ISSN 1022-7954, Russian Journal of Genetics, 2011, Vol. 47, No. 12, pp. 1422–1427. © Pleiades Publishing, Inc., 2011.
Original Russian Text © A.A. Sinjushin, G.A. Khartina, S.A. Gostimskii , 2011, published in Genetika, 2011, Vol. 47, No.12, pp. 1604–1610.

PLANT GENETICS

New Allele of the *COCHLEATA* Gene in Pea *Pisum sativum* L.

A. A. Sinjushin, G. A. Khartina, and S. A. Gostimskii

Department of Genetics, Biological Faculty, Moscow State University, Moscow, 119992 Russia;
e-mail: sinjushin@pisumsativum.org

Received January 20, 2011

Abstract—Analysis with the polymerase chain reaction showed that the Khlorofill-4 pea *Pisum sativum* chlorophyll-deficient mutant with reduced stipules has an altered structure of the *COCHLEATA* (*COCH*) gene, carrying a new mutant *COCH* allele. The phenotype of the mutant was described in comparison with another form having reduced stipules (*stipules reduced*) and the control. Leaves of the *coch* mutant are smaller and have other proportions than in the control; stipules are absent from leaves of the first nodes and are narrow, bandlike, or spoonlike at later ontogenetic stages. It was concluded that the cell number in the stipule epidermis is reduced in the *st* and *coch* mutants compared to the wild type.

DOI: 10.1134/S1022795411120143

INTRODUCTION

Studies of the genetic control of morphogenesis in plants are one of the most actively developing and topical fields in modern biology. Information on the genetic control of floral development, the regulation of activity of the apical meristem, the morphogenesis of various inflorescences has accumulated to date. The regulation of the development of compound leaves is still poorly understood. Lack of data in this field is partly related to the fact that simple leaves are characteristic of the standard model plants used in developmental genetics, *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) and *Antirrhinum majus* L. (Scrophulariaceae). Data obtained for these species cannot be directly extrapolated to plants with compound leaves [1]. Hence, pea *Pisum sativum* L. (Fabaceae) is used as a model to study the development of compound leaves. Leaf morphogenesis in pea (as in all legumes) is rather intricate, and there are many genes whose mutations are known to change the leaf structure [1].

We have previously described the Khlorofill-4 chlorophyll-deficient mutant, which was obtained from the Kapital variety via induced mutagenesis (γ rays) [2]. The initial mutant line carried a translocation and a chlorophyll defect, while more recent studies yielded the subline with the translocation and normal pigmentation and a subline with a normal karyotype and a photosynthesis defect (Khlorofill 4). The mutant and its photosynthetic characteristics were described in [2]. In particular, the mutant is characterized by dramatically reduced stipules and distorted floral development and is normally unviable (can be grown up to flowering via grafting). In addition, we assumed that the mutation is a short deletion and is allelic to the mutation of the known *cochleata* (*coch*) mutant based on the phenotypic similarity of the mutants ([3], cited from [2]).

Several *coch* mutants that differ in origin and result from induction with different mutagens or are spontaneous were described in the literature ([4, 5]; for a review, see [6]). Alterations in the stipule structure were noted. The mutants form either structures that are structurally similar to the compound leaf or narrow spoonlike stipules. Abnormalities were also described for the floral development and the formation of symbiotic nodules on roots [5, 7]. The *COCH* gene is in the long arm of chromosome 3, which corresponds to linkage group V [5, 8]. The Khlorofill 4 mutant can be grown up to flowering via grafting in a greenhouse. However, the mutant has an extremely low seed productivity because of the defects in floral structure, and its crosses with known *coch* mutants are difficult to perform. Zhukov [9] determined the exact location and nucleotide sequence of the *COCHLEATA* gene. With the structural data on the gene available, it is possible to study the nature of the Khlorofill 4 mutant. The objectives of this work were to analyze the phenotype of the Khlorofill 4 mutant in detail and to determine its genotype at the *COCH* gene. In addition, we compared the phenotype for the *coch* mutation and *stipules reduced* (*st*), another mutation resulting in reduced stipules [6].

MATERIALS AND METHODS

The Khlorofill 4 sterile mutant was maintained in our collection in a heterozygous form; self-pollination of heterozygotes yields chlorophyll-deficient forms at a frequency of 1/4 ($\chi^2 = 3.458$, $p < 0.05$). Seeds obtained via self-pollination of heterozygotes and seeds of the original Kapital variety (to be used as a control in measurements) and the Filby (*st*) variety from the collection of the Department of Genetics were grown in a box greenhouse of the Biological Fac-

ulty (Moscow State University). The following conditions were maintained in the greenhouse: the average daily temperature of 25 ± 2°C, illumination with halogen bulbs (7000–8000 lux), and a long light period (16 h). Replicas of the epidermis (see below) were obtained in the same conditions.

DNA was isolated according to a published protocol [10]. Amplification was carried out as in [11]. Two pairs of primers were designed to amplify fragments of the *COCHLEATA* gene, but amplification was optimal only with primers COCH-F2 (5'-GAACAGCTTG-CATTGCTC) and COCH-R1 (5'-TCCACCAT-GCTAGCAAGTTG), which flanked the only intron. The most successful amplification was achieved with an annealing temperature of 62°C. The deduced amino acid sequence of the COCH protein was reported in [9]. The DNA amount in a working solution was measured using a NanoDrop ND-1000 spectrophotometer (PeqLab Biotechnologie GmbH) with ND-100 v. 3.3.0 software. To check the integrity of the template (genomic DNA), we amplified the RAPD marker QR5 (primer 5'-CGGCCCCGGC); amplification was the most efficient at an annealing temperature of 37°C. The amplification products were resolved in 2% agarose gel (Amresco) stained with ethidium bromide and were visualized in UV light.

The structure of the epidermis was studied by scanning electron microscopy. Material was fixed with 70% ethanol (aqueous solution) and dehydrated consecutively in 70% ethanol for 10 min, 80% ethanol for 10 min, 96% ethanol for 10 min, 100% ethanol for 10 min, 100% ethanol–acetone (1 : 1) for 15 min, and acetone (the further treatment and microscopy were described in [12]). In addition, quantitative estimates were obtained using replicas. The leaf surface was covered with transparent colorless cosmetic nail polish (Sally Hansen), and the resulting film was transferred onto a slide and examined under an Olympus CX41 light microscope (Olympus). Microscopic preparations were photographed using an AxioCam HRc camera (Carl Zeiss).

Quantitative parameters of cells (at least 30 cells were examined) were measured using contrasted microphotographs (processed using Adobe Photoshop CS2) and ImageJ 1.44 software [13]. Statistical analysis was performed for the following measurements and computed parameters: total surface area S, perimeter P, round shape coefficient $P/2(\pi S)^{1/2}$ (a parameter that characterized the curvature of the outline), and elongation coefficient $4\pi S/P^2$ (a parameter that characterized the elongation of the leaf shape). Linear measurements of the fifth leaf were performed one the images with the Meazure 2.0 program (C Thing Software) to compare the Khlorofill 4 mutant and the Kapital variety. We measured the length, width, and the ratio of the distance between the leaf tip and the greatest width position to the distance between the greatest width position and the leaf base (the parameter was conventionally termed the shape coefficient). Statistical analysis was carried out using the Statistica 8 package (Statsoft).

The anatomical structure of the first scale leaves was studied using sections obtained manually with a razor and stained with acidic phloroglucin. The sections were examined under a Laboval 4 light microscope (Carl Zeiss).

RESULTS AND DISCUSSION

Nature of the Mutaion in the Khlorofill 4 Line

Amplification with primers flanking the only intron of the *COCH* gene yielded the expected 1124-bp fragment for all control plants of the Kapital variety (Fig. 1a). Similar results were obtained when amplifying a 2073-bp fragment, which included the intron and exon 2 (data not shown). No amplification was observed for all of the pale green plants of the Khlorofill 4 mutant. Amplification of a control marker (QR5) was successful in all samples (Fig. 1b). To rule out possible differences in DNA isolation quality between control and mutant plants, we measured the concentration of template DNA (genomic DNA isolated directly from leaves and diluted 10- to 20-fold). The concentrations of DNA samples from mutant and control plants were no less than 47.9 ng/μl, averaging (mean ± standard deviation) 110.7 ± 40.6 ng/μl for the control and 131.3 ± 60.5 ng/μl for the mutant. A statistical analysis of the measurements (Mann–Whitney test) showed that the difference in DNA concentration between the samples was nonsignificant ($p < 0.05$). When the control marker was amplified, fragments larger in size than the *COCH* gene fragment were observed in the electrophoretic pattern (Fig. 1b). Thus, the difference in amplification with the primers to the *COCH* gene fragment between the control and mutant plants was not related to the quantity and quality of isolated DNAs.

The results indicate that a structural alteration of the *COCH* gene occurred in the Khlorofill 4 mutant to prevent amplification of its major part. In view of the mutagenic factor used to obtain the mutant, the alteration was most likely due to a chromosome rearrangement. The structure of a gene may be altered as a result of a translocation when a translocation breakpoint is within the gene. This hypothesis disagrees with normal fertility of heterozygotes. Pollen fertility in green (normal) plants obtained via self-pollination of heterozygotes was 98.822 ± 0.605%. An alternation of normal and defective plants, which is characteristic of heterozygotes for a translocation, was not observed; the proportion of normally developed seeds in fruits was 74.917 ± 9.141%; and the abortive character was usually observed for the distal and proximal seed anlages, as generally characteristic of pea. Thus, the mutation was most likely a deletion of a small chromosome fragment or an insertion. Additional experiments are nec-

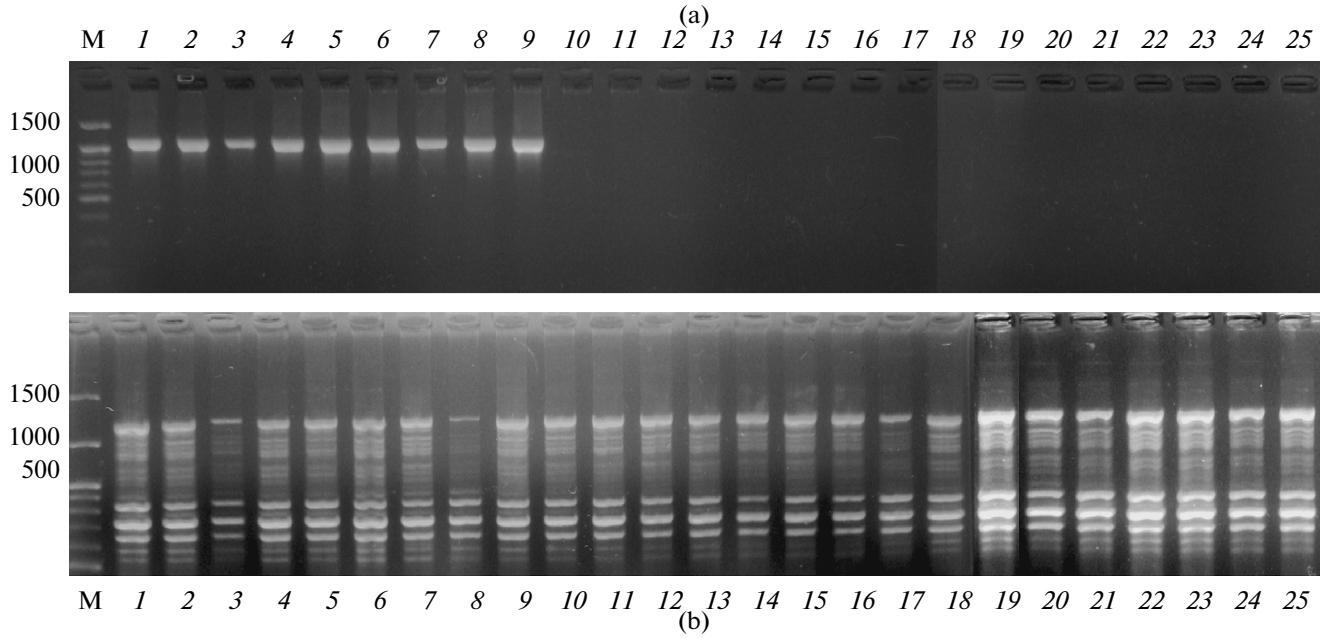

Fig. 1. Electrophoresis of the amplification products of (a) the *COCH* gene and (b) the QR5 RAPD marker. M, molecular weight marker (100 bp + 1.5 kb Ladder, SibEnzyme). Lanes *1–9*, plants of the Kapital variety; *10–25*, homozygous plants of the Khlorofill 4 line. The sizes of the marker fragments are indicated on the left.

essary to further study the character and extent of the alteration.

The pea genome has not been completely sequenced to date, but the linear order of markers on chromosomes proved to be highly similar between *Pisum* and the related legume alfalfa (*Medicago*) [14]. An almost complete nucleotide sequence of the *M. truncatula* Gaertn. genome has been established and is available at http://medicago.org/. A cytogenetic demarcation of the putative deletion was not performed for the Khlorofill 4 mutant; we can only assume that the deletion is relatively small because fertility is normal in heterozygotes for the deletion. Genes whose mutations might cause a chlorophyll defect were not found in the immediate vicinity of the putative *COCH* homolog in alfalfa. A more detailed search by in silico methods is possible when the character and size of the rearrangement are known.

Morphological and Anatomical Analyses of the Phenotype

Several phenotypic features that distinguish the Khlorofill 4 mutant from the original Kapital variety were characterized previously [2]. In this work, we focused on leaf characteristics.

The first two leaves (cataphylls) normally look like scales with three dents in pea (Fig. 2a). The first leaves of the mutant were unusual in morphology (Figs. 2, 3); the mutant developed a flattened awl-shaped structure that was generally similar to the central part of the scale leaf in wild-type plants. An anatomical examination showed that the first leaves of the *coch* mutant had a distinct bifacial structure and had three vascular bundles without a mechanic lining. In wild-type plants, the cataphylls are distinctly tripartite and have many vascular bundles (Figs. 2a, 2c).

The subsequent leaves were generally similar to those of normal plants, but lacked stipules (Fig. 3b). First stipules developed starting from nodes 5–6 (observations were performed with mutant plants grafted onto normal ones because mutants usually die at earlier stages). The stipules of the mutant were band or spoon shaped (Fig. 3c). Epidermal cells of stipules in the *coch* mutant were somewhat smaller than in the control (the difference was nonsignificant) and had significantly less curved anticlinal walls (table).

Plants with a mutation of the *ST* gene are also characterized by reduced (band-shaped) stipules [6]. A comparison of the stipule epidermis structure for plants with the *COCH ST*, *COCH st* and *coch ST* genotypes showed that the cell number of *COCH st* plants (Filby variety) was lower than in wild-type plants. The conclusion was based on the fact that parquet cells of the *COCH st* mutant were nonsignificantly larger than in the control, while the stipule size was dramatically reduced. The anticlinal walls of parquet cells of the mutant were more undulate than in the control. Stoma clefts of plants of the Kapital variety were significantly shorter than in both of the mutants ($p < 0.01$). The elongation coefficient did not significantly differ between the forms under study. It is clear that the number, rather than size, of stipule cells is reduced in the mutants compared with the control.

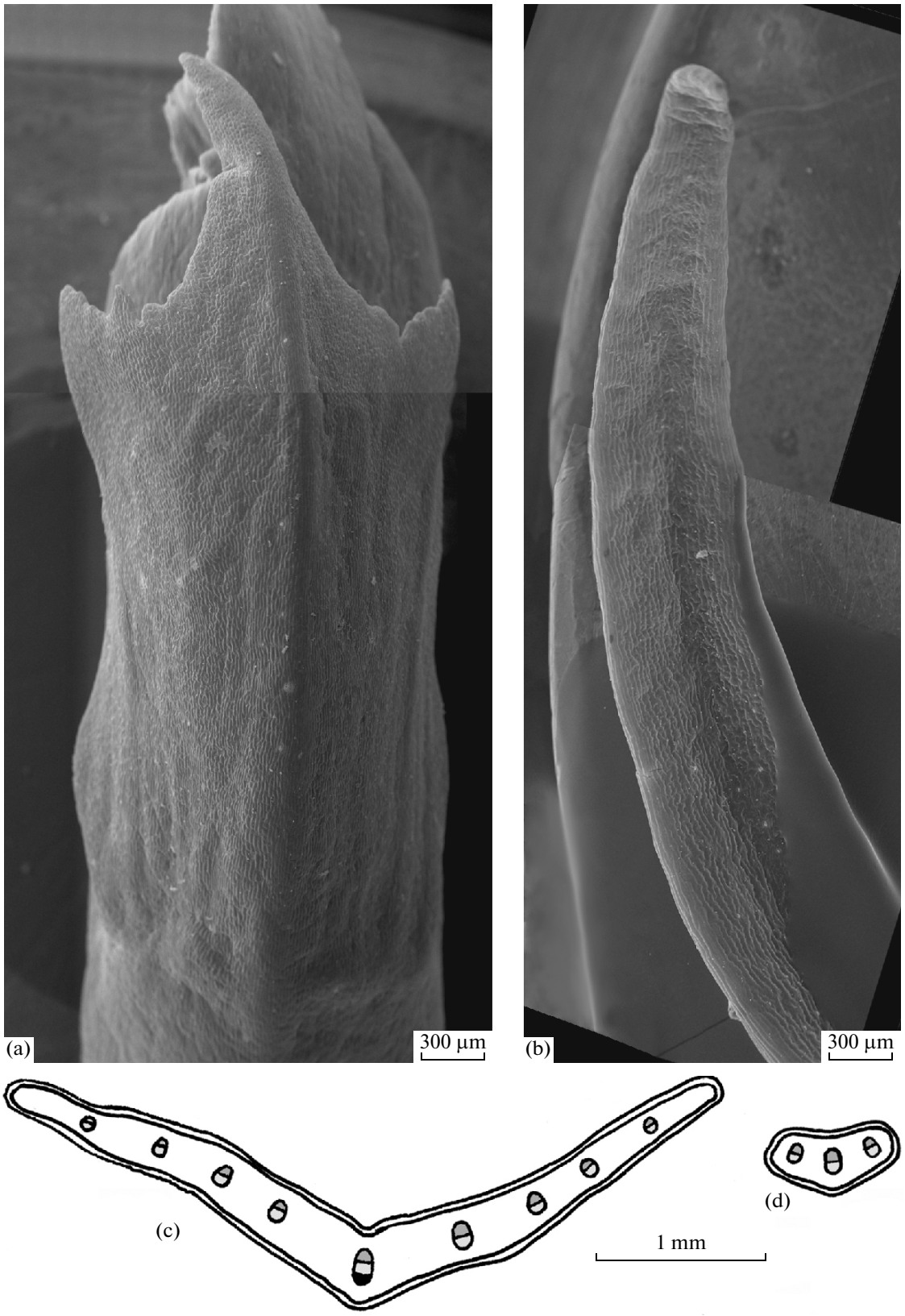

Fig. 2. Structure of the first leaf in (a, c) the Kapital variety and (b, d) the Khlorofill 4 mutant as seen on (a, b) scanning electron microscopic images and (c, d) schemes of transversal anatomical section. Xylem, dark gray; phloem, light gray; mesophyl, white; mechanic tissue, black; epidermis, double line.

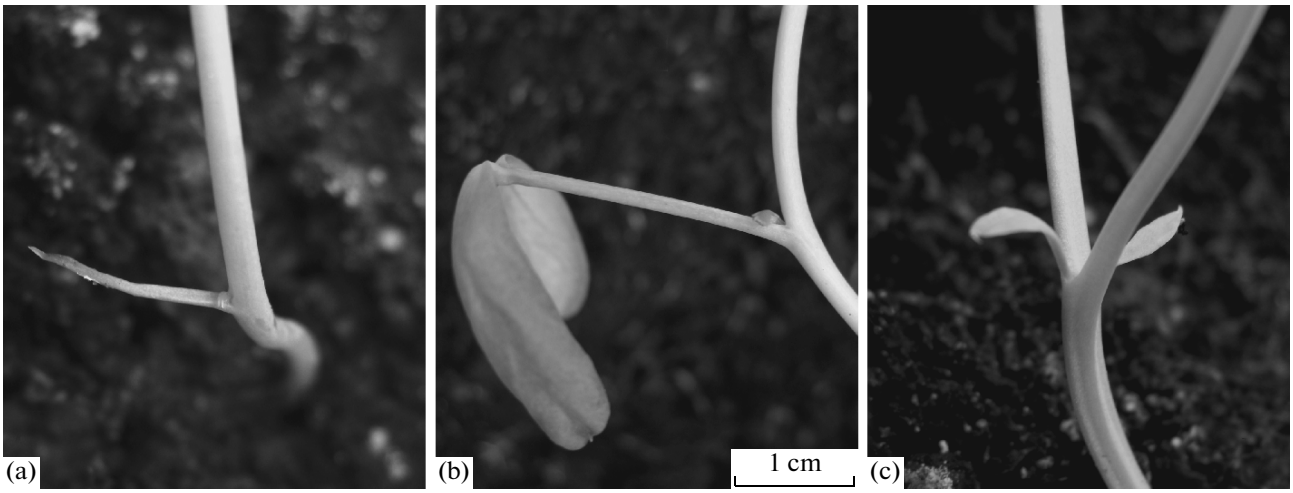

Fig. 3. Specifics of stipule development in the Khlorofill 4 mutant: (a) an awl-shaped structure without stipules in node 2, (b) two-leaflet leaf without stipules in node 3, and (c) spoon-shaped stipules in node 6. A developing lateral shoot is seen in the sheath of the leaf in (b).

We have previously noted that leaves of the Khlorofill 4 mutant seem narrower than in wuld-type plants [2], but the trait was not studied in detail. Analysis of the qualitative traits of leaves showed that leaves of the Khlorofill 4 mutant were significantly shorter and narrower than in wild-type plants (table). In addition, leaves of the mutant have other proportions than leaves of the original variety and are obovate, while oval or ovate leaves are characteristic of the Kapital variety (the forms significantly differed in shape coefficient at $p < 0.01$).

The structural alteration of the *COCH* gene (or its major part) makes the Khlorofill 4 mutant particularly interesting for studying the genetic control of leaf development. The function of the *COCH* gene is normally related to inhibition of expression of the *UNIFOLIATA* (*UNI*) gene, which is a key regulator of the development of a compound leaf, in stipula anlages [15]. This circumstance presumably explains why compound leaves develop in place of stipules in homozygotes for certain *coch* alleles.

The fact that the Khlorofill 4 mutant with an altered structure of the *COCH* gene still develops stipules (meaning true stipules, although altered as they are) indicates that a certain genetic regulatory system backs up the function of the *COCH* gene. Sidorova [4] described a mutant that is nonallelic, but phenotypically similar, to *coch*. It is possible that the *ST* gene acts synergistically with *COCH* because the *coch st* double mutant lacks stipules and the *st* mutants are known to develop compound stipules similar to those of the *coch* mutants [15, 16]. However, Yaxley et al. [17] suggested an epistatic interaction of the *coch* and *st* mutations because stipules of the *coch st* double mutants are similar to those of the *coch* mutant. The available data on the nucleotide sequence of the *COCH* gene make it possible to study in detail the mechanism of this interaction at the level of key gene expression.

Quantitative parameters of leaves and the stipule epidermis in the forms under study

Parameters	Kapital (*COCH ST*)	Khlorofill 4 (*coch ST*)	Filby (*COCH st*)
Leaf length, cm	2.6040 ± 0.3003	1.8417 ± 0.1864*	–
Leaf width, cm	1.9559 ± 0.1999	1.2423 ± 0.1942*	–
Shape coefficient, units	1.166 ± 0.1788	0.7510 ± 0.1268*	–
Stoma cleft length, mm	0.1474 ± 0.0175	0.2071 ± 0.0240*	0.2149 ± 0.0281*
Parquet cell perimeter, mm	4.4238 ± 1.1906	4.0342 ± 1.3569	5.7973 ± 1.6484*
Parquet cell area, mm^2	0.2064 ± 0.0578	0.2206 ± 0.0809	0.3128 ± 0.0875
Round shape coefficient of parquet cells, units	2.7366 ± 0.4277	2.4155 ± 0.4586*	2.9216 ± 0.6054
Elongation coefficient of parquet cells, units	0.1192 ± 0.0317	0.1132 ± 0.0361	0.1054 ± 0.0311

Note: The results are given as mean standard deviation. Significant differences (by the *t* test, $P < 0.01$) from the control (Kapital variety) are indicated with asterisks. A dash indicates that measurements were not performed.

ACKNOWLEDGMENTS

We are grateful to V.A. Zhukov (All-Russian Institute of Agricultural Microbiology, Russian Academy of Agricultural Sciences) for valuable advice and help with references and to staff of the Interdepartmental Laboratory of Electron Microscopy (Biological Faculty, Moscow State University) for technical assistance.

This work was supported by the Russian Foundation for Basic Research (project no. 10-04-01480).

REFERENCES

1. Sinyushin, A.A. and Gostimskii, S.A., Progress and Perspectives of Pea (*Pisum sativum* L.) Usage as a Model Object in Plant Developmental Genetics, *Usp. Sovrem. Biol.*, 2008, vol. 128, no. 6, pp. 531–541.
2. Gostimskii, S.A., Pleiotropic Effect of Chlorophyll Mutation in Pea, *Genetika* (Moscow), 1972, vol. 8, no. 7, pp. 31–35.
3. Gottschalk, W., Neue Aspekte zum Problem der Pleiotropen Genwirkung, *Ber. Dtsch. Bot. Ges.*, 1967, vol. 80, no. 8, pp. 545–553.
4. Sidorova, K.K., Study of Allelism in Phenotypically Similar Pea Mutants in Connection with the Law of Homologous Series in Hereditary Variability, *Genetika* (Moscow), 1970, vol. 6, no. 11, pp. 23–35.
5. Zhukov, V.A., Kuznetsova, E.V., Ovchinnikova, E.S., et al., Gene-Based Markers of Pea Linkage Group V for Mapping Genes Related to Symbioses, *Pisum Genet.*, 2007, vol. 39, pp. 19–25.
6. Blixt, S., Mutation Genetics in *Pisum*, *Agri Hortique Genet.,* 1972, vol. 30, pp. 1–293.
7. Zhukov, V.A., Rychagova, T.S., Sulima, A.S., et al., Gene *COCHLEATA* in *Pisum sativum* L. and Its Ortholog *NOOT* in *Medicago truncatula* Gaertn. Control Meristem Fate of Nitrogen Fixing Nodules, *Biologiya—nauka XXI veka* (Biology—the Science of 21th Century), (Proc. 14th Pushchino Int. School–Conf. Young Scientists), Pushchino, 2010, vol. 2, p. 135.
8. Swiecicki, W.K., A New Gene *heterophyllus (het)* on Chromosome 7, *Pisum Newslett.*, 1989, vol. 21, pp. 75–76.
9. Zhukov, V.A., Cloning of Symbiotic Pea (*Pisum sativum* L.) Genes Using Legume Genome Synteny, *Cand. Sci. (Biol.) Dissertation*, St. Petersburg, 2008.
10. Torres, A.M., Weeden, N.F., and Martin, A., Linkage among Isozyme, RFLP and RAPD Markers in *Vicia faba*, *Theor. Appl. Genet.*, 1993, vol. 85, pp. 937–945.
11. Konovalov, F., Toshchakova, E., and Gostimsky, S., A CAPS Marker Set for Mapping in Linkage Group III of Pea (*Pisum sativum* L.), *Cell Mol. Biol. Lett.*, 2005, vol. 10, pp. 163–171.
12. Sinyushin, A.A. and Gostimskii, S.A., Genetic Control of Fasciation in Pea, *Russ. J. Genet.*, 2008, vol. 44, no. 6, pp. 702–708.
13. Abramoff, M.D., Magalhaes, P.J., and Ram, S.J., Image Processing with ImageJ, *Biophotonics Int.*, 2004, vol. 11, no. 7, pp. 36–42.
14. Kalo, P., Seres, A., Taylor, S.A., et al., Comparative Mapping between *Medicago sativa* and *Pisum sativum*, *Mol. Genet. Genomics*, 2004, vol. 272, no. 3, pp. 235–246.
15. Gourlay, C.W., Hofer, J.M.I., and Ellis, T.H.N., Pea Compound Leaf Architecture Is Regulated by Interactions among the Genes *UNIFOLIATA, COCHLEATA, AFILA*, and *TENDRIL-LESS, Plant Cell*, 2000, vol. 12, pp. 1279–1294.
16. Kumar, S., Mishra, R.K., Kumar, A., et al., Regulation of Stipule Development by *COCHLEATA* and *STIPULE-REDUCED* Genes in Pea *Pisum sativum*, *Planta*, 2009, vol. 230, pp. 449–458.
17. Yaxley, J.L., Jablonski, W., and Reid, J.B., Leaf and Flower Development in Pea (*Pisum sativum* L.): Mutants *cochleata* and *unifoliata, Ann. Bot.*, 2001, vol. 88, pp. 225–234.