AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 3, pp. 465-474
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Symbiotic interactions

UDC 635.64:573.6.086.83:577.21]:58

doi: 10.15389/agrobiology.2021.3.465eng
doi: 10.15389/agrobiology.2021.3.465rus

PRODUCTION AND ANALYSIS OF COMPOSITE TOMATO PLANTS *Solanum lycopersicum* L. CARRYING PEA GENES ENCODING THE RECEPTORS TO RHIZOBIAL SIGNAL MOLECULES

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by the Russian Science Foundation (grant No. 16-16-10043)
Received December 30, 2020

Abstract

The development of legume-rhizobial symbiosis is based on signal exchange between partners, which ensures their mutual recognition and activation of the infection process and the program of nodule organogenesis. In this regard, it is of great interest to study the possibility of acquisition by non-legume plants of the ability to perceive lipochito-oligosaccharide signal molecules of rhizobia, the Nod factors, and subsequent activation of signal transduction pathway. To study this possibility in our work, we carried out the transfer of the genes encoding receptors to Nod factors of legume plant pea *Pisum sativum* L. into tomato *Solanum lycopersicum* L. (Carmello cultivar) using the transformation with *Agrobacterium rhizogenes*. In pea, two receptor kinases, SYM10 and K1, were previously identified, which are necessary for the recognition of Nod factors during the initiation of symbiosis with rhizobia. Upon reception of Nod factors, a complex is formed between these two receptor kinases, which leads to signal transduction. In the present work, we carried out the transfer of two genes encoding LysM-RLK SYM10 and K1 in pea *P. sativum* into tomato plants *S. lycopersicum* using agrobacterial transformation. In composite plants transformed with *PsSym10* or *PsK1* genes, the possibility of expression activation of introduced receptor genes in response to inoculation with a typical rhizobial strain *Rhizobium leguminosarum* bv. *viciae* CIAM1026 was shown. It was also shown that, under the influence of receptors in genetically transformed roots of composite plants, the expression of genes is increased, which can be regulated by components of the "common" signal pathway. The aim of this work was to study the possibility of acquiring the ability of *S. lycopersicum* plants to recognize signal molecules of rhizobia after transfer of the genes encoding receptors for Nod factors in the legume plant *P. sativum*. Two types of constructs in the pKm43GW vector were obtained and used, in which the *PsSym10* or *PsK1* genes encoding receptors were cloned under the pSlEXT1 promotor of tomato extensin gene — pSlEXT1::*PsSym10-3xFLAG*::T35S and pSlEXT1::*PsK1-RFP*::T35S. Young tomato seedlings of *S. lycopersicum* cv. Carmello were transformed with the *Agrobacterium rhizogenes* Arqua 1 strain. The transformed seedlings were placed on Murashige-Skoog (MS) agar medium without sucrose in Petri dishes and cultured in an upright position in a phytotron until callus is appeared. After that, the plants were transferred to MS medium with 3 % sucrose containing 0.3 mg/ml of the antibiotic cefotaxime and incubated until transgenic roots are appeared. Composite plants were transferred into vermiculite poured with 0.5× Fahreus medium and incubated under high humidity conditions for 2-3 days. The plants were then inoculated with *R. leguminosarum* bv. *viciae* CIAM1026 containing the *uidA* glucuronidase gene (*GUS*). For the analysis we used transformed roots of composite tomato plants without rhizobial inoculation (control, 7 days), as well as transformed roots at 7 and 21 days after inoculation. The analysis of gene expression was performed by quantitative PCR combined with reverse transcription (RT-PCR). In genetically transformed roots of tomato plants the expression of both *PsSym10* and *PsK1* genes was observed under the pSlEXT1 promoter, moreover the expression was enhanced under the influence of rhizobial inoculation. A significant (approximately 2.0-2.5-fold) increase in the expression of the *PsSym10* gene was shown in response to inoculation with rhizobia both at 7 and 21 days. The level of *PsK1* expression was found to be the highest 7 days after inoculation in the transformed roots of composite tomato plants as compared to the control. To determine whether the components of the "common" signal pathway will be activated under the influence of transferred receptors in composite tomato plants, the changes in the expression of *S. lycopersicum SlD27*, *SlNSP2*, *SlRAM1*,

and *SlMAPK6* genes were assessed. These genes encode carotenoid isomerase (DWARF27) which regulates the synthesis of the hormones strigolactones, transcription factors NSP2 and RAM1, and mitogen-activated protein kinase (MAPK6). Activation of the expression of two genes, the *SlNSP2* and *SlMAPK6* in response to inoculation may indicate the effect of the introduced pea *K1* gene on the susceptibility of tomato plants to rhizobial inoculation.

Keywords: legume-rhizobial symbiosis, receptor-like kinases, Nod factors, composite plants, gene expression.

The ability for symbioses with *Rhizobiales* nitrogen-fixing bacteria called rhizobia is evolutionarily beneficial and gives plants an advantage with a lack of soil nitrogen. However, the plants involved in symbiotic interactions are restricted to the order *Fabales* and some members of the *Rosales*, namely several *Parasponia* species of the *Cannabaceae* family [1]. New host plants capable of entering symbiosis with rhizobia can be prospective for efficient farming with minimum application of nitrogen fertilizers. A crucial step is to assess the ability of non-leguminous plants' receptors to percept rhizobial signaling molecules and to transmit molecular signals which allow the symbiotic partners to recognize each other.

Lipochito-oligosaccharide signaling molecules (Nod-factors) secreted by rhizobia serve as key mediators of symbiosis in leguminous plants. The organogenesis of nodules and bacterial colonization depend on the recognition of these molecules by the host plant [2]. Compounds of a very similar structure — lipochito-oligosaccharides (Myc-factors) secreted by fungi of arbuscular mycorrhiza (AM) [3], play the role of signaling molecules necessary for the development of another type of symbiosis with AM fungi formed by more than 80% of terrestrial plants. Activation of responses upon recognition of Nod factors may be associated with the presence of signaling pathway components in non-leguminous plants that are common for signal transmission during the development of legume-rhizobial symbiosis and symbiosis of plants with AM fungi [4, 5]. The differences are mainly in the recognition of Nod and Myc factors by different receptors and the activation of transcription factors specific for each pathway which stimulate their target genes [6, 7].

The LysM receptor-like kinases (LysM-RLK family) with specific lysine motifs (LysM) in the extracellular domains are involved in binding Nod and Myc factors. Among LysM- RLK, it is customary to discriminate LYK (LysM-receptor-like kinases) proteins with an active kinase domain and LYR (LYK related) proteins with inactive kinase domain. The presence of LysM motifs determines the ability to bind compounds containing N-acetylglucosamine residues (Nod and Myc factors consist on average of 4-5 N-acetylglucosamine residues and contain a specific fatty acid at the non-reducing end of the molecule) [8-10]. Some members of the LysM-RLK family recognize structurally similar molecules containing N-acetylglucosamine residues, such as chitin, peptidoglycan murein, and their low molecular weight derivatives [11, 12]. It turned out that upon reception of both Nod and Myc factors, a complex should be formed between LYR and LYK proteins to generate the signal transduction in plants [13, 14].

Some non-leguminous plants can also enter symbiosis with nitrogen-fixing rhizobia. LysM-RLK PanNFP, a kinase of LYR class found in *Parasponia andersonii* Planch. (*Cannabaceae*), recognizes signals from both rhizobia and AM fungi [15]. It is assumed that PanNFP forms complexes with different co-receptors (LYK) upon recognition of Nod and Myc factors. LysM-RLK SlLYK10 (LYR), a kinase recently identified in tomato *Solanum lycopersicum* L., shows high homology to the Nod factor receptor of leguminous plants — MtNFP of alfalfa *Medicago truncatula* Gaertn. [16]. The SlLYK10—co-receptor LysM-RLK SlLYK12 (LYK) complex binds Myc factors. Moreover, the introduction of the *SlLYK10* gene under a strong promoter into the *M. truncatula nfp* mutants defective in the

Nod factor receptor gene restores nodulation and the formation of functional nodules [17, 18]. The Nod factor recognition restored in leguminous plants due to the expression of tomato receptor gene explains the responsiveness of the tomato cell culture to exogenous Nod factors and development of the earliest responses to these signals, i.e., alkalization of the growth medium and depolarization of the membrane [19]. It can be assumed that the recognition of Nod factors in tomato plants is associated with the activation of the SlLYK10/SlLYK12 Myc factor receptor complex due to the similarity of the structure of signaling molecules. Indeed, a recent study of LysM-RLK SlLYK10 showed that this receptor binds not only Myc factors but also Nod factors with high affinity [18]. Such susceptibility of non-leguminous plants to signaling molecules of rhizobia suggests that these molecules can activate the components of "common" signaling pathway (CSP). Therefore, it is of interest to introduce genes encoding rhizobial Nod factor receptors highly affine and specific to the rhizobial signaling molecules into non-leguminous plants and to assess the plant response to the rhizobial signals. In particular, it is necessary to find out i) whether the components of the CSP are involved in the signal transduction and ii) what transcription factors and target genes are activated.

In the pea *Pisum sativum* L., two LysM-RLKs, SYM10 and K1 (LYR and LYK), were identified, which are necessary for the recognition of Nod factors during the initiation of symbiosis with rhizobia [20, 21]. Note, pea mutants for the *sym10* and *k1* genes almost completely lack the ability to respond to inoculation with rhizobia and exogenous Nod factors that indicates the important role of these receptors for symbiosis [20, 21].

Here, two *P. sativum* genes encoding LysM-RLK SYM10 and K1 were transferred to tomato plants *S. lycopersicum* using Agrobacterium-mediated plant transformation. It was revealed for the first time that inoculation of the composite plants with a typical rhizobium strain *Rhizobium leguminosarum* bv. *viciae* CIAM1026 activates the expression of *PsSym10* or *PsK1* genes. In genetically transformed roots of the composite plants, the expression of genes that CSP components can regulate also increases due to the receptors.

We transferred *Pisum sativum* genes for Nod factor receptors to *Solanum lycopersicum* to find out if tomato plants will acquire the ability to recognize rhizobial signaling molecules.

Materials and methods. Seeds of tomato (*Solanum lycopersicum*) cv. Carmello were sterilized with 15% NaOCl (0.1 M) for 5 min, washed 6-fold with distilled sterile water, exposed to 10% H_2O_2 for 2 min, and washed 3-fold with large volume of sterile water. Sterilized seeds were placed in Petri dishes on agarized Murashige-Skoog (MS) medium without sucrose [22] and incubated in the dark for 1 day at 4 °C followed by incubation in the dark for 5-7 days at room temperature for germination. Young seedlings were transferred to sterile 400 ml pots with MS medium supplemented with 3% sucrose and grown in a MLR-352H phytotron (Panasonic, Japan) at 21 °C, 60% humidity and a 16 h light/8 h dark regime.

The *Rhizobium leguminosarum* biovar *viciae* CIAM1026 strain was cultured at 28 °C on tryptone yeast agar (TY) with 0.5 mg/ml streptomycin. *Escherichia coli* XLBlue MRF' and TOP10 strains (Thermo Fisher Scientific, USA) were used for standard cloning procedures. The *Agrobacterium rhizogenes* Arqua 1 strain, containing the required construct, was used to obtain composite plants. *A. rhizogenes* Arqua 1 was cultured at 28 °C on TY agar.

Two constructs, the pSlEXT1::*PsSym10-3xFLAG*::T35S and pSlEXT1::*PsK1-RFP*::T35S were generated for plant transformation. The coding full-length

sequence of the *PsSym10* gene lacking a stop codon was amplified using cDNA as a template (total RNA was isolated from nodules of Finale peas collected on day 21 after inoculation) [20]. Amplification was performed using high-precision Phusion polymerase (Thermo Fisher Scientific, USA) [20]. The *PsSym10* gene sequence was fused with the 3xFLAG coding sequence and transferred into the pDONR™ 221 vector (Thermo Fisher Scientific, USA). We also used previously designed construct containing the full-length coding sequence of the *PsK1* gene (without a stop codon) fused with the sequence encoding the RFP fluorescent protein cloned in the pDONR™ 221 vector [20].

pDONR L4-pEXT1-R1r vector containing the pEXT1 tomato extensin gene promoter (1121 bp) was provided by Dr. S. Bensmihen (Institut National de la Recherche Agronomique, Toulouse, France). The pDONR L4-pEXT1-R1r was used for multilocus homologous recombination. The pDONR™ 221 vectors containing the coding sequences of the *PsK1* gene or the PsSym10 gene and the pENTRY R2-T35S-L3 vector with the T35S terminator (Ghent University, Belgium) were also used for recombination. The constructs were cloned into the pKm43GW delivery vector (pDEST4-3) using LR-clonase II (Thermo Fisher Scientific, USA). pKm43GW was used as the final vector for homologous recombination cloning. The resulting constructs in the pKm43GW vector (pKm43GW-pSlEXT1::*PsSym10-3xFLAG*::T35S and pKm43GW-pSlEXT1::*PsK1-RFP*::T35S) were electroporatically transferred into *A. rhizogenes* Arqua 1 strain.

For transformation, young tomato seedlings cut off at the hypocotyl were treated with *A. rhizogenes* Arqua 1 suspension. Four or five transformed seedlings were placed on MS agar medium without sucrose [22] in a Petri dish between two sheets of filter paper soaked in sterile distilled water. The root area was covered with foil. The dishes were placed in the phytotron and cultured upright for 10-14 days at 21 °C, 60% humidity, and 16 h light/8 h dark until the appearance of callus. Then the plants were incubated under the same conditions for 5-10 days on the MS medium with 3% sucrose and 0.3 mg/ml cefotaxim until the appearance of transgenic roots. The composite plants covered with transparent plastic bags were grown at high humidity, 21 °C, and 16 h light/8 h darkness on vermiculite poured with 0.5× Fahreus medium (0.132 g/l $CaCl_2$, 0.12 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l KH_2PO_4, 0.075 g/l $Na_2HPO_4 \cdot 2H_2O$, 5 mg/l Fe-citrate, 0.07 mg/l $MnCl_2 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnCl_2$, H_3BO_3 and $Na_2MoO_4 \cdot 2H_2O$, pH 7.5). In 2-3 days, the plants were inoculated with *R. leguminosarum* bv. *viciae* CIAM1026 carrying the *uidA* glucuronidase gene (*GUS*) (2 ml of OD_{600} = 0.5 suspension per plant). After cultivation for 7 and 21 days, total RNA was isolated from the transformed roots carrying the pSlEXT1::*PsSym10-3xFLAG*::T35S or pSlEXT1::*PsK1-RFP*::T35S construct to synthesize cDNA. Transgenic plant roots without rhizobia inoculation were used as a control.

In all experiments, total RNA was extracted using the PureZOL™ RNA Isolation Reagent (Bio-Rad Laboratories, USA) according to the manufacturer's protocol. DNAseI treatment (Thermo Fisher Scientific, USA) was applied to remove genomic DNA. cDNA was synthesized using reverse transcriptase RevertAid H Minus (Thermo Fisher Scientific, USA) with oligo(dT18) primer (Sileks, Russia) as per the manufacturer's protocol.

Gene expression was analyzed by quantitative reverse transcription PCR (RT-PCR) (a CFX96 Real-Time System amplifier, Bio-Rad Laboratories, USA) with iQ SYBR Green Super Mix (Bio-Rad Laboratories, USA). RT-PCR conditions: 30 s at 95 °C, 30 s at 54 °C, 40 s at 72 °C (40 cycles). All primers were designed using the DNAStar program and synthesized by the Evrogen company (Russia, http://www.evrogen.com).

To calculate the averaged values of the relative gene expression and the

standard error of the mean (±SEM), the built-in functions of Microsoft Excel were used.

Results. We used the *Agrobacterium rhizogenes* Arqua 1 strain containing the pSlEXT1::*PsSym10-3xFLAG*::T35S or pSlEXT1::*PsK1-RFP*::T35S constructs to transform tomato plants of cv. Carmello with pea genes *PsSym10* and *PsK1* for receptors of Nod factors.

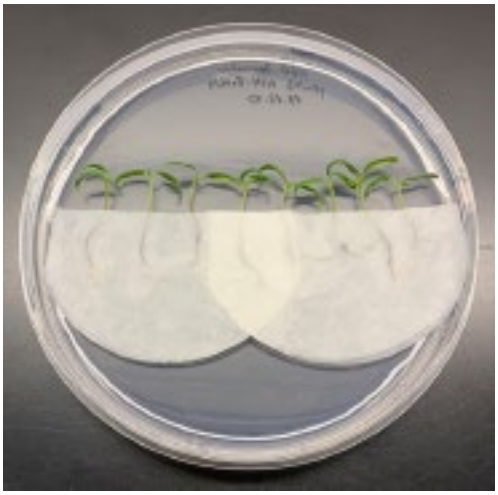

Fig. 1. Agrobacterium-mediated transformation of tomato (*Solanum lycopersicum* L., cv. Carmello).

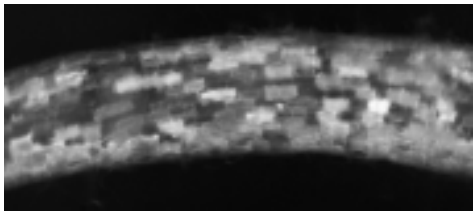

Рис. 2. Root of a composite tomato plant (*Solanum lycopersicum* L., cv. Carmello) transformed with the pSlEXT1::*PsK1-RFP*::T35S construct (a fluorescent binocular microscope. Zeiss, Germany; luminescence of the fluorescent protein RFP in cells).

We reveal a selective luminescence of genetically transformed roots with the pSlEXT1::*PsK1-RFP*::T35S construct in which the gene of K1 receptor was fused with the sequence encoding the fluorescent protein RFP (Fig. 2). This indicated the expression of the transferred gene in the transformed tomato roots. We transformed the plants with genes encoding full-length protein sequences with N-terminal signal peptides. The degree of RFP luminescence confirmed normal function of the signal peptides.

The next step was to assess the expression of the *PsSym10* and *PsK1* genes in the roots of composite tomato plants possessing SlEXT1::*PsSym10-3xFLAG*::T35S or pSlEXT1::*PsK1-RFP*::T35S constructs upon inoculation with *R. leguminosarum* bv. *viciae* CIAM10267 (in 7 and 21 days) and without inoculation (in 7 days, the control). The table shows sequences of the primers in the RT-qPCR assay.

Nucleotide sequences of the primers used in the RT-qPCR assay

Gene	Nucleotide sequences
PsK1-F	5′-CGCGATGTAAAATCAGCAAACATATTG-3′
PsK1-R	5′-CGTCACCATATTGAGCATATTCTGG-3′
PsSym10-F	5′-GTACTTCATTGGCGGAGACTG-3′
PsSym10-R	5′-CCATAAGTTTCACAAGATTTCCAT-3′
SlNSP2-F	5′-AAGGCCGATAGGAGACGAAGAAGG-3′
SlNSP2-R	5′-CCCCACCCCACTCAACCACTC-3′
SlMAPK6-F	5′-CGCGCTTGCTCATCCTTACCTA-3′
SlMAPK6-R	5′-GTGCTGGTATTCGGGATTAAATG-3′
SlD27-F	5′-GCTACCACAGGATTAAGAAACAAG-3′
SlD27-R	5′-CCAACTAGCCAAGGAAAGAAGAT-3′
SlRAM1-F	5′-GGAAGCGGTCAGGGAAACAGG-3′
SlRAM1-R	5′-CCAGGAACCGACCCAGGAAATAC-3′
SlGADH-F	5′-TGAGAATCAACACACTTCTCCAAGG-3′
SlGADH-R	5′-GCATTAAGAATTTCCCCAGAGGTC-3′

The RT-qPCR assay revealed the *PsSym10* gene expression only in the roots of plants transformed with the pSlEXT1::*PsSym10-3xFLAG*::T35S construct (Fig. 3). Similarly, the expression of the *PsK1* gene was detected only in the roots of plants transformed with the pSlEXT1::*PsK1-RFP*::T35S construct (see Fig. 3). Therefore, agrobacteria-mediated transformation and transfer of the *PsSym10* and

PsK1 pea genes into the DNA of tomato root cells stimulated the expression of these genes.

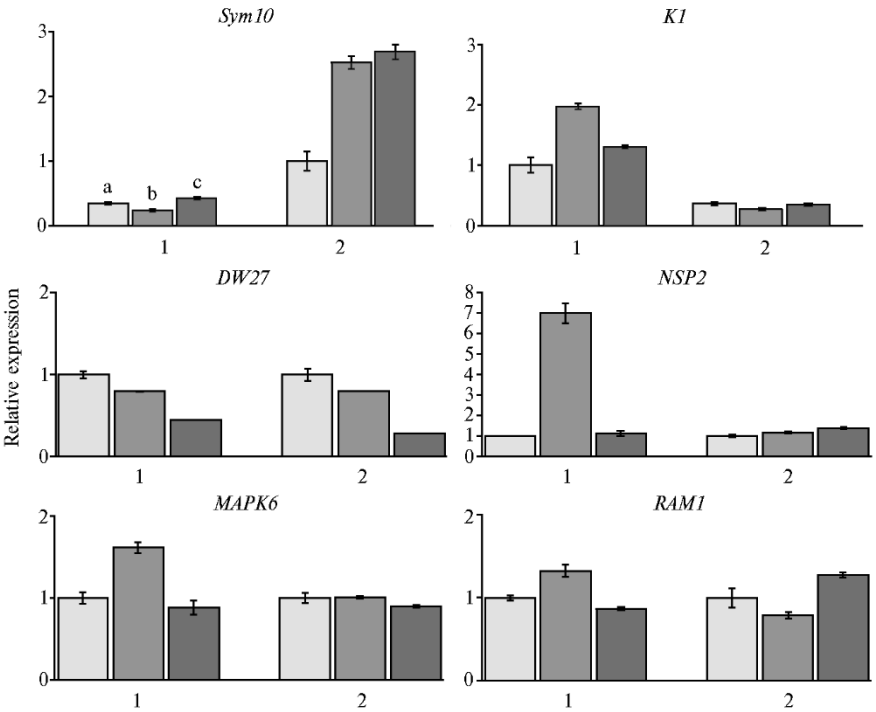

Fig. 3. Relative expression of the genes *PsSym10*, *PsK1*, *SlD27*, *SlNSP2*, *SlMAPK6*, and *SlRAM1* in the roots of composite tomato (*Solanum lycopersicum* L., cv. Carmello) transformed with the constructs pSlEXT1::*PsK1-RFP*::T35S (1) **and pSlEXT1::*PsSym10-3xFLAG*::T35S** (2) upon inoculation with ***Rhizobium leguminosarum* bv. *viciae* CIAM1026:** a — control (without inoculation), b — 7 days after inoculation, c— 21 days after inoculation. The expression levels are normalized to the tomato gene *GADH* expression, the values are shown as a relative expression level upon inoculation to the control without inoculation. Bars mean standard errors of mean (±SEM) for three analytical replicates. For each assay, RNA was extracted from 3-5 transgenic roots.

In the roots of composite tomato plants, there was a significant (approximately 2.0-2.5-fold) increase in the expression of the *PsSym10* gene both 7 days and 21 days after inoculation with rhizobia as compared to control. The increase in the expression of the *PsK1* gene turned out to be the most significant 7 days after inoculation. After 21 days, the expression of the *PsK1* gene decreased, but remained higher than in the control roots without inoculation. Therefore, the pSlEXT1 extensin gene promoter can activate both *PsSym10* and *PsK1* pea genes in composite tomato plants.

It is known that the extensin gene promoter is regulated by ethylene [23]. It also was found that the inoculation of leguminous plants with rhizobia in the roots can increase the ethylene production [24]. An increase in the ethylene content in plant roots upon rhizobia inoculation can explain the relatively high expression of pea genes under the extensin gene promoter. This promotor can be convenient for studying the effect of transferred legume genes on tomato plants upon inoculation with rhizobia.

It is known that binding of signaling molecules to receptors localized in the plasma membrane of plant root cells activates the components of the "common" signaling pathway (CSP) [25]. The roots of tomato plants are capable of perceiving Nod factors, and in this case, under the influence of signaling molecules, a change in the ion flux through the membrane and its depolarization can

be activated [18]. However, it remained unknown whether Nod factors can activate CSP components in non-leguminous plants.

It was of interest to reveal i) whether the transfer of pea genes encoding two receptors for Nod factors will affect the expression of markers normally activated by CSP components and ii) whether the CSP components will be activated in the composite tomato plants under the influence of transferred receptor genes in response to recognition of rhizobia Nod factors. We compared the expression of marker genes for plant symbiosis with AM fungi activated by signal transduction, using the transformed tomato roots without inoculation and upon inoculation with rhizobia. Previously, we carried out a search for gene sequences in tomato, which may be homologues of genes of leguminous plants activated in symbiosis with AM fungi. These are the genes of β-carotene isomerase DWARF27 (D27), transcription factor Nodulation Signaling Pathway 2 (NSP2), transcription factor Required for Arbuscular Mycorrhization (RAM1), and Mitogen-Activated Protein Kinase (MAPK6) [12]. The expression of marker genes was analyzed in transgenic roots of the composite tomato plants on days 7 and 21 upon inoculation with the *R. leguminosarum* bv. *viciae* CIAM1026 (see Fig. 3).

In the roots of tomato plants with the pSlEXT1::*PsSym10-3xFLAG*::T35S construct, there were no significant changes in the expression of the *SlD27*, *SlNSP2*, *SlRAM1*, and *SlMAPK6* genes (see Fig. 3). In plants transformed with the pSlEXT1::*PsK1-RFP*::T35S construct, the expression of *SlD27* and *SlRAM1* genes also did not change significantly in response to inoculation, while the expression of *SlNSP2* and *SlMAPK6* genes significantly increased (see Fig. 3). The activation of these markers may indicate the effect of the transferred pea gene *PsK1* on the susceptibility of tomato plants to inoculation with rhizobia. In the future, a more detailed analysis of morphological changes in plants transformed with the pSlEXT1::*PsK1-RFP*::T35S construct will be carried out. It may be of great interest to analyze plants transformed simultaneously with two genes, the *PsSym10* and *PsK1* in one genetic construct, since in peas both of these receptors (in the complex) are involved in the binding of Nod factors.

Thus, we managed to obtain composite tomato plants of the Carmello cultivar transformed with pea genes *PsSym10* and *PsK1* which encode receptors for Nod factors. Integration of the *Sym10* and *K1* genes into the tomato genome was carried out using the vector constructs pSlEXT1::*PsSym10-3xFLAG*::T35S and pSlEXT1::*PsK1-RFP*::T35S and confirmed by PCR analysis. Inoculation with the *Rhizobium leguminosarum* bv. *viciae* CIAM1026 enhanced the expression of both the *PsSym10* gene and the *PsK1* gene in the transgenic roots of composite plants. In the roots transformed with the pSlEXT1::*PsK1-RFP*::T35S construct, the expression of the *SlNSP2* and *SlMAPK6* genes which are normally activated under the influence of the components of the "common" signaling pathway selectively increased. Activation of the expression of two genes, *SlMAPK6* and *SlNSP2*, in response to inoculation may indicate the effect of the transferred pea receptor gene on the susceptibility of tomato plants to inoculation with rhizobia.

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