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Research Article

Diversity of *PsSym29* and *PsNRLK1* genes in the VIR germplasm collection of pea (*Pisum sativum* L.)

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BACKGROUND: N.I. Vavilov Institute of Plant Genetic Resources (VIR) (Saint Petersburg, Russia) maintains a large collection of pea (*Pisum sativum* L.). Earlier, several growth and yield parameters were recorded for plants of 99 accessions grown under inoculation with nodule bacteria and arbuscular mycorrhizal fungi.

MATERIALS AND METHODS: Polymorphism of genes encoding symbiotic receptor kinase PsSym29 [participating in the autoregulation of nodulation (AON) system] and closely related receptor kinase PsNRLK1 (with yet unknown function in symbiosis) was assessed in 99 pea genotypes from the VIR collection. Nucleotide diversity, Tajima's D, and Fay and Wu's H statistics were calculated using DNAsp 5.0 software. The significance of associations of allelic state of the sequenced genes with the growth and yield parameters was tested by two-way ANOVA followed by FDR correction and by regression analysis.

RESULTS: Nucleotide diversity and the ratio of synonymous to non-synonymous substitutions was greater in *PsNRLK1* as compared to *PsSym29*. The analysis of Fay and Wu's H in sliding window revealed signatures of positive selection in one site of *PsSym29* and in three sites of *PsNRLK1* gene sequences located in 1st exons encoding LRR (leucine rich repeat) domains. No significant associations of allelic state of neither *PsSym29* nor *PsNRLK1* genes was found with plant growth and yield parameters.

CONCLUSIONS: The sequences of both *PsSym29* and *PsNRLK1* genes undergo positive selection, but the conditions in which specific allelic states of the genes become adaptive are to be elucidated in future.

Keywords: *Pisum sativum*; pea; symbiotic genes; nodule bacteria; arbuscular mycorrhiza; autoregulation of nodulation; receptor kinase; polymorphism; selection pressure.

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Научная статья

Разнообразие последовательностей генов *PsSym29* и *PsNRLK1* в коллекции гороха (*Pisum sativum* L.) Всероссийского института генетических ресурсов растений им. Н.И. Вавилова (ВИР)

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Во Всероссийском институте генетических ресурсов растений им. Н.И. Вавилова (ВИР) поддерживается обширная коллекция образцов гороха посевного (*Pisum sativum* L.). Ранее для 99 генотипов гороха, выращенных в условиях инокуляции клубеньковыми бактериями и грибами арбускулярной микоризы, были определены значения ростовых параметров и показателей урожайности и качества семян. В настоящем исследовании был оценен полиморфизм генов, кодирующих симбиотическую рецепторную киназу PsSym29 (компонент системы авторегуляции клубенькообразования) и гомологичную киназу PsNRLK1 с пока неизвестной функцией, на указанной выборке из 99 генотипов гороха. В программе DNAsp 5.0 были определены параметры нуклеотидного разнообразия и значения критериев D Таджимы и H Фэя и By. С использованием двухфакторного дисперсионного анализа (two-way ANOVA) с поправкой FDR, а также методов регрессионного анализа была исследована достоверность ассоциации аллельного состояния секвенированных генов с ростовыми параметрами и показателями урожайности и качества семян. Было показано, что нуклеотидное разнообразие и отношение частот синонимичных и несинонимичных замен выше в случае гена *PsNRLK1* по сравнению с *PsSym29*. Анализ значений критерия H Фэя и By методом скользящего окна выявил признаки позитивного отбора в одном сайте последовательности *PsSym29* и в трех сайтах последовательности *PsNRLK1*. Все эти сайты локализуются в первых экзонах генов, кодирующих домены LRR (leucine rich repeat). Статистически достоверных ассоциаций аллельного состояния генов *PsSym29* и *PsNRLK1* с ростовыми параметрами и показателями урожайности и качества семян выявлено не было. Таким образом, продемонстрировано, что последовательности генов *PsSym29* и *PsNRLK1* испытывают действие позитивного отбора, однако для выяснения условий, при которых определенные аллели изученных генов придают эволюционное преимущество, требуются дальнейшие исследования.

Ключевые слова: *Pisum sativum*; горох; симбиотические гены; клубеньковые бактерии; арбускулярная микориза; авторегуляция клубенькообразования; рецепторная киназа; полиморфизм; давление отбора.

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BACKGROUND

Garden pea (*Pisum sativum* L.) is an important crop plant and a useful model for studying symbiotic interactions between legume plants and nodule bacteria [1]. Over 70,000 specimens of pea are maintained in large genetic collections [2], and for several of them, agriculturally important traits have been characterized. The collection of cultivated peas of N.I. Vavilov Institute of Plant Genetic Resources (VIR) (St. Petersburg, Russia) contains more than 8000 accessions, most of which were characterized with respect to agriculturally valuable features [3]. Among them, for a subset of 99 genotypes, growth and yield parameters were recorded for plants grown in symbiotic conditions (namely, under inoculation with either nodule bacteria (Rhizobia, Rh) or nodule bacteria plus arbuscular mycorrhizal fungi (Rh+AM) [4, 5].

The number of symbiotic nodules, as well as the rate of arbuscular mycorrhizal colonization, is controlled in legumes via a long-distance signaling system, called autoregulation of nodulation (AON). The AON system involves CLE (CLAVATA3/EMBRYO SURROUNDING REGION-RELATED) peptides that migrate from nodulated roots into shoots, where they are perceived by a receptor kinase homologous to CLAVATA1 of *Arabidopsis thaliana* (L.) Heynh. (encoded by the gene *MtSUNN* (*Super Numeric Nodules, Medtr4g070970*) in *Medicago truncatula* Gaertn. and *PsSym29* in *P. sativum*) [6]. The genome of *M. truncatula* contains 10 genes related to *MtSUNN*, among which only a single gene is up-regulated by rhizobia [7]. This gene, named *Nodulation related LRR kinase 1* (*MtNRLK1, Medtr5g090100*), is considered a component of the AON system due to its nodule-specific expression profile; however, its detailed role in symbiosis is unclear [7].

The polymorphism of the genes that participate in AON may influence the amount of fixed nitrogen by modulation of number of nodules formed, and consequently, may contribute to the yield. Thus, to address a possible link between pea yield parameters and the AON components, we studied in detail the polymorphism of the *PsSym29* gene, as well as its homologue *PsNRLK1* with nodule-specific expression, and analyzed the association between non-synonymous nucleotide variations and earlier obtained data on plant yield and growth parameters on the set of 99 pea cultivars from the VIR collection.

MATERIALS AND METHODS

The set of 99 pea cultivars from the Pea World Germplasm Collection of the N.I. Vavilov Institute of Plant Genetic Resources (VIR, St. Petersburg, Russia) was initially selected as representing the diversity of the species *Pisum sativum* L. [4, 5]. This set contains genotypes from more than 25 countries and territories, and includes accessions belonging to *P. sativum* subsp. *sativum* (85), *asiaticum* (8), *abyssinicum* (3) and *transaucasicum* (3) (for detailed information see Supplementary files to V.A. Zhukov et al. [5]). In previous studies, the yield and growth parameters (PW — plant shoot weight, PL — plant shoot length, PN — pod number, SN — seed number per plant, SW — seed weight per plant, TSW — thousand-seed weight, NtS — seed nitrogen content, PhS — seed phosphorus content) were determined for plants grown under inoculation with rhizobia or under complex inoculation with rhizobia and AM fungi [4, 5]. For the present work, the DNA was extracted from one week old seedlings of the 99 genotypes using CTAB method with modifications, as described in A.S. Sulima et al. [8], and was used for genotyping.

The fragments of *PsSym29* and *PsNRLK1* genes were amplified using specific primers, which were designed with help of Primer3Plus program [9] (Table S1), and were sequenced in Evrogen company (Moscow, Russia). The sequences of the pea genes, along with *MtSUNN* (AY769943 in NCBI database) or *MtNRLK1* (Medtr5g090100, *M. truncatula* genome v4.0) served as the outgroups, were aligned in Mega 5.0 [10], and the polymorphism analysis was performed in DNAsp 5.0 [11]. Tajima's D and Fay and Wu's H criteria were calculated in sliding window (size 100 bp, step 25 bp) using DNAsp 5.0 [11]. The statistical significance of the departure from the neutral evolution model was determined by coalescent simulations in DNAsp 5.0 [11]. Codon based Z test of neutrality (Nei–Gojobori method [12]) was performed in Mega 5.0 [10].

Single nucleotide variants (SNVs) were obtained by comparison of the aligned sequences, and for non-synonymous substitutions the significance of associations with the trait values was tested by two-way ANOVA with interaction analysis followed by FDR correction. Further, the association between the non-synonymous substitutions and AM-caused increments of traits (calculated as the increase of the trait values in Rh+AM conditions as compared to Rh conditions) were performed by regression analysis using models described in V.A. Zhukov et al. [5]. Likelihood Ratio Test for variable significance testing and FDR correction were used. All statistical analyses were performed using R Statistical Software (version 3.5.3). The association analysis was performed if the minor allele occurred in at least five cultivars. The effect of amino acid substitutions corresponding to the detected SNVs on the protein function (deleterious or not) was predicted by SIFT algorithm using the web-based tool (https://sift.bii.a-star.edu.sg) [13].

RESULTS AND DISCUSSION

The genes *PsSym29* (*Psat7g183240*) and *PsNRLK1* (*Psat2g019520*) consist of two exons separated by an intron and encode proteins possessing 20 LRR (leucine rich repeat) domains, a transmembrane domain, and a protein kinase domain (Fig. 1). On the protein level, PsSym29 and PsNRLK1 share 57.6% similarity and 44.1% identity; PsSym29 shows 88.0% similarity and 83.6% identity to MtSUNN, and PsNRLK1 shows 91.1% similarity and 86.7% identity to MtNRLK1. According to the pea gene expression atlas [14], *PsSym29* has maximal expression level in leafs and tendrils, whereas *PsNRLK1* has maximal expression level in symbiotic nodules (Fig. S1).

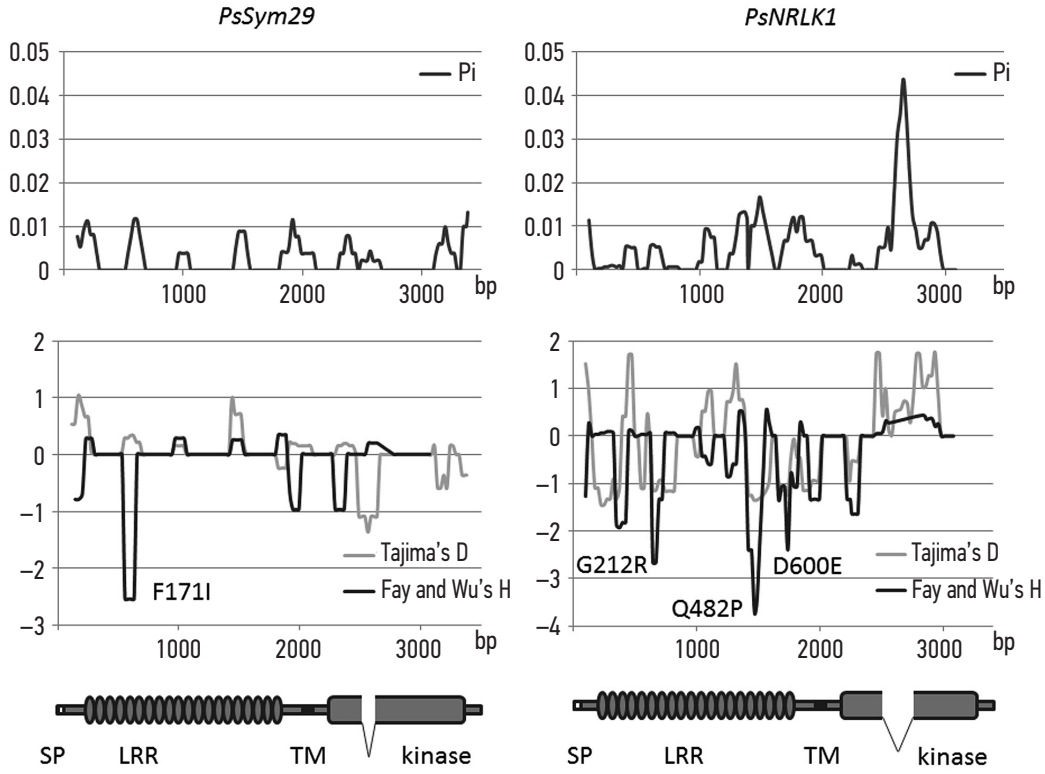

Fig. 1. Nucleotide diversity, Tajima's D, and Fay and Wu's H statistics calculated for *PsSym29* and *PsNRLK1* gene sequences, and the domain structure of the proteins encoded by the genes. Amino acid substitutions in sites with detected signals of positive selection are indicated. SP — signal peptide, LRR — leucine rich repeats, TM — transmembrane domain, kinase — protein kinase domain

Table 1. Nucleotide diversity of *PsSym29* and *PsNRLK1*

Gene	π	π_s	π_a	π_a / π_s
PsSym29	$2{,}87 \cdot 10^{-3}$	$7{,}17 \cdot 10^{-3}$	$1{,}13 \cdot 10^{-3}$	0,158
PsNRLK1	$4{,}48 \cdot 10^{-3}$	$6{,}94 \cdot 10^{-3}$	$1{,}98 \cdot 10^{-3}$	0,285

Note. π — nucleotide diversity, π_s — nucleotide diversity calculated for synonymous substitutions, π_a — nucleotide diversity calculated for non-synonymous substitutions, π_a / π_s — ratio of non-synonymous to synonymous substitutions.

We sequenced the coding parts of the genes *PsSym29* and *PsNRLK1* in the set of 99 pea cultivars (the list is presented in Supplementary materials of V.A. Zhukov et al. [5]) and calculated the nucleotide diversity (π) of the gene sequences (Table 1). The gene *PsNRLK1* appeared to be more diverse within the tested set of cultivars than *PsSym29*. Interestingly, the nucleotide diversity calculated for synonymic changes (π_s) is comparable for the two genes, whereas the nucleotide diversity calculated for non-synonymic changes (π_a) is greater for *PsNRLK1* (Table 1). Consequently, the π_a / π_s ratio is greater for *PsNRLK1* than for *PsSym29*, too. This fact may reflect stronger purifying selection pressure upon the *PsSym29* gene sequence, as compared with *PsNRLK1*. In *M. truncatula*, mutations in *MtSUNN* (orthologue of *PsSym29*) lead to supernodulation phenotype of the plants [15], whereas mutations in *MtNRLK1* do not affect any aspect of nitrogen-fixing symbiosis or plant development [7], thus indicating the higher importance of *MtSUNN* as compared to *MtNRLK1*. However, codon-based Z-test showed no deviations from the model of neutral evolution for both genes (for *PsNRLK1*: $Z = -1.819$, p-value = 0.071; for *PsSym29*: $Z = -0.909$, p-value = 0.365).

In order to examine the polymorphic sites distribution within the gene sequences, and in order to find possible sites with specific selection signatures, we calculated nucleotide diversity (π) along with Tajima's D and Fay and Wu's H criteria in a sliding window. As seen in Fig. 1, the value of π for *PsNRLK1* sequence increases remarkably in intronic part of the gene, indicating that the intron of *PsNRLK1* is quite polymorphic, which is not the case for the *PsSym29* gene, for which the π is low in both coding and non-coding parts of the gene. However, the nucleotide diversity of *PsSym29* and *PsNRLK1* sequences was at least five times lower than the π calculated in our previous study of the same pea cultivars for 1st exons of symbiotic receptor kinase genes *PsSym37*, *PsK1* and *PsLykX* [8]. The Tajima's D calculated either for the whole sequences (0.023 for *PsSym29* and –0,607 for *PsNRLK1*) or for sliding windows of 100 bp length does not indicate any departure from neutral evolution model. The analysis of Fay and Wu's H, however, revealed signatures of

positive selection in one site of *PsSym29* and in three sites of *PsNRLK1* gene sequences (Fig. 1). This finding indicates that the sequences of both *PsSym29* and *PsNRLK1* are evolving, and newly arisen alleles of the genes are substituting the older ones. The identified sites containing non-synonymous substitutions (Fig. 1) are located within the first exons of the genes, which encode LRR domains that bind ligands (CLE peptides and, probably, other related peptides). Further studies based, for instance, on mathematical modeling of ligand-receptor interactions are required to elucidate whether the detected amino acid substitutions in LRR domains affect the binding properties of the receptors.

The number of polymorphic sites in the sequences of the studied genes, which result in non-synonymous substitutions, was 8 for *PsSym29* and 20 for *PsNRLK1*. After exclusion the rare alleles from the analysis, the number of analyzed sites remained was six for *PsSym29* and seven for *PsNRLK1* genes (Table S2). According to prediction of the SIFT program [13], all amino acid changes, except for Sym29.43PH, were considered 'tolerated', i. e., not changing the function of the proteins. The amino acid substitution P43H in PsSym29 sequence is located upstream of the LRR domain, and the corresponding amino acid in this site of orthologous MtSUNN is deleted, so it is unlikely that this amino acid change can affect the function of the PsSym29 protein.

For all analyzed polymorphic sites, no statistically significant impact of allelic states on growth and yield parameters was obtained (Table S2), and no influence of the allelic variants on the AM-caused increments of the parameters was observed (Table S3). In our previous work, allelic state of the non-symbiotic gene *Le* showed strong association with plant yield, and among the symbiotic genes, only *PsLykX* gene encoding receptor kinase participating in legume-rhizobial symbiosis showed association with TSW (thousand seed weight) [5]. This implies that the effect of amino acid substitutions was minor in the conditions of the experiment where the yield parameters were determined.

CONCLUSION

The pea gene *PsSym29* with known role in autoregulation of nodulation and mycorrhization was less polymorphic than its homologue *PsNRLK1*, for which the function in plant growth and development is unknown to date. However, allelic states of both genes did not show associations with yield and growth parameters of pea plants grown in symbiotic conditions (i.e., under inoculation with either nodule bacteria or nodule bacteria and arbuscular mycorrhizal fungi). Remarkably, we found signatures of positive selection in 1st exons of the studied genes, which implies that some alleles of the genes can be advantageous in some conditions; however, if these conditions are symbiotic or non-symbiotic, remains to be analyzed in future.

ADDITIONAL INFORMATION

Author contribution. Thereby, all authors made a substantial contribution to the conception of the study, acquisition, analysis, interpretation of data for the work, drafting and revising the article, final approval of the version to be published and agree to be accountable for all aspects of the study. Contribution of each authors: V.A. Zhukov — research concept and design, analysis of gene polymorphism, writing text; A.I. Zhernakov — associative analysis, mathematical processing of the data obtained, writing text; M.Yu. Belozerova — amplification of fragments of studied genes, writing text, literature review; I.E. Dodueva — amplification of fragments of studied genes, writing text; M.A. Lebedeva — analysis of gene polymorphism, literature review, text writing; L.A. Lutova, I.A. Tikhonovich — research concept and design, discussion of results, text writing.

Competing interests. The authors declare that they have no competing interests.

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Appendix

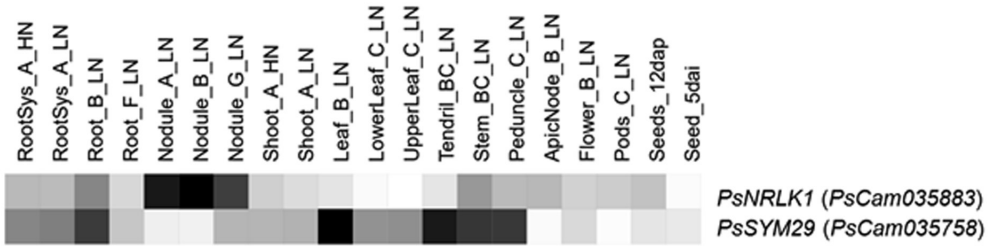

Fig. S1. The heatmap diagram illustrating expression levels (normalized RPKM values) of the *PsNRLK1* and *PsSYM29* genes in different pea organs according to transcriptomic data by S. Alves-Carvalho et al. [14]. The heatmap was generated using Morpheus software (https://software.broadinstitute.org/morpheus)

Table S1. Primers used for amplification and sequencing of the genes *PsSym29* and *PsNRLK1*

Gene	Name of the primer	Sequence, 5'-3'	Length of the PCR product, bp
PsNRLK1	PsNRLK1-F1	ATATTCCTATGTCTTCTTTCACCTTTCTTC	704
	PsNRLK1-R1	TTCACCTTTCAAACCACAGTTAGCAAG	
	PsNRLK1-F2	GGTAATCTTGTTAGTTTGGTTCATCTTGA	803
	PsNRLK1-R2	CCTATATCTTGAGGAATTTCACCTGAGA	
	PsNRLK1-F3	CAGATTCTGCTTCTTCATGGAAATAGAT	837
	PsNRLK1-R3	AAGGCATAGATACATTGTGAAGTTGTTG	
	PsNRLK1-F4	TAACAAAGGTAGTTCTTATGATAATGGTT	763
	PsNRLK1-R4	GTAAAGCAACCAAATATCTTTCATGTGTAG	
PsSym29	PsSYM29-F1	CGTGTTATTTGCTGGTATTCTTCTGC	987
	PsSYM29-R1	CGCCTATGAATGCCTGAACAGAA	
	PsSYM29-F2	GCAAATGAACAACCTCACCG	1066
	PsSYM29-R2	CGAGGGAAAGGTATAGGAAG	
	PsSYM29-F3	CGTTTTTCGGAAACCCTAACCTCTGT	1084
	PsSYM29-R3	GGTGGATTAGTGAGCACATGAACTACTTC	

Table S2. FDR-corrected *p*-values of F-tests for significance of the SNV (single nucleotide variation) factor's effect on growth and yield parameters values in two-way ANOVA-tests

SNV	PW, Plant shoot weight, g	PL, Plant shoot length, cm	PN, Pod number	SN, Seed number per plant	SW, Seed weight per plant, g	TSW, Thousand seed weight, g	NtS, Seed nitrogen content, mg/g	PhS, Seed phosphorus content, mg/g
NRLK1.19SL	0.8975	0.8975	0.8975	0.8975	0.8975	0.9013	0.8975	0.8975
NRLK1.30SP	0.5255	0.9871	0.8602	0.5255	0.5255	0.3465	0.9871	0.9871
NRLK1.212RG	0.8226	0.8226	0.8226	0.1208	0.8226	0.2551	0.8226	0.8226
NRLK1.356LQ	0.7879	0.0188	0.8651	0.7879	0.7879	0.7879	0.6018	0.6721
NRLK1.443GR	0.6138	0.0670	0.7739	0.6138	0.6292	0.6138	0.6138	0.6138
NRLK1.875FY	0.6113	0.6113	0.6113	0.6113	0.6765	0.6113	0.6113	0.9087
NRLK1.890GE	0.2631	0.2728	0.2365	0.2631	0.2816	0.2599	0.2728	0.3020
Sym29.16FS	0.2778	0.1792	0.8113	0.3173	0.4688	0.8113	0.8113	0.2778
Sym29.43PH	0.4068	0.7399	0.8814	0.8814	0.5393	0.1745	0.7858	0.8814
Sym29.53AGV	0.4022	0.7395	0.8792	0.8792	0.5575	0.2034	0.7608	0.8792
Sym29.171IF	0.7670	0.6475	0.6475	0.8005	0.8005	0.6475	0.6475	0.6676
Sym29.S618P	0.4857	0.3378	0.4857	0.3378	0.4857	0.4857	0.3378	0.4857
Sym29.T623I	0.5105	0.4421	0.7061	0.6451	0.5105	0.8590	0.4021	0.5105

Table S3. FDR-corrected *p*-values of Likelihood-Ratio Tests for significance of the SNV (single nucleotide variation) factor's effect on AM-caused increments of growth and yield parameters

SNV	PW, Plant shoot weight, g	PL, Plant shoot length, cm	PN, Pod number	SN, Seed number per plant	SW, Seed weight per plant, g	TSW, Thousand seed weight, g	NtS, Seed nitrogen con-tent, mg/g	PhS, Seed phosphorus content, mg/g
NRLK1.19SL	0.3444	0.7264	0.7264	0.7264	0.4034	0.3444	0.4034	0.3444
NRLK1.30SP	0.4384	0.8388	0.8003	0.8388	0.6873	0.4384	0.6873	0.4384
NRLK1.212RG	0.8517	0.8517	0.8517	0.8861	0.8517	0.3277	0.8517	0.8861
NRLK1.356LQ	0.1350	0.8911	0.7722	0.8412	0.1350	0.1209	0.1350	0.5528
NRLK1.443GR	0.2186	0.9221	0.9221	0.9221	0.2186	0.0901	0.1646	0.9221
NRLK1.875FY	0.2499	0.2499	0.4094	0.4488	0.2499	0.4453	0.7828	0.4094
NRLK1.890GE	0.3769	0.9540	0.8308	0.9591	0.4788	0.3769	0.4788	0.4788
Sym29.16FS	0.8836	0.8998	0.8998	0.8836	0.8836	0.6663	0.8836	0.8836
Sym29.43PH	0.1674	0.8405	0.7943	0.9994	0.3782	0.1674	0.7943	0.7943
Sym29.53AGV	0.9035	0.7123	0.5304	0.5304	0.9035	0.5304	0.7123	0.7123
Sym29.171IF	0.8495	0.8495	0.8495	0.8495	0.8495	0.8495	0.8495	0.8495
Sym29.S618P	0.5022	0.9898	0.5022	0.9898	0.5853	0.5022	0.9898	0.5022
Sym29.T623I	0.7992	0.7992	0.5987	0.8889	0.8042	0.7992	0.7992	0.5987

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