directed mutagenesis kit (Thermo Fisher Scientific) according to the manufacturer's protocol with corresponding primers (Supplementary Table S1). All verified constructs were transferred into the *Agrobacterium tumefaciens* LBA 4404, GV3101 strains, or *A. rhizogenes* strain Arqua1.

To detect protein–protein interactions in yeast two-hybrid system, the partial gene sequences of *Sym37*, *K1* and *Sym10,* encoding the extracellular domains and flanked with attb1/attb2 sequences were inserted in the pDONR221 vector (Thermo Fisher Scientific) with BP Clonase enzyme (Thermo Fisher scientific) and finally into the pDEST22 (PREY) or pDEST32 (BAIT) using the LR clonase enzyme (Thermo Fisher Scientific). Domain structure of proteins was determined using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM). Since the interaction between proteins should be in yeast nucleus, the sequences corresponding to signal peptides (http://www.cbs.dtu.dk/services/SignalP) have been excluded from each protein. Thus, we cloned *Sym37-ECD* (fragment encoding the amino acids from 22 to 223), *K1-ECD* (24–222) and *Sym10-ECD* (32–238). All verified constructs were transferred into *Saccharomyces cerevisiae* MaV203 yeast strain.

Transient gene expression in *Nicotiana benthamiana* leaves

Agrobacterium tumefaciens strains LBA 4404 or GV3101 carrying the vectors for expression of full-size genes encoding receptor-like kinases were used for infiltration in *N. benthamiana* leaves. The additional expression of the silencing suppressor P19 was shown to enhance the expression of the transiently expressed *Sym10*, *K1,* and *Sym37* genes in our study. Bacterial cultures were grown at 28 °C overnight, then centrifuged at 3000 *g,* and resuspended in 10 mM Mes-KOH, 10 mM $MgCl_2$, and 0.5 mM acetosyringone up to culture density $OD_{600} = 0.5$.

Bacterial cells were infiltrated into the leaves of 3-week-old *N. benthamiana*. Plants were analyzed 48–96 h after infiltration.

Western-blot analysis of leaf tissues

1–2 transformed leaves were homogenized in a mortar with liquid nitrogen, resuspended in 0.1 M Mes-KOH buffer, 0.1 mM PMSF, 1 mM $MgCl_2$, 1 mM EDTA, 0.1% sucrose, 3% ethylene glycol, and protease inhibitor cocktail (Sigma-Aldrich, Munich, Germany) centrifuged at 16,000*g* for 30 min; the pellet was resuspended in sample buffer for SDS-PAGE electrophoresis. The proteins were separated in SDS-PAGE and blotted onto a nitrocellulose membrane (Bio-Rad Laboratories). Proteins were detected using monoclonal anti-FLAG M2 conjugated with horseradish peroxidase (HRP) (Sigma-Aldrich) at 1:5000 or rabbit polyclonal anti-RFP antibodies (Thermo Fisher Scientific) at 1:2000, followed by secondary anti-rabbit antibodies linked with HRP (Sigma-Aldrich) at 1:5000. The anti-GFP antibodies linked with HRP (Thermo Fisher Scientific) were also used for detection at 1:5000. Bovine serum albumin was used for membrane saturation and incubation with antibodies. Peroxidase activity was revealed by chemiluminescence using the Immobilon Western Chemiluminescent (Merck Millipore, Darmstadt, Germany). Molecular weights were estimated by comparison with the PageRuler prestained protein ladder (Thermo Fisher Scientific).

Yeast two-hybrid assay (GAL4 transcription factor-based assay)

The pDEST22 (PREY) and pDEST32 (BAIT) vectors (ProQuest Two-Hybrid System, Thermo Fisher Scientific) have been used for analysis. The *S. cerevisiae* strain MaV203 (Thermo Fisher Scientific) was transformed simultaneously with pDEST22 and pDEST32 vectors. To generate *S. cerevisiae* MaV203 transformants, the protocol for preparation of electrocompetent cells was used (Thermo Fisher Scientific). The two-hybrid interactions were determined by growth or growth absence on different selective media: synthetic complete media (SD) lacking of leucine (Leu) and tryptophan (Trp); SD media lacking Leu, Trp, histidine (His), and uracil (Ura); SD media lacking Leu and Trp containing Ura and 0.2% 5-fluoroorotic acid (Thermo Fisher Scientific); SD media lacking Leu, Trp, His, and containing various concentrations of 3-aminotriazole. As controls, a few pairs of vectors (pEXP32/Krev1 and pEXP22/RalGDS wild type, and pEXP22/RalGDS-m1 and pEXP22/RalGDS-m2) suggested by the manufacturer were used for strong, weak, and not detectable interactions (Thermo Fisher Scientific).

Agrobacterium-mediated pea plant transformation

Pea seeds were sterilized as described above and germinated on 1% water agar in the dark. 4–5-day-old seedlings were transferred to the light in sterile plastic boxes and incubated for 2–3 days. The pea roots were cutoff in the area of hypocotyl and transformed with fresh grown *A. rhizogenes* Arqua1 strain. Plants were put in special plastic jars on Jensen agar; the place of cutoff was covered with wet wool and foil. Plastic jars were incubated in a growth chamber; callus was formed on 10–14 days, and then, the plants were transferred onto Emergence medium containing 150 mg/ml cefotaxime. Plants were incubated on Emergence medium up to 5–7 days until the transgenic roots have been formed. Emerging roots have been analyzed using epifluorescent stereomicroscope (Stereo Discovery V8, Carl Zeiss, Oberkochen, Germany); non-transgenic roots were removed. The composite plants have been placed into vermiculite and kept under a vented plastic cap for a few days (16 h light/8 h dark cycle, 21 °C, 60% humidity). The plants were inoculated with *R. leguminosarum* bv. *viciae* RCAM 1026 (OD_{600} = 0.1 – 0.2) and checked for nodule formation in 21–28 days.

Targeting-induced local lesions in genomes (TILLING) screens

The analysis was made with help of TILLING approach relied both on the construction of high-quality pea cv. Cameor mutant collection (Dalmais et al. 2008) (https://www6.dijon.inra.fr/umragroecologie_eng/Research-Cluster/GEAPSI) available at UMR Agroécologie, INRA, France and on the mutation detection system by sequencing developed at IPS2, INRA, France. The alignment of the K1 protein sequences from cv. Cameor and cv. Finale showed only two replacements in ECD (cf. Supplemental Fig. S4). Searching for mutations was performed using the next-generation sequencing technology (method unpublished) to detect putative point mutation within four amplicons of the *K1* gene (Supplementary Table S2). DNA libraries from 2500 mutated lines were constructed for each PCR fragment. MiSeq runs were performed with the MiSeq V3-kit (600 cycles) and 2 × 300 cycles' pair-end sequencing was done. Row data were analyzed by our bioinformatics pipeline (unpublished) to detect rare SNPs. Prediction of the amino acid changes that affect protein function was made using the SIFT program (sift.jcvi.org/). Mutant lines were evaluated in M4 and M5 generations for *K1* gene homozygosity. Totally, for 2265 mutant line, 13 plants, for 817 line, 18 plants, and for 885 line, 7 plants were taken for analysis. 4–5-day-old seedlings of homozygous mutant plants were inoculated with *R. leguminosarum* bv. *viciae* 1026 strain expressing *gusA* as described above. Phenotypic analysis

was performed in 14 and 21 days after inoculation (dai). Wild-type pea plants cv. Cameor were used as control.

Histochemical staining and microscopy

Root segments and nodules were collected and stained with X-Gluc (0.2 M Na_2PO_4, pH7.0; 0.5 M EDTA, pH8.0; 20 mM K ferricyanide; 20 mM K ferrocyanide; 20 mM X-Gluc; 0.01% Triton X-100). Tissues were incubated in staining due overnight at 37 °C. Colored tissues were fixed in the freshly prepared fixative solution (3% paraformaldehyde, 0.25% glutaraldehyde, 0.3% Tween-20, and 0.3% Triton X-100) in phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, and 5 mM KH_2PO_4, pH 7.3) (Voroshilova et al. 2009). For optimal fixative penetration, air from tissues was pumped out for 7 min at 0.9 bar five times (Kitaeva et al. 2016). Fixation was performed at 4 °C overnight. Root pieces or nodules were washed in PBS 20 min three times, molded in 3% agarose gel blocks, and sliced into 50- or 60-µm sections using a vibratome Microm HM 650 V (Microm International GmbH, Walldorf, Germany). Sections were mounted in distilled water on glass slides for light microscopy. Pictures were taken using an Olympus BX51 microscope (Olympus Optical Co. Europa GmbH, Hamburg, Germany) equipped with a ColorView II digital camera and analySIS FIVE analytical software (Olympus Soft Imaging Solution GmbH). Immunolabeling was made as described (Kitaeva et al. 2016); for bacteria visualization, antibodies MAC57 were used (Brewin et al. 1986); as second antibody Alexa conjugated antibody: goat anti-mouse IgG Alexa Fluor 488 were used. For ITs or infection droplet visualization, MAC265 antibodies were used (van den Bosch et al. 1989; Tsyganova et al. 2009) followed by second Alexa conjugated antibody (anti-mouse IgG Alexa Fluor 488) which were also used (Kitaeva et al. 2016). After washing, sections were incubated with propidium iodide (0.5 µg/ml) for 10 min. Sections were analyzed using the laser-scanning confocal systems LSM 510 META (Carl Zeiss). Roots were collected 14 days after inoculation (three biological repeats). For each repeat, 4–5 upper roots (1.5 cm length) from 3 plants were collected, sliced into a lot of pieces, and analyzed.

Results

Quantitative RT-PCR analysis of *K1* expression upon nodulation in pea

To analyze the role of *K1* in symbiosis, we monitored its expression level in response to inoculation with *R. leguminosarum* bv. *viciae* RCAM 1026 for up to 28 days by qRT-PCR (Supplemental Fig. S1). Previously, expression pattern of *K1* gene in different organs and roots upon inoculation for up to 7 days was monitored (Zhukov et al. 2008). As a comparison, we studied the expression of two other *Sym10* and *Sym37* genes encoding the LysM-RLKs (Supplemental Fig. S1) as well as *NIN* gene encoding transcription factor (Supplemental Fig. S2); they are involved in the control of symbiosis development, as has been previously shown (Schauser et al. 1999; Borisov et al. 2003; Madsen et al. 2003; Zhukov et al. 2008). A time-course analysis revealed that *K1* expression slightly increased (1.8-fold) at the early stages of symbiosis development in response to rhizobial inoculation (2 dai), but then its transcript level did not significantly change upon nodule development (Supplemental Fig. S1). Nevertheless, in nodules (14 dai), the *K1* expression was 1.5-fold higher when compared with uninoculated roots (Supplemental Fig. S1).

Similarly, the expression level of *Sym10* started to increase from 1 to 2 days in response to inoculation, and it remained high at the subsequent stages of nodule development and in mature nodules. The expression level of *Sym37* also slightly increased at the symbiosis initiation and was noticeably upregulated at 5 dai (fivefold increase). At later stages, *Sym37* expression continued to increase up to the maximum at 14 dpi. The level of *NIN* transcripts increased after inoculation starting from 2 dpi and reached the highest level at mature stages (at 21–28 dpi). Thus, in our experimental conditions, all four genes *K1*, *Sym10*, *Sym37,* and *NIN* showed activation at the initial stages of symbiosis development and increased level in mature nodules. This may suggest a requirement for increased levels of these genes at symbiosis initiation and in functional nodules. Nevertheless, minor changes in *K1* transcript level were caused by inoculation with *R. leguminosarum* bv. *viciae* as compared with other genes. Activation of *K1*, *Sym10, Sym37,* and *NIN* genes in mature nodules is consistent with other experimental data (Zhukov et al. 2008).

TILLING analysis and mutant phenotype characterization

A search for mutants in the *P. sativum k1* gene was conducted using the TILLING platform (Dalmais et al. 2008). Screening revealed 17 mutant lines (Supplemental Table S2); of them, 3 lines were chosen with mutations that are predicted to lead to amino acid changes and disruption of protein function (according to in silico predictions; the SIFT program) (Ng and Henikoff 2003). These were the 885 line (*k1-1*), which carries mutation that results in G332 → D replacement in the kinase domain (the nucleotide-binding glycine-rich loop); the 817 line (*k1-2*), which carries mutation that results in P169 → S replacement in the LysM3 motif of ECD; and the 2265 line (*k1-3*), which carries mutation that results in S59 → F replacement in the LysM1 motif of

the ECD (Table 1). A nodule formation analysis was performed at 14 and 21 dai.

No nodules were observed on the roots of the *k1-1* and *k1-2* mutant lines (Nod^- phenotype) (Figs. 1, 2). Hence, two *k1* mutants with loss-of-function phenotypes were identified in pea. The *k1-3* mutant line showed total number of developing nodules at 14 dai comparable with wild type (38.7 ± 3.1 per plant on the *k1-3* line and 41.7 ± 5.2 in wild-type plants); however, only part of them (22.3 ± 3.7) were infected or at the initial stage of infection (Fig. 3b). At 21 dai, a number of infected nodules were comparable with the wild-type (43 ± 0.97 on the 2265 line and 46.57 ± 1.23 in wild-type plants; $P < 0.05$, *t* test) and their structure did not differ from that of wild-type nodules (Fig. 3c, d).

A comparative light microscopy analysis of the non-nodulating roots of the *k1-1* and *k1-2* lines showed blocking of symbiosis development at the earlier stage in the *k1-1* line. Analysis of the *k1-1* mutant line revealed only rare root-hair deformations (Table 2); however, with a very low frequency, we observed growth of short-aborted ITs connected with small root hairs (Fig. 1). In such cases, the IT development was blocked after reaching the epidermal layer (Fig. 1). In the same conditions, normal nodules were formed on the roots of wild-type plants cv. Cameor-inoculated *R. leguminosarum* bv. *viciae* RCAM 1026-*gusA*.

Microscopic analysis of the roots of the *k1-2* mutant line (P169 → S replacement in the LysM3 motif of ECD) showed the development of the early symbiosis stages, such as the curling of root hairs, formation of infection pockets, and IT formation; however, most ITs have lost their polar mode of growth forming the sac-like structures and were blocked in the epidermal layer (Fig. 2I). The activation of cortical cell division and nodule primordium formation took place (Fig. 2Ic), but no infected primordia were found, because no bacteria had released from the ITs (Fig. 2II), as it was shown using immunochemical approach with antibodies MAC57

Table 1 Mutations in the *k1* gene and their effects in mutant plants

Mutant line	Mutation	DNA position	Protein position	Localization
885 (*k1-1*)	G → A	1445	G332D	Kinase domain
817 (*k1-2*)	C → T	571	P169S	LysM3 motif of ECD
2265 (*k1-3*)	C → T	242	S59F	LysM1 motif of ECD

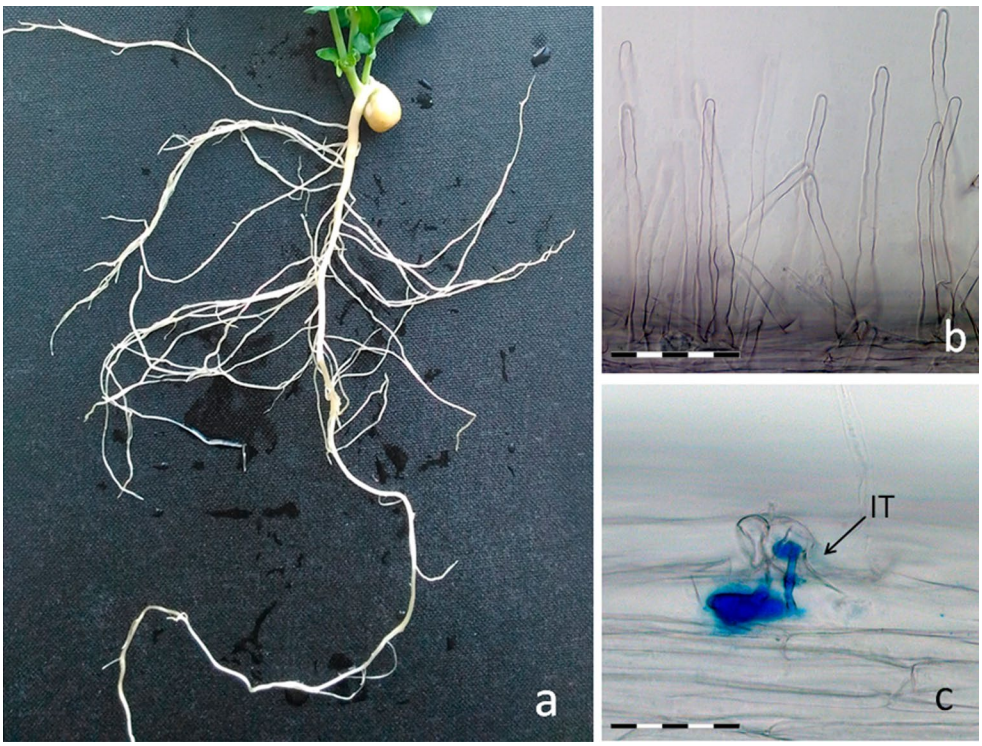
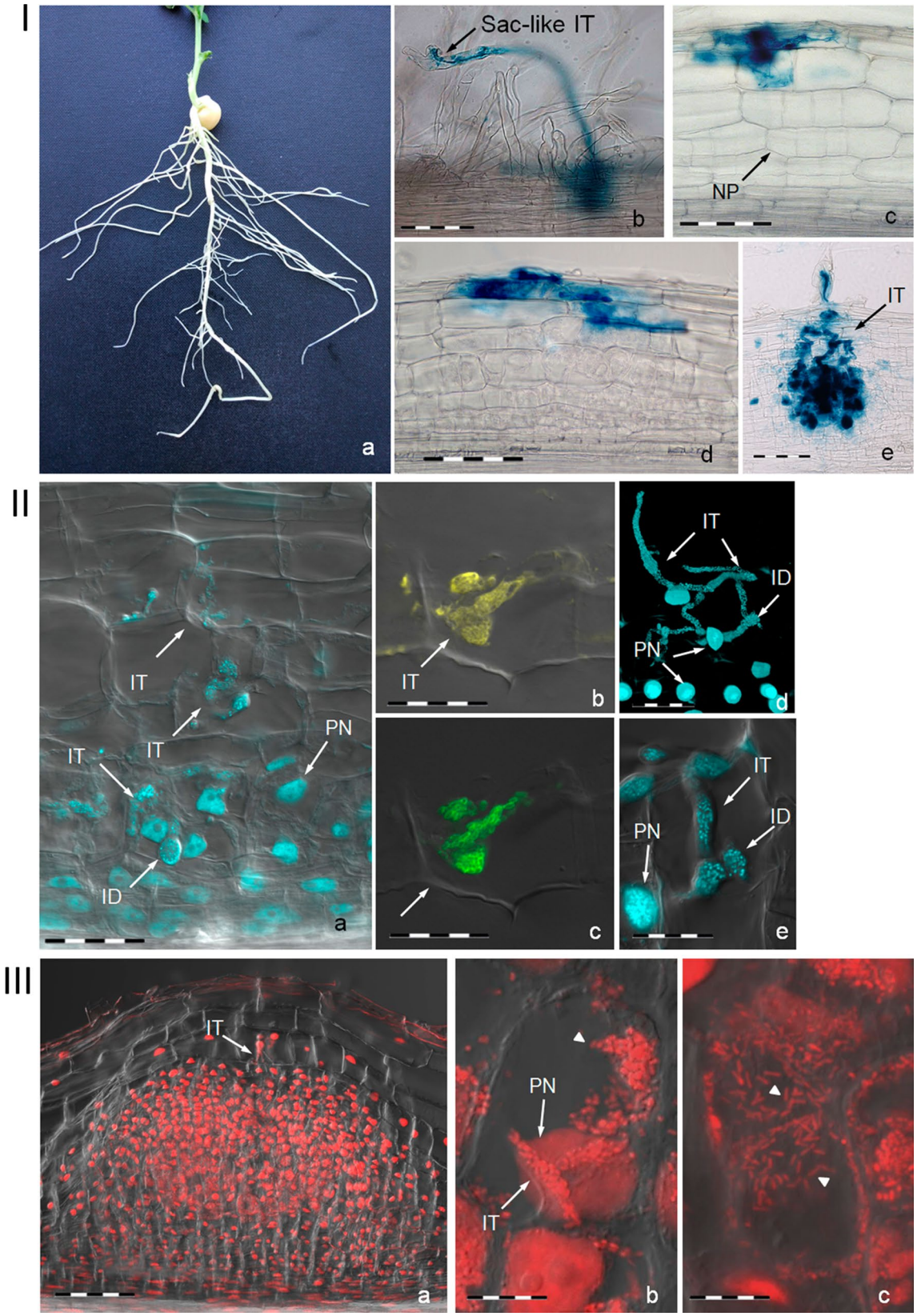


Fig. 1 Histochemical staining and microscopy analysis of *k1-1* mutant line. **a** Light micrograph of *k1-1* mutant roots inoculated with *R. leguminosarum* bv. *viciae* RCAM 1026-*gusA* at 21 dai. No nodule formation was observed. **b** Typical view of root hairs without deformations. **c** Light micrograph of rare short-aborted IT in small root hair. Scale bars = 200 µm (**b**) and 50 µm (**c**). *IT* infection thread


I
Sac-like IT
NP
IT
a
b
c
d
e
II
IT
IT
IT
PN
ID
IT
ID
PN
a
b
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e
III
IT
PN
IT
a
b
c

◂**Fig. 2** **I** Microscopical analysis of *k1-2* mutant line. **a** Roots of *k1-2* mutant were inoculated with *R. leguminosarum* bv. *viciae* RCAM 1026-*gusA* at 21 dai. No nodule formation was observed. **b** Light micrograph of a root-hair curling containing microcolony and sac-like IT. **c**, **d** Light micrographs of nodule primordium formation and agarose sections. **e** Light micrographs of transcellular growth of sac-like IT with no bacteria release, agarose sections. Scale bars = 50 µm (**b**, **c**), 100 µm (**d**, **e**). **II** Histochemical staining of *k1-2* mutant line. **a** IT and infection droplet (ID) (bacteria and nuclei are blue) showed the absence of bacterial release from the IT, white arrows indicate ITs and ID. **b**, **c** IT and absence of bacterial release from the IT (IT is yellow; bacteria are green). **d** 3D reconstruction of IT growth (bacteria and nuclei are blue) showed the absence of bacterial release from the IT; white arrows indicate ITs and ID. **e** IT and absence of bacterial release into plant cell from the IT. Scale bars = 20 µm (**a**), 10 µm (**b**, **c**), 30 µm (**d**), and 20 µm (**e**). **III** Histochemical staining of Cameor wild-type roots. **a** Nodule primordium formation and IT growth. **b** IT and bacterial release from the IT into plant cell. **c** bacteria into plant cell (white arrowheads indicate bacteria). Scale bars = 100 µm (**a**) and 15 µm (**b**, **c**). *NP* nodule primordium, *IT* infection thread, *ID* infection droplet, *PN* plant nuclei

against bacterial cell wall components (Brewin et al. 1986) and MAC265 against arabinogalactan protein extensin as a major component of the infection thread lumen (van den Bosch et al. 1989; Tsyganova et al. 2009). It was based on viewing a large number of roots (see Materials and methods). Previously, immunochemical approach with antibodies MAC57 and MAC265 was helpful to indicate the defect in bacteria endocytosis in *M. truncatula* and pea mutants (Kitaeva et al. 2016). At the same time, analysis of wild-type roots revealed normal primordium development, IT growth, and bacteria release (Fig. 2III).

To check specificity of the LysM-RLK K1, we have also tested the effect of *R. leguminosarum* bv. *viciae* strain TOM carrying *nodX* and producing Nod factors with additional acetyl group on reducing end on nodulation of *k1-2* line. No nodules were observed on the roots of mutant line 3 weeks after inoculation, while 47.4 ± 9.1 nodules were found on the roots of wild-type plants.

Analysis of the *k1-3* mutant line (S59 → F replacement in the LysM1 motif) showed defects in development of the ITs due to the loss of their polar growth (sac-like structures) (Fig. 3a). Apparently, it resulted in a significant delay of infection and the emergence of a large number of developing nodules at the initial stage of infection at 14 dai. Total amount of ITs were higher in *k1-3* mutant line (322 ± 25.1 per plant on the *k1-3* line and 115.8 ± 12.3 in wild-type plants) at 14 dai.

Analysis of receptor interactions in various systems

The ability of LysM receptors from model legume plants to form protein complexes (like NFR5/NFR1 in *L. japonicus* and *Mt*NFP/*Mt*LYK3 in *M. truncatula*) (Madsen et al. 2010; Pietraszewska-Bogiel et al. 2013) encouraged us to investigate the possibility of hetero- and homo-oligomerization for different pea RLKs—SYM10, K1, and SYM37. Two different methods, such as transient leaf transformation and the yeast two-hybrid system (Y2H), have been used to analyze the interactions between different pea LysM-RLKs.

As was previously shown, attempts to carry out FRET analysis for LysM receptors in the leaves of *N. benthamiana* were unsuccessful, as co-expression resulted in the development of a strong HR, but this reaction itself provides evidence for the direct or indirect cooperation between proteins. The infiltration of constructs for the synthesis of pea proteins fused with fluorophores like YFP and RFP allowed us to check for correct subcellular localization in leaves. To follow the protein distribution in leaf tissues after mono-transformation, confocal laser-scanning microscopy was applied (Fig. 4a). All the analyzed full-sized K1-RFP/YFP, SYM10-YFP/RFP, and SYM37-YFP LysM-RLKs were localized at the plasma membrane of leaf cells, supporting their function as transmembrane receptors in pea plants (Fig. 4a, b). Synthesis of all tested SYM10, K1, and SYM37 LysM-RLKs at mono-transformation did not result in HR development. Three independent experiments were performed (10–12 plants in each experiment for variant). No HR development was found at the mono-transformation—K1-RFP 0/42 leaves, SYM10-YFP—0/44 leaves, and SYM37-YFP—0/37 leaves. To confirm the presence of synthesized proteins in the cells of *N. benthamiana*, Western-blot analysis with antibodies against RFP and YFP was carried out (Fig. 4b). The proteins with expected molecular weights were detected on the blots.

The next step was to perform the co-expression of LysM-RLKs in various combinations. It was shown that the co-expression of LysM-RLK pairs, like SYM10/K1 and SYM10/SYM37, resulted in the development of HR 48 h after transformation, followed by cell disruption in these zones in 5–6 days: SYM10-YFP/K1-RFP—45/46 leaves and SYM10-YFP/SYM37-YFP—42/44 leaves (Fig. 5). As a control, another receptor with an active kinase domain, SYM19 (orthologue of *Lj*SYMRK/*Mt*DMI2), normally activated at symbiosis initiation and during signal transduction was used (Schneider et al. 1999; Stracke et al. 2002). However, while the co-expression of SYM10 and SYM19 was noted, HR development was not observed (SYM10-YFP/SYM19-YFP—0/48 leaves) (Fig. 5). Since mono-expression did not result in cell death, we may consider the HR as a specific response to the co-expression of tested LysM-RLKs.

HR development in *N. benthamiana* leaves during SYM10/K1 and SYM10/SYM37 co-expression suggests that these LysM-RLKs may cooperatively participate in the control of definite stages of symbiosis development.

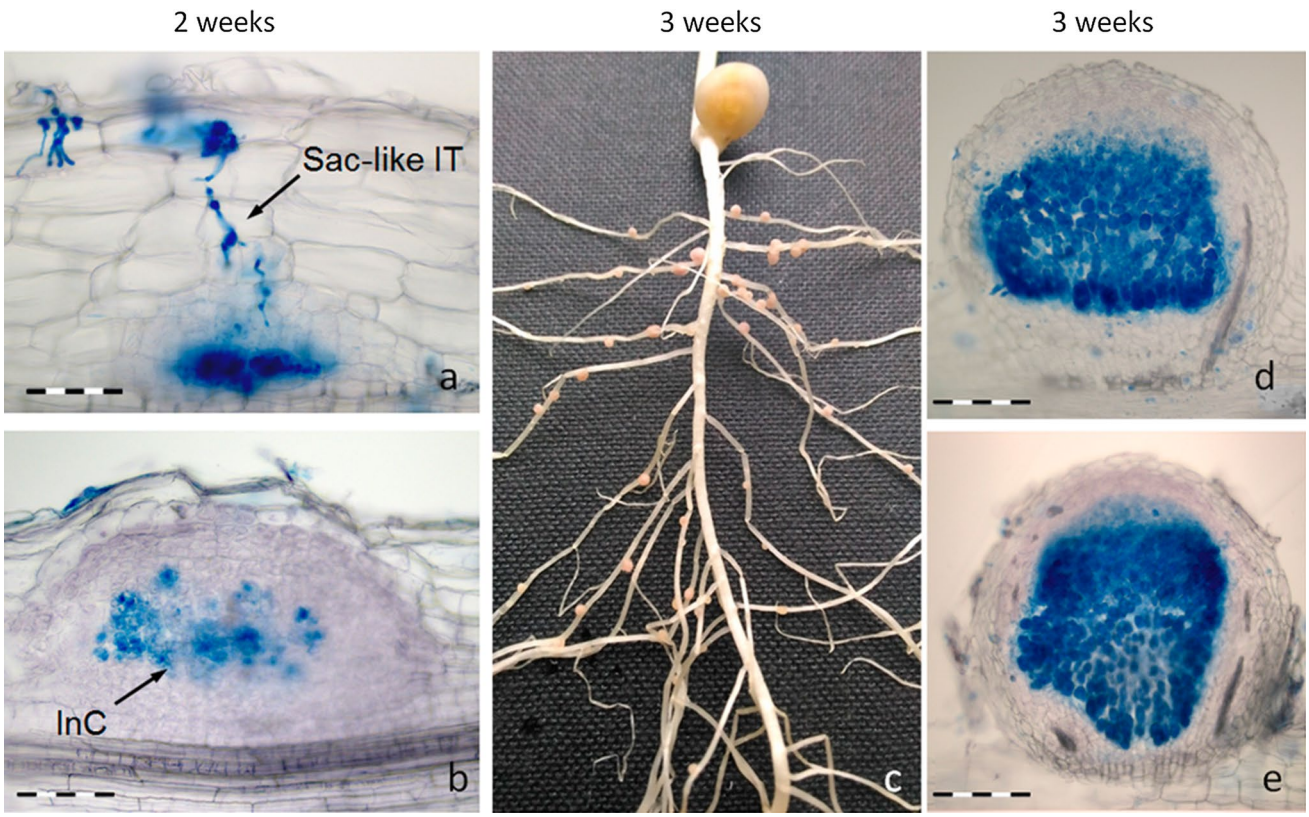

Fig. 3 Histochemical staining and microscopy analysis of *k1-3* mutant line. **a** Light micrograph of longitudinal agarose sections (50 µm) of *k1-3* mutant line at 14 dai, formation of nodule primordium, and sac-like IT development. **b** Infected developing nodules on *k1-3* mutant roots inoculated with *R. leguminosarum* bv. *viciae* RCAM 1026-*gusA* at 14 dai. **c** Roots of *k1-3* line inoculated with *R. leguminosarum* bv. *viciae* RCAM 1026-*gusA* at 21dai. **d** Light micrograph of a longitudinal agarose sections (60 µm) of *k1-3* mutant line nodule at 21 dai. **e** Longitudinal agarose sections (60 µm) of wild-type Cameor nodule inoculated with rhizobia expressing *GUS* at 21 dai. Scale bars = 50 µm (**a**, **b**), 100 µm (**d**, **e**). *IT* infection thread, *InC* infected cell

Table 2 Root-hair deformations and curling, infection thread (IT) growth in wild-type Cameor and *k1-1*, *k1-2,* and P56 (*sym10*) mutant lines during inoculation with *R. leguminosarum* strain RCAM 1026—*gusA*

Variant	Root-hair deformations and curling	Infection thread growth	Nodules per plant
Wild-type Cameor	301.7 ± 13.4	18.3 ± 2.8	46.6 ± 1.2
885 (*k1-1*)	4.8 ± 1.3	2.8 ± 0.9[a]	0
817 (*k1-2*)	125.2 ± 11.2	142.2 ± 4.7 (101.8 ± 6.6)[b]	0
P56 (*sym10*)	1.45 ± 0.46	0	0

Data represent the number (± SE) of deformed and curled root hairs as well as ITs at 21 dai in 100 visual fields of the microscope. Mutants differ significantly ($P > 0.95$) from wild-type

[a]For *k1-1* line, only short-aborted ITs were found in small root hairs and no nodules (Nod$^-$ phenotype)

[b]For *k1-2* line, most of ITs were shown to be early aborted in epidermis (enclosed in brackets) and no nodules (Nod$^-$ phenotype). P56 mutant line (*sym10*) inoculated with *R. leguminosarum* strain RCAM 1026—*gusA* was used as control

Yeast two-hybrid system (Y2H)

To examine protein–protein interactions, we also used the Y2H, which allows us to estimate the strength of such interactions. Previously, the Y2H system was successfully applied to prove hetero- and homo-oligomerization between rice LysM-RLK *Os*CERK1 and *Os*CEBiP (Shimizu et al. 2010), and between *Os*CERK1 and other pattern recognition receptors *Os*LYP4 and *Os*LYP6 (Kouzai et al. 2014).

Pea LysM-RLKs SYM10, K1, and SYM 37 have transmembrane domains and predicted signal peptides that can be recognized by the yeast transport system. To be quite sure that the pea RLKs are expressed in the yeast nucleus and not transferred into the yeast membrane, we performed

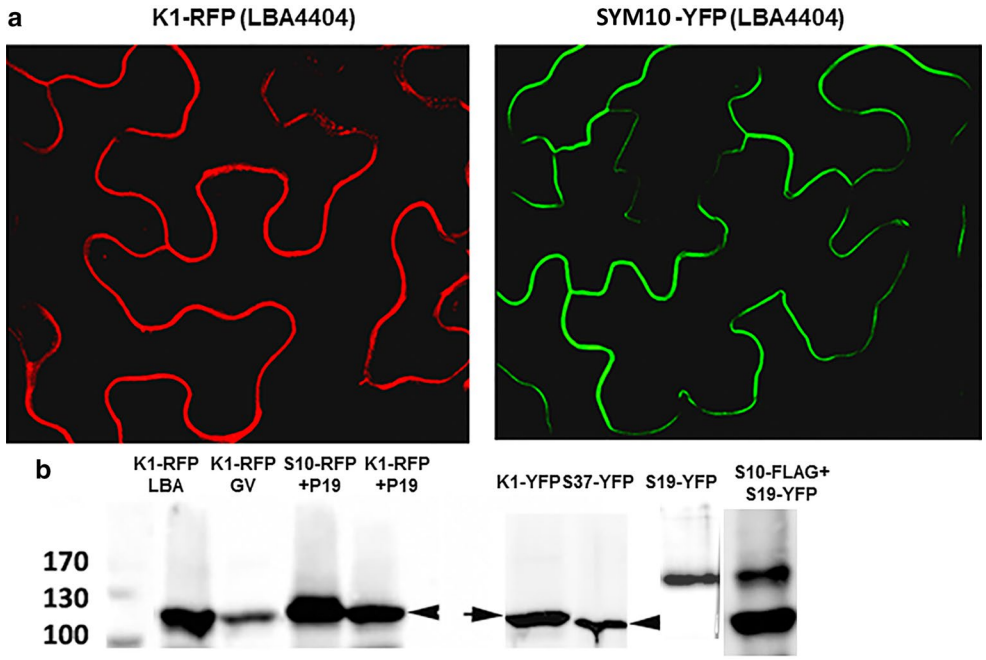

Fig. 4 Expression of K1-RFP and SYM10-YFP in leaves of *N. benthamiana* using the constructs under CaMV35S promoter. **a** The localization of synthesized full-size LysM-RLKs was connected with the plasma membrane of cells. No hypersensitivity reaction (HR) development was found at the mono-transformation. **b** Western-blot analysis of K1, SYM10, and SYM37 LysM-RLKs in leaves of *N. benthamiana* with anti-RFP and anti-YFP antibodies. For protein detection in *N. benthamiana* leaves, the solubilized proteins were separated by gel electrophoresis in the presence of SDS and visualized using HRP-coupled antibodies together with Immobilon staining solutions. Two different *A. tumefaciens* strains, LBA 4404 and GV3101, were used for the analysis. We found that the *A. tumefaciens* strain LBA4404 was more effective than GV3101

an interaction analysis using only the ECDs of SYM10, K1, and SYM37 (without signal peptides and transmembrane domains) (Fig. 6). It was shown that the ECD of SYM10 used as BAIT can interact with both K1-ECD and SYM 37-ECD. Similarly, the ECDs of K1 and SYM37 used as BAIT were able to interact with SYM10-ECD (data not shown). No capacity to form the K1-ECD/K1-ECD homo-oligomers was found (Fig. 6). At the same time, only a weak capacity to form the SYM37-ECD/SYM37-ECD homo-oligomers was demonstrated using the Y2H system. These data imply that pairs SYM10/K1 and SYM10/SYM37 have the potential to form hetero-oligomers through an interaction of their LysM ECDs. These results are in agreement with the data on transient leaf transformation for full-sized proteins.

Complementation test

To verify the impact of mutation in the *K1* gene on nodulation, the complementation of *k1-2* mutant line was performed with the wild-type *K1* gene. *A. rhizogenes*-mediated transformation of pea plants was carried out using pB7WG2D plasmid with the full-length *K1* gene under 35S promoter (*p35S::K1*). The same plasmid, pB7WG2D with *GUS* (*p35S::GUS*), was applied as a negative control. Transformation resulted in recovering the ability to form nodules (Fig. 7a, Table 3). This suggests that Nod$^-$ phenotype of this line is determined by the mutation in *K1* gene, but not by the influence of other mutations. When plants of *k1-2* mutant line have been transformed with pB7WG2D (*p35S::GUS*), they were not able to form nodules (Fig. 7c, Table 3). Moreover, transformation of the *k1-2* mutant line with the *K1* cDNA, which was constructed by site-directed mutagenesis and carried mutation resulting in P169 → S replacement, was performed. As it was expected, this construct failed to complement the *k1-2* line, confirming the importance of this mutation (Fig. 7d).

The proteins encoded by *Sym37* and *K1* genes have a high level of similarity (alignment of protein sequences showed that their kinase domains are up to 95% identical, while the predicted extracellular regions are more diverse—75% identical). Since we have demonstrated the possibility of the formation of two complexes (SYM10/K1 and SYM10/SYM37), it raises the question of whether the SYM37 and K1 receptors are functionally interchangeable in symbiosis. To assess this suggestion, the transformation for *k1-2* line defective in the *K1* gene with wild-type *Sym37* gene was also performed (Fig. 7b, Table 3). In addition, we transformed pea mutants Ris-Nod4 and K24 (Nod$^-$ phenotype), which were impaired in *Sym37* gene (Engvild 1987; Postma et al. 1988; Borisov

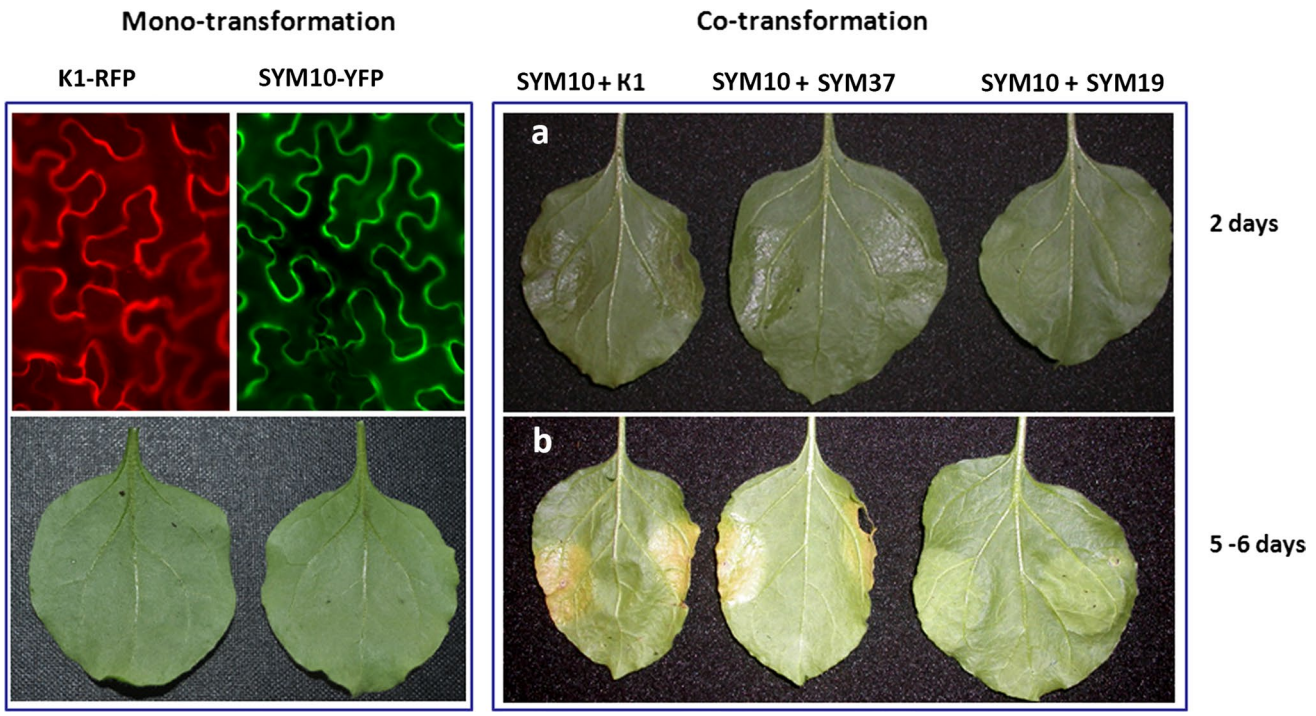

Fig. 5 Analysis of hypersensitivity reaction (HR) development at the transient expression of pea LysM-RLKs SYM10, K1 and SYM37 in *N. benthamiana* leaves. The receptors were expressed as fusion proteins using *A. tumefaciens*-mediated transient transformation of leaves *N. benthamiana.* SYM10-RFP and K1-YFP were localized at the plasma membrane when expressed alone (left panel, mono-transformation). The development of HR at the co-expression of SYM10 + K1 and SYM10 + SYM37 [right panel, 2 days after transformation (**a**); 5–6 days after transformation (**b**)]. No HR was found at the co-expression of SYM10 + SYM19. Three independent experiments were performed (10–12 plants in each experiment for variant). No HR development was found at the mono-transformation K1-RFP—0/42 leaves, SYM10-YFP—0/44 leaves, SYM10-YFP/K1-RFP—45/46 leaves, SYM10-YFP/SYM37-YFP—42/44 leaves, and SYM10-YFP/SYM19-YFP—0/48 leaves

et al. 2007) with wild-type *Sym37* and *K1* genes (Fig. 8a, b, d, e, Table 3).

Following inoculation with *R. leguminosarum* bv. *viciae* RCAM 1026, no nodule formation was observed on the transgenic roots of *k1-2* pea plants carrying the pB7WG2D (*p35S::Sym37*) construct (Fig. 7b, Table 3). It suggests that *Sym37* was not probably able to restore the function of *K1* in symbiosis or regulated in a specific way through regulatory elements in its promoter upon rhizobial infection.

As it was expected, nodule formation was observed on the transgenic roots of RisNod4 and K24 (*sym37*) pea plants carrying the pB7WG2D (*p35S::Sym37*) construct (positive control) (Fig. 8b, e, Table 3). However, we were surprised that nodule development was observed on the roots of both RisNod4 and K24 mutant plants transformed with *p35S::K1* (Fig. 8a, b, Table 3). Thus, when *K1* had been introduced into *sym37* mutant plants and expressed at high level, it was able to restore the function of *Sym37*.

Discussion

Here, we describe a transmembrane receptor-like kinase necessary for pea-rhizobial symbiosis development. It has been proposed that Nod factor perception in pea is mediated by two receptors differing in specificity (Walker and Downie 2000). Recent findings in investigations of most membrane receptors in plants, including the LysM-RLKs, demonstrate that the formation of homo- and heteromeric complexes might be essential for ligand binding and the phosphorylation of kinase domains that result in the initiation of downstream signaling (Kaku et al. 2006; Miya et al. 2007; Shimizu et al. 2010; Willmann et al. 2011; Liu et al. 2012a, b; Kouzai et al. 2014; Miyata et al. 2014; Cao et al. 2014). Pea mutants in the *sym10* gene (P5, P56, RisFixG, and N15) (Duc and Messager 1989; Kneen et al. 1994; Sagan et al. 1994; Schneider et al. 2002) were completely blocked in all responses to inoculation, suggesting

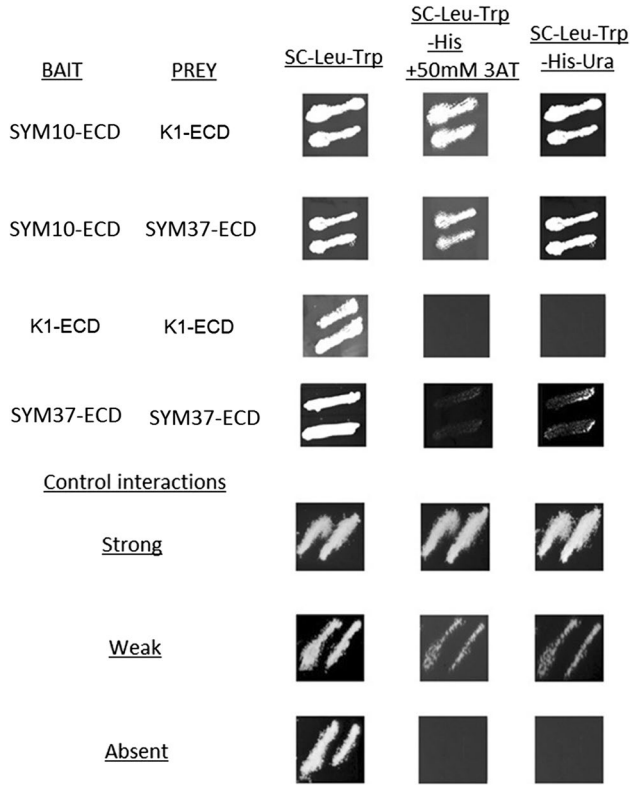

Fig. 6 Yeast two-hybrid analysis for pea LysM-RLKs. A yeast two-hybrid assay was performed using the extracellular domains of K1-ECD, SYM37-ECD and SYM10-ECD as bait or prey. Yeast growth on selective medium-lacking leucine and tryptophan SD-Leu-Trp indicated the presence of both bait and prey constructs. Interaction was tested on medium additionally lacking histidine and uracil (SD-Leu-Trp-His-Ura) or supplemented with 3-amino-1,2,4-triazole (3AT) (SD-Leu-Trp-His + 50 mM 3AT). As controls, a few pairs of vectors (pEXP32/Krev1 and pEXP22/RalGDS-wild type, pEXP22/RalGDS-m1, and pEXP22/RalGDS-m2) suggested by the manufacturer were used for strong, weak, and not detectable interactions (Thermo Fisher Scientific)

the involvement of this receptor in symbiosis initiation. However, the evidence that SYM10 has an apparently inactive kinase necessitates an explanation of how SYM10 might function at symbiosis initiation. The most probable explanation is that SYM10 associates into the complex with an additional receptor that has a functional kinase for signal transduction. The recently identified LysM-RLK K1 became a possible candidate for such analysis. K1 showed a high level of protein sequence homology with *Ps*SYM37 (85.7%), as well as with *Mt*LYK3 (83.7%) and *Mt*LYK2 (80.3%), *Lj*NFR1 (78%), *Gm*NFR1a (75.7%), and *Gm*NFR1b (71.6%) from *Glycine max* (Supplemental Fig. S3). In addition, K1 has specific YAQ motif in the kinase domain that makes it suitable candidate for a role of symbiotic receptor (Nakagawa et al. 2011).

To analyze the role of *K1* in symbiosis, we detected mutants in this gene and studied their phenotypes. Two non-nodulating Nod$^-$ mutant lines (*k1-1* and *k1-2*), defective in the *K1* gene, were identified in pea. This fact has confirmed the hypothesis about the essential role of *K1* in symbiosis development. Moreover, when wild-type *K1* gene was introduced in *k1-2* mutant line, it was able to restore the nodulation. The construct with the same mutation resulting in P169 → S replacement failed to complement the *k1-2* line. The mutation in the *k1-1* line resulted in G332→D replacement in the K1 LysM-RLK kinase domain (the nucleotide-binding glycine-rich loop). Similarly, the replacement G334→E in the *Mt*LYR3 kinase domain was found in B56 mutant (*hcl-1*), which was impaired in the *Mtlyk3* gene, and led to a complete loss of activity (Smit et al. 2007; Klaus-Heisen et al. 2011). However, in contrast to the B56 mutant (*hcl-1*) (Catoira et al. 2001), no extensive root-hair branching or swelling of the root-hair tips were found in the pea *k1-1* mutant line. Pea non-nodulating mutants, RisNod4 and K24, are affected in the homologous *sym37* gene, but they have also demonstrated extensive root-hair curling, as well as microcolony formation (Tsyganov et al. 2002; Zhukov et al. 2008). Thus, blocking of symbiosis development seems to occur in earlier stages in the *k1-1* line, when compared with the B56 (*hcl-1*) or RisNod4 and K24 (*sym37*) mutants. Therefore, the phenotype of the *k1-1* line was more similar with that of the *Lotus nfr1-1* and *nfr1-2* mutants, which were impaired in almost all responses to rhizobial inoculation (Radutoiu et al. 2003). As a very rare event, the appearance of short-aborted ITs connected with individual small root hairs and blocked in the epidermal layer was found in the *k1-1* mutant line. Previously, in pea plants inoculated with the *R. leguminosarum* bv. *viciae* deletion mutant Δ*nodFELMNTO* that retained only the *nodD* and *nodABCIJ* genes and secreted the most basic Nod factors, the intracellular accumulation of rhizobia was revealed at the base of root hairs (Walker and Downie 2000). Such aberrant infection seems to be the result of intracellular epidermal penetration; moreover, a *nodE nodO* double mutant in contrast to deletion mutant Δ*nodFELMNTO* formed a few short-aborted ITs per root. Probably, the low stringency conditions for rhizobial entry in pea may be the result of some differences in phenotypic manifestations between the *k1-1* mutant line and the *Lotus nfr1-1* and *nfr1-2* mutants. At the same time, the differences in phenotypic manifestations may be due to the fact that the *k1-1* line has a missense, not nonsense mutation. If it would be a case, the synthesis of truncated protein in mutant line should result in more severe phenotype. In addition, it is impossible to exclude the possibility of partial concomitant activation of the signal transduction pathway through common components by other receptors in *k1-1* mutant (due to the functional redundancy).

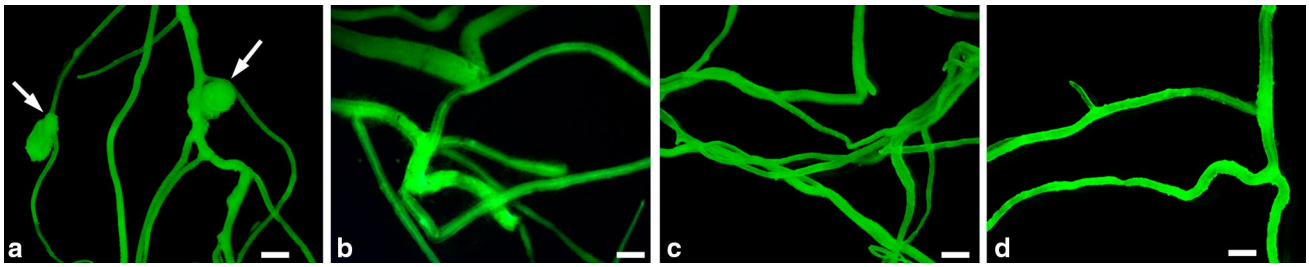

Fig. 7 Complementation test for *k1-2* mutant line with the wild-type *K1* gene using pB7WG2D (*p35S::K1*) (**a**), pB7WG2D (*p35S::Sym37*) (**b**), pB7WG2D (*p35S::GUS*) (negative control (**c**), and pB7WG2D (*p35S::k1-2)* (**d**). Transformed plants were inoculated with *R. leguminosarum* bv. *viciae* RCAM 1026. Nodulation was estimated at 28 dai. Totally, 17 roots of pB7WG2D (*p35S::K1*) (8.52 ± 1.05 nodules per root) ($P < 0.05$ in *t* test), 54 roots of pB7WG2D (*p35S::Sym37*) (no nodule formation), 15 roots of pB7WG2D (*p35S::GUS*) (no nodule formation), and 35 roots of pB7WG2D (*p35S::k1-2*) (no nodule formation) were analyzed in two–three independent experiments. White arrows indicate nodules. Scale bars = 2 mm

Table 3 Complementation for pea 817 (*k1-2*), RisNod4 (*sym37)* and K24 (*sym37*) mutant lines with the wild-type *K1* and *Sym37*

Variant	Construct	Number of plants with transgenic roots	Number of transgenic roots	Number of nodules per root
Cameor	pB7WG2D (p35S::GUS)	10	18 roots	9.67 ± 1.73
817 (*k1-2*)	pB7WG2D (*p35S::K1*)	12	17 roots	8.52 ± 1.05
	pB7WG2D (*p35S::Sym37*)	17	54 roots	No nodules
	pB7WG2D (*p35S::κ1-2*)	13	35 roots	No nodules
	pB7WG2D (*p35S::GUS*)	10	15 roots	No nodules
Finale	pB7WG2D (p35S::GUS)	14	21 roots	8.95 ± 1.58
RisNod4 (*sym37*)	pB7WG2D (*p35S::K1*)	14	23 roots	9.54 ± 1.71
	pB7WG2D (*p35S::Sym37*)	15	20 roots	9.72 ± 1.55
	pB7WG2D (*p35S::GUS*)	11	25 roots	No nodules
K24 (*sym37*)	pB7WG2D (*p35S::K1*)	15	21 roots	9.73 ± 2.05
	pB7WG2D (*p35S::Sym37*)	16	22 roots	9.92 ± 2.16
	pB7WG2D (*p35S::GUS*)	12	27 roots	No nodules

An alternative explanation may be suggested that, in *k1-1* mutant, the infection process was interrupted at later stage after initiation of IT development, but a negative feedback inhibition of root-hair deformations had already activated. However, the percent of deformed root hairs should be comparable with wild type, but it was not the case for *k1-1* mutant, as its phenotyping showed.

The mutation in the *k1* gene (*k1-2* line) resulted in P replacement by S, which is located in the LysM3 motif. As a result, an absence of nodules and multiple sac-like ITs without bacterial output has been found. Therefore, this amino acid seems to be important for the overall structure of this LysM3 motif or the entire extracellular region. Alternatively, it may be important for protein–protein interactions at complex formation. A similar phenotype with *k1-2* line was observed in the *M. truncatula* AC6 (*hcl-4*) mutant impaired in the *Mtlyk3* gene (Smit et al. 2007), which efficiently formed root-hair curls containing microcolonies of *S. meliloti.* As in the *k1-2* line, IT formation had initiated; however, sac-like infection structures filled with *S. meliloti* had also formed. It should be noted that *hcl-4* (a weak allele) is able to form 8.2% of normal nodules (Smit et al. 2007), but no nodules were observed in the pea *k1-2* mutant line, which correlates with the absence of rhizobia penetration in the *k1-2* line. Therefore, although *MtLYK3/Ps*Sym*37* and *PsK1* are highly homologous, the mutants in these genes with weaker phenotypes seem to also have different manifestation.

Our data showed the possibility of a cooperative effect between full-length SYM10 and K1 during the co-expression in a heterologous system, such as in the leaves of *N. benthamiana*, which resulted in the development of HR, causing a local effect of cell death. This allows us to conclude that the interaction between these two LysM-RLKs may take place, as a similar response was observed at the co-expression of the LysM-RLKs *Lj*NFR1/*Lj*NFR5 and *Mt*NFP/*Mt*LYK3 (Madsen et al. 2011; Pietraszewska-Bogiel et al. 2013), which are capable of forming a heterodimeric complex (Moling et al. 2014).

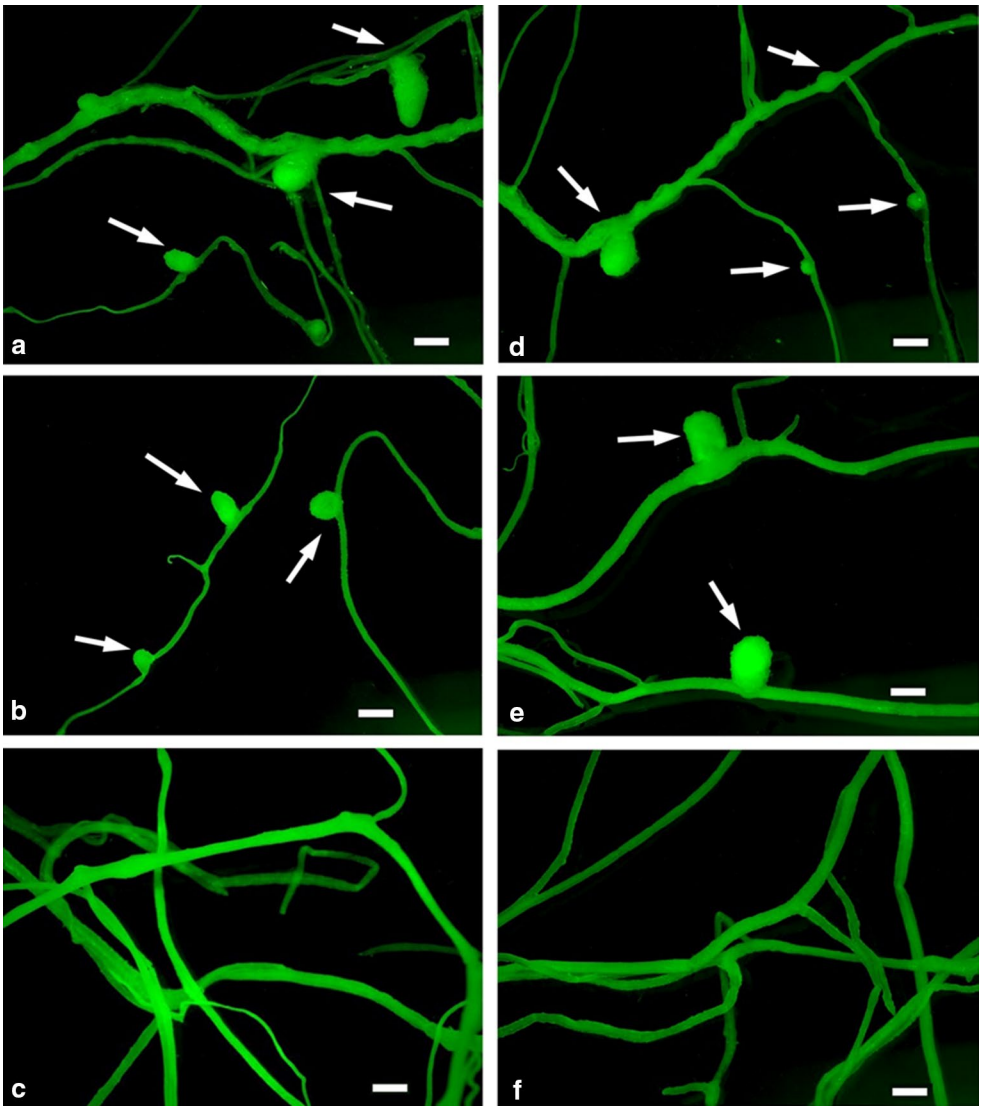

Fig. 8 Complementation test for pea K24 (**a**–**c**) and RisNod4 (**d**–**f**) mutant lines with the wild-type *Sym37* gene using pB7WG2D (*p35S::K1*) (**a**, **d**), pB7WG2D (*p35S::Sym37*) (**b**, **e**) (positive control), and pB7WG2D (*p35S::GUS*) (**c**, **f**) (negative control). Transformed plants were inoculated with *R. leguminosarum* bv. *viciae* RCAM 1026. Nodulation was estimated at 28 dai. For K24 line, in total, 21 roots of pB7WG2D (*p35S::K1*) (9.73 ± 2.05 nodules per root), 22 roots of pB7WG2D (*p35S::Sym37*) (9.92 ± 2.16 nodules per root), and 27 roots of pB7WG2D (*p35S::GUS*) (no nodules formation) ($P < 0.05$ in *t* test) were analyzed in two independent experiments. For RisNod4 line, totally, 23 roots of pB7WG2D (*p35S::K1*) (9.54 ± 1.71 nodules per root), 20 roots of pB7WG2D (*p35S::Sym37*) (9.72 ± 1.55 nodules per root) ($P < 0.05$ in *t* test), and 25 roots of pB7WG2D (*p35S::GUS*) (no nodules formation) were analyzed in two independent experiments. White arrows indicate nodules. Scale bars = 2 mm (**a**, **b**, **d**, **e**), 1 mm (**c**, **f**)

At the same time, we observed the development of HR at the co-expression of *Sym10* and *Sym37*, indicating that SYM10 is capable of interacting with two structurally similar LysM-RLKs: K1 and SYM37. If the SYM10 protein is capable of forming a complex with SYM37, which is necessary for infection, it should also be involved in infection control. There is no direct evidence indicating that SYM10 is involved in such process; however, there is support for the ortholog of *Sym10*, *MtNFP*. It is known that *nfp* mutants, as well as pea *sym10* mutants, are characterized by a complete blockage of symbiosis development; however, the analysis of knockdown transgenic plants in the *MtNFP* gene, as well as swap experiments, showed that this LysM-RLK controls not only the early stages of symbiosis, but it also regulates IT growth, as well as *Mt*LYK3 (Arrighi et al. 2006; Bensmihen et al. 2011). Furthermore, FRET analysis recently showed that *Mt*NFP and *Mt*LYK3 are, indeed, able to form a complex in vivo, and that this complex controls infection development (Moling et al. 2014).

Using a two-hybrid system, we observed an interaction between SYM10-ECD and K1-ECD, as well as between SYM10-ECD and SYM37-ECD, what is correlated with the results of transient leaf expression. Although the cooperative effect of whole proteins was estimated at the leaf expression, while the interaction of the reduced ECDs was checked in a Y2H, the data of two experiments regarding protein production in heterologous systems suggest a possible interaction between SYM10 and K1, as well as between SYM10 and SYM37. Therefore, the most likely current working model includes two different receptor complexes, SYM10/K1 (I) and SYM10/SYM37 (II), formed during the course of symbiosis development in pea, and apparently activated at different stages of symbiosis. Although this does not exclude the possibility that the receptors may work independently, but their concomitant signaling is necessary. In this case, two-receptor complexes can be activated at the same time and complement the work of each other.

In *sym37,* mutant infection was blocked at the stage of IT initiation in root hairs (Zhukov et al. 2008). A similar function was suggested for hypothetical protein SYM2 (Geurts et al. 1997). Interaction of *R. leguminosarum* bv *viciae* strains lacking *nodX* with *sym2*^{A}-harboring peas was specifically arrested in the infection process initiation. It may suggest more complicated nature of the complex SYM10/SYM37 and involvement of an additional component like SYM2 in its formation (Fig. 9). Similarly, in *M. truncatula,* LysM-RLK LYK4 may play an additional role in the regulation of IT development together with LYK3 (Limpens et al. 2003).

The interaction of LysM-RLKs was observed in the absence of the ligand Nod factor. In our experimental conditions, we expressed the LysM-RLKs genes under a cauliflower mosaic virus (CaMV)35S promoter that may provide a high level of protein synthesis at the membrane and increases the possibility of their physical contact. However, in yeast, the level of protein synthesis was not very high, but we were able to show an interaction between ECD of SYM10/K1 and SYM10/SYM37. This suggests that the shape complementarity of protein interfaces may be more important and it enables their mutual recognition. To assess the accuracy of this suggestion, it is necessary to identify those amino acids that may be important for protein/protein interactions, as well as for possible interactions with the ligand Nod factor. Nod factor ligand may stabilize the receptor complex in this case.

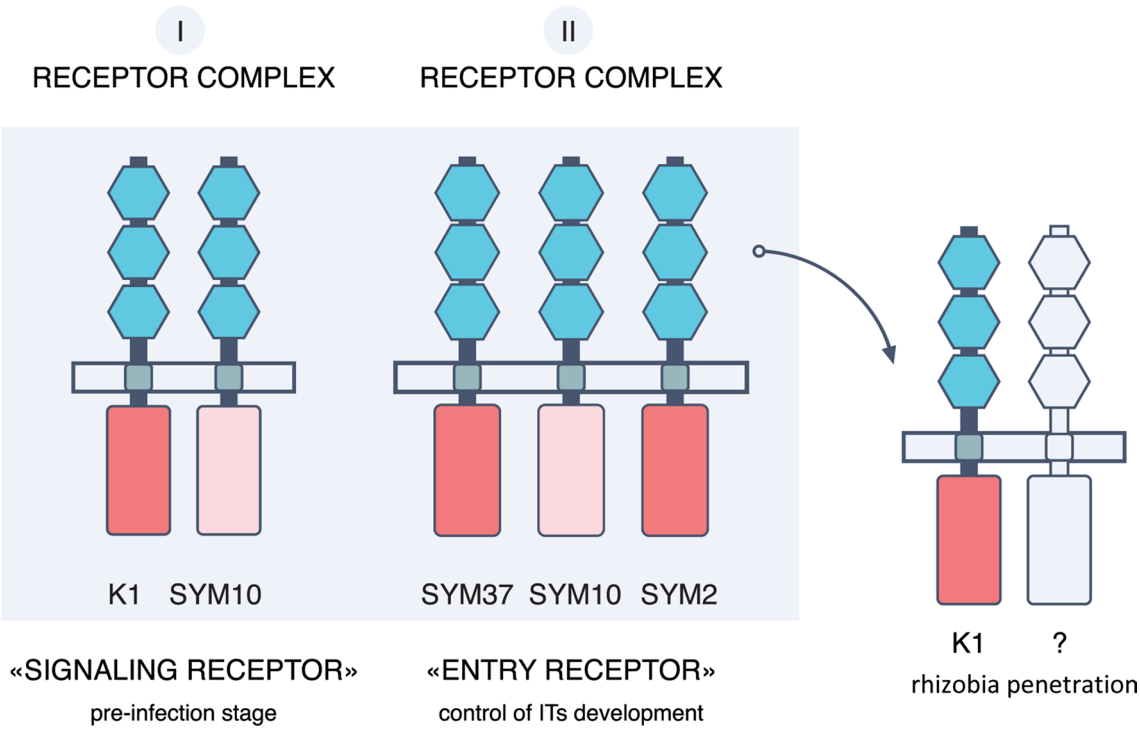

Fig. 9 Model of receptor complexes organization in *P. sativum* L. Complex SYM10/K1 (I) is required for initiation of symbiosis. SYM10 and SYM37 (II) may participate in the regulation of infection process and development. Since interaction of *sym2*^{A}-harboring peas (Afghan lines) and *R. leguminosarum* bv *viciae* strain-lacking *nodX* was specifically arrested in the infection process initiation, it may suggest a more complicated nature of the complex II and involvement of an additional component like SYM2 in formation of SYM10/SYM37/SYM2 (II). This model does not exclude the possibility that the receptor complexes may work independently, but their concomitant signaling is necessary. An activation of these complexes may be related to the appearance of an additional signal, which is required for bacteria output from ITs. This stage may be under control of K1 and its hypothetical co-receptor (III)

The structural similarity between SYM37 and K1 raises the question of whether these receptors are functionally interchangeable in symbiosis. To test this, a transformation of non-nodulating *k1-2* mutant and *sym37* mutants with a construct for the synthesis of wild-type full-length K1 and SYM37 proteins under the CaMV35S promoter was performed. Transformation of *k1-2* mutant with the wild-type *Sym37* gene did not result in recovering the ability of this mutant to form nodules. However, as the full-length *K1* gene was introduced to *sym37* mutants, these plants were able to form symbiotic nodules. Therefore, we can conclude that the receptors K1 and SYM37 fulfill various functions or activated at different stages of symbiosis. The alternative interpretation of such effect could be due to the differences in the expression levels of the *K1* and *Sym37* genes in the complemented pea lines and original mutants. It could be a probable explanation, although there were no significant variations in the synthesis of K1 and SYM37 using constructs under CaMV35S promoter in other system like *N. benthamiana* leaves. At the same time, it cannot be ruled out that the *Sym37* gene is regulated in a specific way through regulatory elements in its promoter during symbiosis. Therefore, the future experiments should clarify the interplay between K1 and SYM37 receptor proteins in regulation of various stages of symbiosis.

Based on our experimental work, it currently can be explained that K1 is involved not only in initiation of the symbiosis in pea (in the complex with SYM10), but also controls development of ITs in cortex and release of bacteria into plant cells (probably in the complex with unknown receptor) (Fig. 9). It can be assumed that K1 is repeatedly reactivated after SYM10/K1 (I) and SYM10/SYM37/SYM2 (II) complexes formation and, therefore, able to restore the function of the SYM37 (Fig. 9). However, in *sym37* mutants, *K1* gene is not impaired, but not able to functionally replace *sym37*, because these mutants are non-nodulating (in contrast to *sym37* mutant plants transformed with *p35S::K1*). We suggest that the lack of recoverability in *sym37* mutants by *K1* may be due to the fact that activation of SYM10/K1 and SYM10/SYM37/SYM2 receptor complexes should provide signal, which is involved in the activation of events that lead to the release of bacteria with the participation of K1. In accordance with this suggestion, the analysis of the *k1-2* and *k1-3* mutant lines showed the importance of K1 for ITs distribution in root cortex and rhizobial release from ITs. The appearance of additional signal and participation of new receptors is well correlated with the previous data about that the cortical cell division and pre-ITs formation in cortex in pea can strictly dependent on the Nod factors' specific acyl chain C18:4 and O-acetyl group (Spaink et al. 1991; van Spronsen et al. 1994). To address this question in detail, the inoculation of the *k1* mutants by rhizobial strains like *nodE* and *nodO* producing modified Nod factors is clearly necessary to resolve the specificity of the receptor complexes. As a first step, we have initiated the experiments with TOM strain-carrying *nodX* that was not able to restore the phenotype of the *k1-2* mutant. It may suggest that K1 is not involved in the recognition of an additional acetyl group at the reducing end of *R. leguminosarum* bv. *viciae* Nod factors.

Author contribution statement ANK and EAD conceived and designed the experiments; MD, CLS, RT, CS, AB performed the screening of TILLING collection and SIFT analysis; ANK and EAD drafted the manuscript; YBP, NVM and GAA contributed materials for experiments; IAT and EAD coordinated experiments and edited the manuscript.

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Compliance with ethical standards

Conflicts of interest No conflicts of interest declared.

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