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Features of Expression of the *PsSst1* and *PsIgn1* Genes in Nodules of Pea (*Pisum sativum* L.) Symbiotic Mutants

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Abstract—The sequences of the *PsSst1* and *PsIgn1* genes of pea (*Pisum sativum* L.) homologous to the symbiotic *LjSST1* and *LjIGN1* genes of *Lotus japonicus* (Regel.) K. Larsen are determined. The expression level of *PsSst1* and *PsIgn1* genes is determined by real-time PCR in nodules of several symbiotic mutants and original lines of pea. Lines with increased (Sprint-2Fix⁻ (*Pssym31*)) and decreased (P61 (*Pssym25*)) expression level of both genes are revealed along with the lines characterized by changes in the expression level of only one of these genes. The revealed features of the *PsSst1* and *PsIgn1* expression allow us to expand the phenotypic characterization of pea symbiotic mutants. In addition, *PsSst1* and *PsIgn1* cDNA is sequenced in selected mutant lines, characterized by a decreased expression level of these genes in nodules, but no mutations are found.

INTRODUCTION

Mutation analysis is a powerful tool for studying the genetic basis of various biological processes, including the development of a nitrogen-fixing symbiosis between leguminous plants and nodule bacteria. Since this type of symbiosis is optional for plants (some authors determine it as "ecologically obligate" [1]), mutations in symbiotic genes usually affect the development of nodules, but not the viability of plants. Owing to this fact, large collections of symbiotic mutants were obtained for many legumes, including model (*Lotus japonicus* (Regel.) K. Larsen and barrel medic *Medicago truncatula* Gaertn.) and agricultural (garden pea, *Pisum sativum* L.) species [2–4]. In the course of their study, more than two dozen symbiotic genes were cloned and sequenced, and the list of these genes still continues to be replenished [5].

Nodules of leguminous plants have a pink color owing to the presence of a nodule-specific protein, leghemoglobin [6]. Plants carrying mutations in genes responsible for the early symbiotic stages are characterized by the lack of nodules (Nod⁻ phenotype). Mutations in genes controlling late symbiotic stages result in the absence of nitrogen fixation in the formed nodules (Fix⁻ phenotype). Fix⁻ mutants are only able to form underdeveloped white nodules or prematurely senescent nodules of green or grayish green color determined by leghemoglobin degradation products [7].

A comparative characterization of mutant phenotypes makes it possible to draw a conclusion about the role of genes affected by mutations in the development and functioning of symbiotic processes. In addition to the phenotype assessment by light and electron microscopy, another important feature that should be studied is the expression level of known symbiotic genes ("symbiotic markers"). The results of such studies make it possible to reveal a relation between the expression of marker genes and the specific stages of symbiosis development and, therefore, determine the order of functioning of symbiotic genes and their products in the course of a nodule development [8].

In the case of garden pea, at least 40 genes responsible for the development of a symbiotic nodule were identified by experimental mutagenesis; 11 of these genes were cloned and sequenced [9, 10]. The cloned genes encode receptor kinases, which perceive the signal molecules of nodule bacteria, and the components of a signal cascade leading to initiation of nodule formation [11, 12]. The role of other genes identified in symbiotic pea mutants is still unclear, especially in the case of "late" symbiotic genes, i.e., genes, mutations in which result in failures in the late stages of symbiosis.

The sequences of two "late" symbiotic genes, *LjSST1* and *LjIGN1*, were first identified in *L. japonicus* [13, 14] (here and below, the initial letters of a gene name, *Lj* or *Ps*, indicate the corresponding plant species *Lotus japonicus* or *Pisum sativum*, respectively).

Mutants in these genes have a Fix⁻ phenotype (lack of a nitrogen fixation in developed nodules). The *LjSST1* gene (Symbiotic Sulfate Transporter 1) encodes a transporter of sulfate ions, which is specific to nodules and required for their normal functioning [13]. The *Ljsst1* mutants form prematurely senescent pink-green nodules, which do not fix nitrogen [13]. The expression of the *LjSST1* gene is nodule-specific: the expression level of this gene in roots and leaves reaches only 4 and 1% of that in nodules, respectively [13]. The *LjSST1* expression is induced at the relatively late stage of nodule development, after the induction of a leghemoglobin gene expression [13]. The LjSST1 protein probably provides a transfer of sulfate ions from the cytoplasm of a plant cell to bacteria, where they are used for the synthesis of nitrogenase, an enzyme providing the biological fixation of nitrogen [13, 15].

The *LjIGN1* (ineffective greenish nodules 1) gene encodes a protein containing transmembrane domains and ankyrine repeats [14]. The expression of this gene is not confined only to the symbiotic organs (the corresponding transcripts were observed in nodules, roots, leaves, and flowers); however, *Ljign1* mutants are characterized by the defective functioning of the nodules only (formation of pale green nodules, which do not fix nitrogen) [14]. The nodules manifest a premature senescence starting from the stage at which the nitrogen fixation starts in normal nodules [14]. The possible function of the LjIGN1 protein is the anchoring of other proteins on a cell membrane. This function is probably connected with the control of plant defense reactions, since the activity of plant defense systems in the absence of the LjIGN1 protein results in the death of bacteria and degradation of symbiotic structures [14].

Genetic systems of leguminous plants responsible for the nodule formation are highly conservative. Therefore, it is quite possible that the orthologous genes of garden pea (*PsSst1* and *PsIgn1*) also can be involved in the development and functioning of nitrogen-fixing nodules. The corresponding genes in *L. japonicus* determine two different processes occurring in the course of the symbiosis development (formation of symbiotic structures (*LjSST1*) and modification of plant defense reactions (*LjIGN1*)) and are expressed at the late stages of the nodule development. For this reason, the *PsSst1* and *PsIgn1* genes were selected as marker genes, and their expression level was evaluated in a series of mutant pea lines with failures in the late stages of symbiosis that provided an additional characterization of the phenotypes of these lines. In addition, since mutations within ORF are able to result in a transcript degradation via the nonsense-mediated mRNA decay [16], we expected that the evaluation of the expression level of the *PsSst1* and *PsIgn1* genes would allow us to reveal mutant lines carrying mutations in these genes (if such mutants are present in the available collection of pea symbiotic mutants).

Table 1. Phenotypes of pea lines used in the study

Line name*	Mutant gene	Nodule phenotype
Finale	–	Pink
RisFixK	*PsfixK*	Pale pink
RisFixV	*Pssym42*	White
Frisson	–	Pink
P59	*Pssym23*	White
P61	*Pssym25*	Grayish pink
P63	*Pssym26*	Pale green
Rondo	–	Pink
FN1	*Psfn1*	White and pink-white
SGE	–	Pink
SGEFix⁻-8	*Pssym25*	Grayish pink
Sparkle	–	Pink
E135f	*Pssym13*	White and pale green
Sprint-2	–	Pink
Sprint-2Fix⁻	*Pssym31*	White

* The name of a wild type line used to obtain mutant lines listed below is indicated in bold.

MATERIALS AND METHODS

Biological Material

Garden pea lines used in this study were obtained from the collection of the Laboratory of Genetics of Plant-Microbe Interactions of the All-Russia Research Institute for Agricultural Microbiology (Table 1). Plants were grown under controlled conditions (21°C and 16/8 h light/dark photoperiod) and inoculated with nodule bacteria (*Rhizobium leguminosarum* bv. *viciae* strain RCAM 1026). Each experimental variant included five plants. Seeds were sterilized with concentrated sulfuric acid (15 min under constant mixing using a laboratory shaker) and washed 5−10 times with sterile distilled water. Seeds were germinated for three days in Petri dishes filled with sterile vermiculite and then individually planted in 0.5-L plastic glasses filled with heat-sterilized quartz sand. During the planting, seedlings were inoculated with a water suspension of nodule bacteria (10^6 CFU per plant) and watered with a nitrogen-free mineral salt solution [17].

Four weeks after the inoculation, plants were taken out of the substrate. After the visual control of the nodule phenotype (color and number), nodules were detached from roots by tempered pincers, fixed in liquid nitrogen, and ground. The samples obtained were stored at −80°C.

Molecular Analysis

DNA was isolated from fresh plant leaves by a CTAB method with some modifications [18]. At the

Table 2. Sequences of primers used for real-time PCR

Primer name	Sequence, 5'–3'
PsIgn1_real_time_FW	TAACACCGCATTACACGTGGCAA
PsIgn1_real_time_RV	CCCGTACCGAGAAAGGACGT
PsSst1_real_time_FW	AATTATATTTGGCTAGACCTGCAT
PsSst1_real_time_RV	CCGGAAGAGCTTTGTTCGCT
PsActin2_real_time_FW	CTCAGCACCTTCCAGCAGATGTG
PsActin2_real_time_RV	CTTCTTATCCATGGCAACATAGTTC
PsGAPDH_real_time_FW	CAAAGAGTTGGGTTACAGTACCC
PsGAPDH_real_time_RV	CATGATAAGAGGCCTAACTGCCGCT

first stage, fragments of the *PsSst1* and *PsIgn1* genes were amplified and sequenced using degenerate primers; then the primers specific to the already studied gene regions were used. PCR fragments were sequenced using an ABI Prism 3500 xL Genetic Analyzer (Applied Biosystems, United States).

For RNA isolation, nodules were collected from five plants, mixed, and ground. Total RNA was isolated using a TRIzol reagent (Life Technologies, United States) and treated with DNase I (Thermo Scientific, United States). The end sequences of the *PsSst1* and *PsIgn1* transcripts were determined using a Mint RACE cDNA amplification kit (Evrogen, Russia). The fragments obtained were cloned into a pAL-TA vector (Eurogen, Russia) and sequenced with M13 primers using an ABI Prism 3500 xL Genetic Analyzer (Applied Biosystems, United States).

To synthesize cDNA on the base of total RNA, a M-MuLV reverse transcriptase and an oligo-dT primer (both manufactured by Thermo Scientific, United States) were used. Real-time PCR was performed in three to five replicates using an iCycler iQ™ amplifier equipped with an optical module (Bio-Rad, United States) and a SYBR® Green Master Mix (Bio-Rad, United States). The following PCR conditions were used: one cycle for 30 s at 95°C and 35 cycles for 20 s at 95°C, 20 s at 60°C, and 20 s at 72°C. To determine the relative expression level of the studied genes, we used two reference genes, *Actin2* and *GAPDH* (NCBI accession numbers X68649 and L07500, respectively). The sequences of primers used for real-time PCR are shown in Table 2. The statistical analysis of PCR results, including the normalization of a cDNA amount, was performed using iQ™5 Optical System Software (Bio-Rad, United States). The significance of differences between the expression levels of genes in mutant and wild type lines was evaluated by the Student's *t*-test.

Computer Analysis

The search for homologous genes was performed using BLASTN software [19] and the following databases: NCBI (http:/blast.ncbi.nlm.nih.gov/Blast.cgi), *Medicago*

truncatula HAPMAP project (http://www.medicagohap-map.org/tools/blastform), and *Medicago truncatula* Gene Expression Atlas (http://mtgea.noble.org/v2/blast_search_form.php). The sequence alignment was performed using Multalin online software (http://multalin.toulouse.inra.fr/multalin/) [20]. Primers were designed using OligoCalc online software (http://www.basic.northwestern.edu/biotools/oligocalc.html) [21].

RESULTS

Amplification of the PsSst1 and PsIgn1 Genes

In the course of this study, the sequences of pea genes homologous to the *LjSST1* and *LjIGN1* genes were determined. First, since the degree of relationship between pea and barrel medic is higher than between pea and *L. japonicus*, a search for *M. truncatula* genes homologous to the *LjSST1* and *LjIGN1* genes was performed in available nucleotide sequence databases. As a result, we found sequences with a high similarity (>70%) to cDNA of the *LjSST1* and *LjIGN1* genes (Table 3). These sequences represent either described barrel medic genes located in the sequenced part of its genome (Medtr6g086170.1, Medtr1g071530.1, and Medtr7g100430.1) or unique cDNA used to create microarrays for the gene expression analysis (Mtr.37708.1.S1_at, Mtr.11594.1.S1_at, and Mtr.34129.1.S1_at). The maximum similarity to the *LjSST1* cDNA was revealed for Mtr.37708.1.S1_at and Medtr6g086170.1 (note that Mtr.37708.1.S1_at is a part of the Medtr6g086170.1 sequence); therefore, the Medtr6g086170.1 gene is probably orthologous to the *LjSST1* gene (Table 3). The maximum similarity to the *LjIGN1* cDNA was shown for the cDNA of Mtr.11594.1.S1_at and Mtr.34129.1.S1_at genes, whereas the Medtr7g100430.1 gene, which was revealed as the closest homologue of the *LjIGN1* gene in the sequenced part of the barrel medic genome, is obviously not orthologous to it (Table 3).

The identified sequences from *M. truncatula* were aligned with those from *L. japonicus*; according to the results, we developed degenerate primers suitable for

Table 3. Transcripts of barrel medic (*Medicago truncatula* Gaertn.) and garden pea (*Pisum sativum* L.) similar to the *LjSST1* and *LjIGN1* genes of *Lotus japonicus* (Regel.) K. Larsen

Lotus japonicus	*Medicago truncatula*		*Pisum sativum*		
gene	sequence name*	similarity to the *L. japonicus* gene, %	gene	similarity to the *L. japonicus* gene, %	similarity to the *M. truncatula* gene, %
LjSST1	**Mtr.37708.1.S1_at**	80	*PsSst1*	80	87
	Medtr6g086170.1	80			87
	Medtr1g071530.1	70			64
LjIGN1	**Mtr.11594.1.S1_at****	90	*PsIgn1*	85	89
	Mtr.34129.1.S1_at	82			93
	Medtr7g100430.1	71			71

* Sequences of *M. truncatula* corresponding to the closest homologues (orthologues) of the *LjSST1* and *LjIGN1* genes are indicated in bold.

** According to the new version of the sequenced *M. truncatula* genome Mt4.0verl (www.phytozome.jgi.doe.gov/) published after the completion of this manuscript, a full-size transcript with a maximum similarity to the *LjIGN1* gene has been identified and is called Medtr1g115575.1.

the amplification of Medtr6g086170.1, but not Medtr1g071530.1, and for the amplification of Mtr.11594.1.S1_at and Mtr.34129.1.S1_at, but not Medtr7g100430.1. The use of these primers for the PCR analysis of cDNA isolated from nodules of the pea line Finale allowed us to amplify and sequence transcript fragments homologous to *LjSST1* and *LjIGN1*. The full sequences of the *PsSst1* and *PsIgn1* transcripts were then obtained using a RACE (rapid amplification of cDNA ends) approach. The alignment of these sequences with cDNA of the *LjSST1* and *LjIGN1* genes and with similar sequences from barrel medic showed that the revealed *PsSst1* sequence is more similar to *LjSST1* and Medtr6g086170.1 than to Medtr1g071530.1, whereas the *PsIgn1* sequence is more similar to *LjIGN1*, Mtr.11594.1.S1_at, and Mtr.34129.1.S1_at than to Medtr7g100430.1 (Table 3). On the basis of the revealed sequences of *PsSst1* and *PsIgn1* cDNA, we developed pea-specific primers and used them to amplify and sequence full sequences of the *PsSst1* and *PsIgn1* genes on the genomic DNA of Finale line. A comparison of these sequences with cDNA sequences allowed us to reveal the exon−intron structure of the *PsSst1* and *PsIgn1* genes (Fig. 1). The *PsSst1* and *PsIgn1* sequences were deposited in the NCBI database (accession numbers KR047193 and KR047192, respectively).

Evaluation of the Expression Level of the PsSst1 and PsIgn1 Genes in Nodules of Mutant Pea Lines

On the basis of the revealed exon−intron structure of the *PsSst1* and *PsIgn1* genes, we developed primers for real-time PCR (Table 2). To avoid annealing of the primer caused by possible genomic DNA traces in cDNA samples, the sequences of these primers were chosen to correspond to exon junction regions. Using these primers, we evaluated the expression level of the *PsSst1* and *PsIgn1* genes in nodules of mutant pea lines and the corresponding wild type lines (Table 1). Since mutant lines were obtained from six original pea lines (Finale, Frisson, Rondo, SGE, Sparkle, and Sprint-2) [4], the expression level of the studied genes in nodules of each of these original lines was normalized to the expression level of reference genes and then taken to be equal to 1. The expression levels obtained for mutant lines were recalculated according to the normalized values obtained for the corresponding original lines.

An abnormal *PsSst1* expression was observed in nodules of seven out of nine mutant lines (Fig. 2). Mutant lines P59 (*Pssym23*), P61 (*Pssym25*), RisFixK (*PsfixK*), RisFixV (*Pssym42*), and SGEFix⁻-8 (*Pssym25*) demonstrated a decreased expression level as compared with the corresponding wild type lines, whereas lines E135F (*Pssym13*) and Sprint-2Fix⁻ (*Pssym31*) were characterized by a significant increase in this parameter (by 20 and 50 times, respectively). At the same time, the expression level of the *PsSst1* gene in the nodules of mutant lines FN1 (*Psfn1*) and P63 (*Pssym26*) remained unchanged.

An abnormal expression of the *PsIgn1* gene was observed only in the nodules of three out of nine mutant lines. In the case of the line P61 (*Pssym25*), the expression level of this gene was decreased, whereas in the lines P63 (*Pssym26*) and Sprint-2Fix⁻ (*Pssym31*) it was increased (Fig. 3). In other mutant lines, we did not observe any significant changes in this parameter as compared with wild type lines.
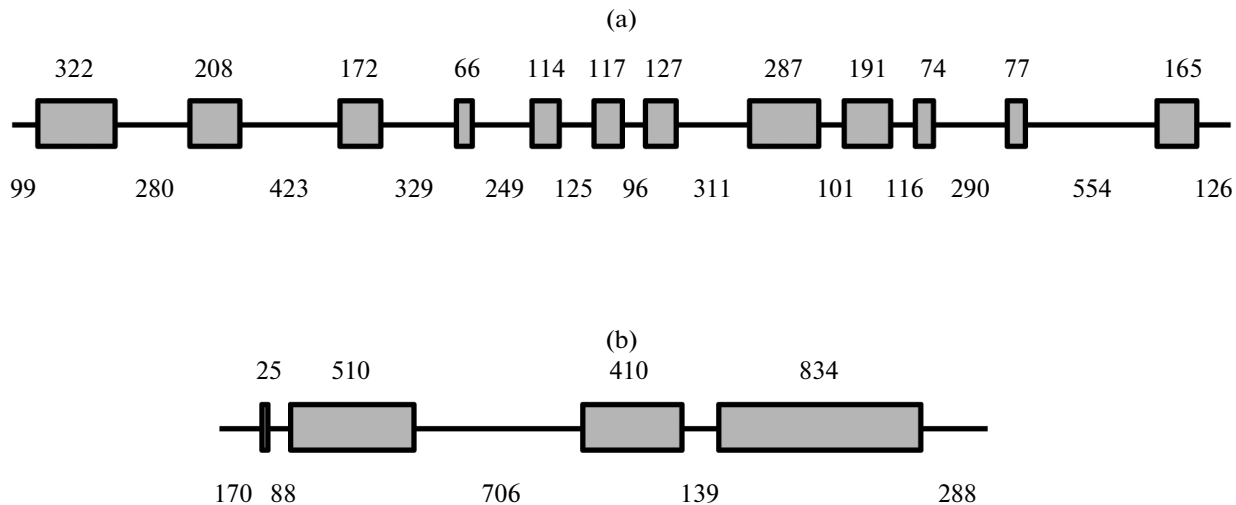
(a)

(b)

Fig. 1. Exon−intron structure of the (a) *PsSst1* and (b) *PsIgn1* genes of garden pea. Exons are indicated with rectangles, whereas noncoding regions (5'-untranslated region, introns, and 3'-untranslated region) are indicated with lines; upper and lower numbers indicate the size (bp) of exons and noncoding regions, respectively.

Sequencing of PsSst1 and PsIgn1 cDNA in Lines with a Decreased Level of Expression of These Genes

One of the possible reasons for the gene expression level decrease is a degradation of a mutation-containing transcript according to the nonsense-mediated mRNA degradation mechanism [16]. To search for any possible mutations in the *PsSst1* ORF, we sequenced PCR fragments obtained for cDNA from the nodules of mutant lines SGEFix⁻-8 (*Pssym25*), P59 (*Pssym23*), P61 (*Pssym25*), RisFixK (*PsfixK*), and Ris-FixV (*Pssym42*) and the corresponding wild type lines (SGE, Frisson, and Finale) (Table 1). We also sequenced *PsIgn1* cDNA from the lines P61 (*Pssym25*) and Frisson (wild type). The results of this work did not reveal any differences between the *PsSst1* and *PsIgn1* ORF sequences of selected mutant lines with decreased expression level of the studied genes and corresponding wild type lines.

DISCUSSION

In this study we identified sequences of pea genes homologous to the *LjSST1* and *LjIGN1* genes. The revealed genes showed a maximum similarity only to those barrel medic genes which are the closest homologues of the *LjSST1* and *LjIGN1* genes (Table 3); therefore, one can assert that, among the whole pea genome, only the identified genes are the closest homologues of the mentioned *L. japonicus* genes. The final confirmation of this assertion can be obtained after the sequencing of the pea genome, which at present is just being planned (http://www.coolseason-foodlegume.org/pea_genome). Nevertheless, the search in the Transcriptome Shotgun Assembly database performed for garden pea at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the search in our own sequencing data for the pea nodule transcriptome [22] using the *LjSST1* and *PsSst1* (or *LjIGN1* and *PsIgn1*) sequences retrieved the same results. This fact indicates that only the *PsSst1* and *PsIgn1* genes of pea have the structure close to that of the *LjSST1* and *LjIGN1* genes, respectively.

Mutations occurring in many symbiotic pea genes are able to change the expression level of the *PsSst1* and *PsIgn1* genes in nodules. The expression levels of both genes in the nodules of the line Spring-2Fix⁻ (*Pssym31*) are increased, which probably indicates the presence of some compensatory mechanism stimulating the expression of symbiotic genes in response to the abnormal functioning of nodules in the mutant line Sprint-2Fix⁻ (*Pssym31*). It is interesting that the line Sprint-2Fix⁻ (*Pssym31*) demonstrates the blocking of nodule development at a very early stage of differentiation of bacteria into a symbiotic form called bacteroids [17]. It seems that the expression of symbiotic genes in the line Sprint-2Fix⁻ (*Pssym31*) can be induced by a certain compound excreted by bacteria which were not differentiated into bacteroids. This compensatory mechanism can also be the reason for a heightened expression level of the *PsSst1* and *PsIgn1* genes in the nodules of the lines E135F (*Pssym13*) and P63 (*Pssym26*), respectively, in which the development of symbiosis is blocked at the later stages.

The decreased expression level of *PsSst1* and *PsIgn1* genes in the nodules of mutant lines probably reflects a key role of genes affected by mutations in the normal development and functioning of nodules. In the case of a nonfunctional mutant gene, the development of symbiotic compartments is blocked and, therefore, expression of nodule-specific symbiotic genes is suppressed. In most cases, such ineffective nodules undergo premature senescence and then die
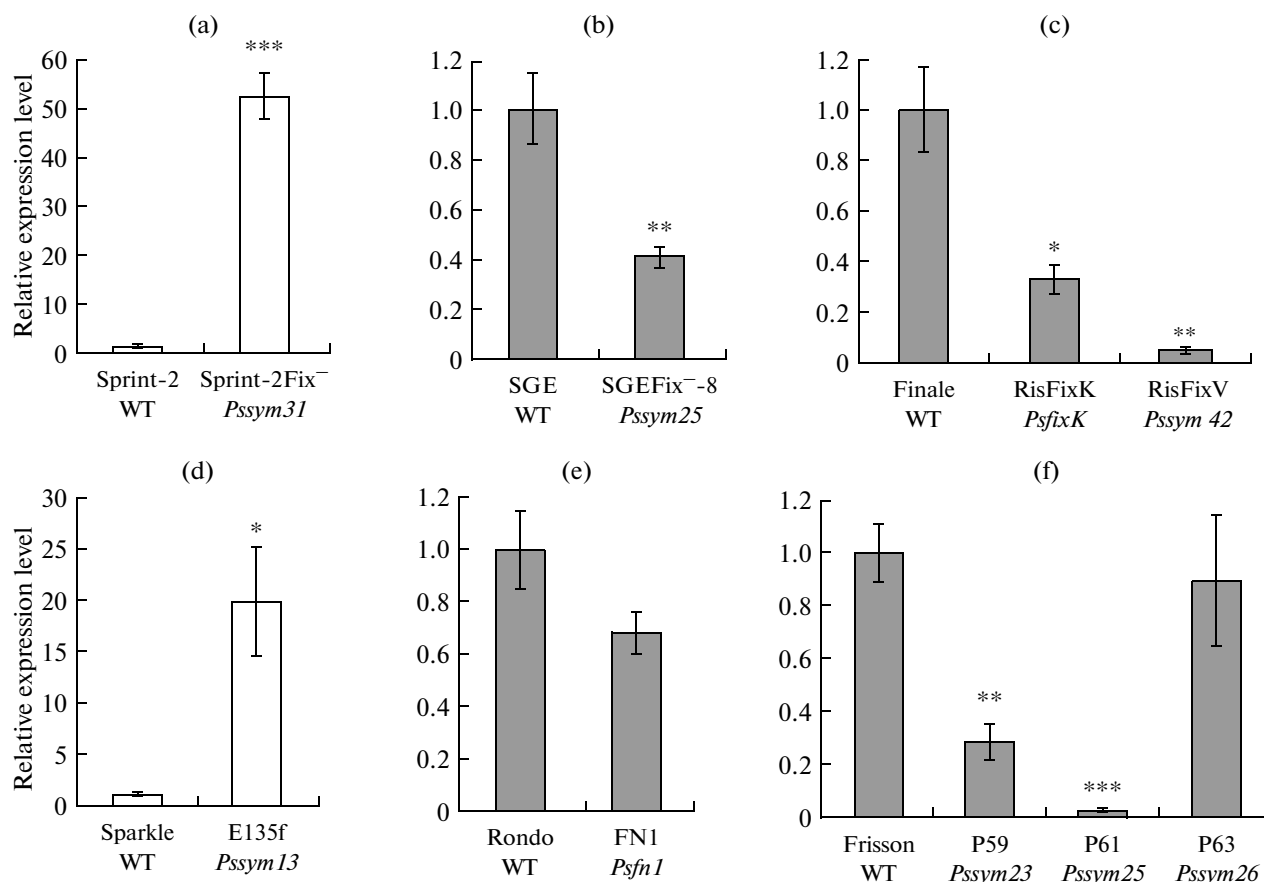
Fig. 2. Expression level of the *PsSst1* gene in nodules of various mutant and original lines. Mutants were obtained using different original lines: (a) Sprint-2; (b) SGE; (c) Finale; (d) Sparkle; (e) Rondo; (f) Frisson. Statistically significant differences in the expression level of the *PsSst1* gene between the mutant and the corresponding original lines were determined by the Student's *t*-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bar = SE.

[23]. However, this mechanism, which implies the suppression of gene expression in defective nodules, is not universal for all mutant lines, because the expression level of the *PsSst1* gene in the nodules of the mutant lines FN1 (*Psfn1*) and P63 (*Pssym26*) was maintained at the same level as in the wild type line.

Mutations in symbiotic genes affect the expression of the *PsSst1* gene more frequently than the *PsIgn1* gene. This fact can probably be explained by a low level of the *PsIgn1* expression in nodules and, therefore, a greater error of its evaluation by real-time PCR, which did not allow us to reveal any significant difference between the nodules of mutant and original lines. It is also possible that the observed phenomenon of premature senescence of nodules, typically accompanying the activation of plant defense reactions [23], is mediated in many mutant lines more by unclear mechanisms involved in the regulation of defense reactions rather than by abnormal expression of the *PsIgn1* gene. At the same time, the decrease in the *PsIgn1* expression level in the nodules of the line P61 (*Pssym25*) probably indicates a key role of the

PsSym25 gene in the pathway, which regulates plant defense reactions and involves the *PsIgn1* gene.

The study of the expression level of symbiotic genes in mutant pea lines makes it possible to identify a line with mutations in the studied gene by the decreased expression of this gene. For example, a microarray-based analysis of total gene expression made it possible to identify the sequences of the symbiotic *MtDMI3* and *MtNSP2* genes in barrel medic [24, 25]. In our study, in spite of finding several mutant lines with decreased expression level of the *PsSst1* and *PsIgn1* genes, we did not reveal any lines with mutations in these genes. Apparently, the available collection of pea mutants, which includes more than 100 lines belonging to more than 40 complementation groups, does not represent all genes required for the development of nitrogen-fixing symbiosis. According to some estimates, the existing collections of barrel medic mutants contain at least twenty new symbiotic genes not identified earlier [3, 26]. Thus, in the very near future, one can expect a significant expansion of the modern concepts of the symbiotic system in legumes.
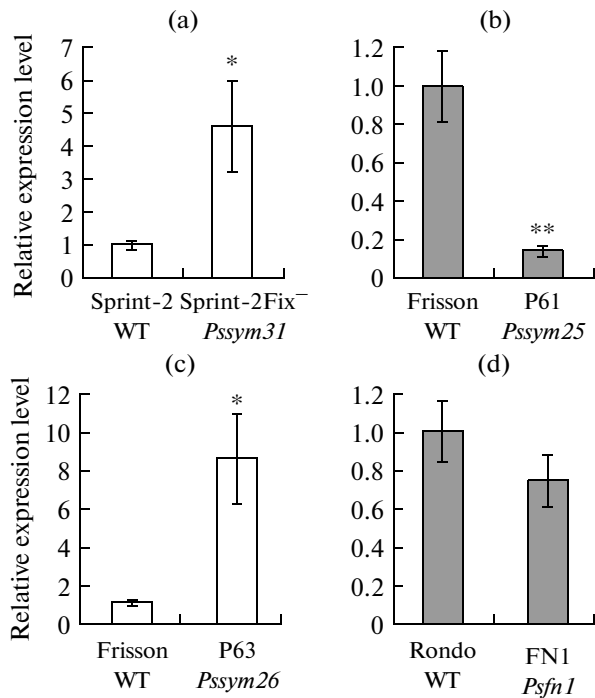
Fig. 3. Expression level of the *PsIgn1* gene in nodules of various mutant and original lines. Mutants were obtained using different original lines: (a) Sprint-2; (b, c) Frisson; (d) Rondo. Statistically significant differences in the expression level of the *PsIgn1* gene between the mutant and the corresponding original lines were determined by Student's *t*-test: * $P < 0.05$, ** $P < 0.01$. Error bar = SE.

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