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ORIGINAL PAPER

Inheritance and genetic mapping of two nuclear genes involved in nuclear–cytoplasmic incompatibility in peas (*Pisum sativum* L.)

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Abstract Genetic analysis was performed to finely map and assess the mode of inheritance of two unlinked nuclear genes *Scs1* and *Scs2* involved in incompatibility of the nuclear genome of the cultivated pea *Pisum sativum* subsp. *sativum* with the cytoplasm of the wild pea of the subspecies *P. sativum* subsp. *elatius*, accession VIR320. Based on the segregation of genotypes in the progeny of the test-crosses, we concluded that if the cytoplasm was inherited from the wild pea VIR320, the *Scs1* allele from the cultivated pea was gametophyte lethal and sporophyte recessive lethal. The *Scs2* allele from the cultivated pea reduced male gametophyte viability. In homozygote, *Scs2* from cultivated parent brought about nuclear–cytoplasmic conflict manifested as chlorophyll deficiency, reduction of blade organs, and low pollen fertility of about 20%. In heterozygote, *Scs1* and *Scs2* genes reduced pollen fertility by ca 50 and 30%, respectively. The *Scs1* and *Scs2* genes involved in nuclear–cytoplasmic incompatibility were genetically mapped. The distance between the markers bordering *Scs1* comprised about 2.5 cM on linkage group III. The map distance between the bordering markers in the neighborhood of *Scs2* varied substantially from cross to cross in the range of 2.0–15.1 cM on linkage group V.

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Introduction

Earlier we described nuclear–cytoplasmic incompatibility in the garden pea occurring when a pea line VIR320, referring to a wild subspecies, *Pisum sativum* subsp. *elatius* (Bieb.) Schmahl, was crossed with domesticated pea lines. The nuclear–cytoplasmic conflict manifested as chlorophyll deficiency, chlorophyll variegation, and reduction of leaflets and stipules (Bogdanova and Berdnikov 2001). Analysis of plastid DNA markers showed that this conflict was associated with plastids inherited from VIR320 (Bogdanova and Kosterin 2006; Bogdanova 2007). Genetic analysis of this case of nuclear–cytoplasmic incompatibility showed that the typical phenotype of an incompatible nucleus–cytoplasm combination was conditioned by two unlinked nuclear genes denoted as *Scs1* and *Scs2*. The former was located on linkage group (LG) III closely linked to the gene *PhlC* coding for phospholipase C, while the latter was located on LGV closely linked to the gene *gp* (*luteo-legumina*) which determines yellow coloration of young pods (Bogdanova et al. 2009).

This situation resembles the two-gene inheritance described for nuclear–cytoplasmic incompatibility among *Triticum* and *Aegilops* species (Ohtsuka 1991; Anderson and Maan 1995). The difference is that, in pea, nuclear–cytoplasmic conflict is conditioned by incompatibility of the nuclear and plastid genomes but not the mitochondrial genome (Bogdanova 2007), while in cereals, the mitochondrial genomes are primarily involved (Aksyonova et al. 2005). The genetic basis of nuclear–cytoplasmic conflict

has been studied in cases of incompatibility of *Triticum* species with the cytoplasms of *Aegilops squarrosa* (Ohtsuka 1991) and *Ae. longissimum* (Anderson and Maan 1995). Gene analysis suggested that two kinds of nuclear genes were responsible for the compatibility with *Ae. squarrosa* cytoplasm. One of these genes led to incomplete development of the endosperm and chlorophyll variegation (incomplete development of chloroplasts), the other was related to the vigor of F1 plants (Ohtsuka 1991). Unlike tetraploid wheat with genomic composition AABB, the *Triticum timopheevii* AAGG genome was compatible with the cytoplasm of *Ae. squarrosa*. The corresponding factor providing this compatibility, *Ncc-tmp*, was located on the 1A chromosome (Asakura et al. 1997a, b), and a functional homoeoallele was also present in the G-genome (Asakura et al. 2000).

Another series of genetic analyses mapped genes conferring nuclear–cytoplasmic compatibility with *Aegilops longissimum* cytoplasm. An alloplasmic line was obtained via back-crosses which combined the nucleus of *T. turgidum* and cytoplasm of *Ae. longissimum.* This line produced viable seeds due to introgression of the compatibility gene from *T. timopheevii* (Maan 1992a). Genetic analysis using RFLP markers showed this gene (scs^{ti}) to be located on chromosome arm 1AL (Anderson and Maan 1995), virtually the same position as in the independently obtained introgressed line of *T. durum* (Asakura et al. 1997a), suggesting that the *T. timopheevii*-derived *Ncc* gene conferring compatibility with the *Ae. squarrosa* cytoplasm, and *scs* conferring compatibility with the *Ae. longissimum* cytoplasm, represented the same locus. Although the scs^{ti} gene improved embryo–endosperm compatibility resulting in plump viable seeds, another gene *Vi* was required to restore male fertility (Maan 1992b). This gene was mapped to chromosome 1BS (Anderson and Maan 1995).

In pea, a well-defined species, *Pisum fulvum*, is known to have limited crossability with *Pisum sativum*, producing viable hybrids only when used as the pollen parent (Ben-Ze'ev and Zohary 1973). Even in the compatible crosses between these pea species, one encounters segregation of gene(s) conferring incompatibility of *P. fulvum* nucleus with *P. sativum* cytoplasm. A substantial segregation distortion in the F2 generation resulted in complete absence of some genotypes. This was probably due to sporophyte lethality linked to the satellite or neighboring regions of chromosome 7 and additionally of chromosome 5 (De Martino et al. 2000). This type of incompatibility may be referred to as "recessive" (Greiner et al. 2011).

In this study, we assessed whether the *Scs1* and *Scs2* loci, conferring incompatibility of the *P. sativum* subsp. *sativum* nuclear genome with the cytoplasm of the wild subspecies, *P. sativum* subsp. *elatius*, were associated with gametophyte and/or sporophyte lethality and performed genetic mapping of these loci.

Materials and methods

Plant material

The wild pea accession VIR320, originating in Palestine, was obtained from the collection of the Vavilov All-Russian Institute of Plant Breeding in St. Petersburg, Russia. The line is classified as *Pisum sativum* subsp. *elatius* (Bieb.) Schmahl. The accession L100, which belongs to the same subspecies originates in Israel and appears to be identical to line 712 from (Ben-Ze'ev and Zohary 1973). The cultivated lines WL1238, WL1072, WL851 were obtained from the Weibullsholm collection, Landscrona (Sweden). These lines are fully compatible with other *P. sativum* subsp. *sativum* germplasm.

The alleles of the genes of interest, inherited from VIR320 or cultivated pea in the lines employed, are listed in Table 1. Visible markers on linkage groups (LG) III and V linked to compatibility genes were employed *st* (reduced stipules, LGIII), *gp* (yellow pod, LGV). Semidominant visible marker *tl* (LGV) converts tendrils to leaflets. WL1238 is marked by *gp*, *tl-w*, WL1072 is marked by *st*, and WL851 is marked by *st*, *tl-w*. The description of the markers can be found at http://data.jic.bbsrc.ac.uk/cgi-bin/pgene. PCR primers used to analyze molecular markers employed are listed in Supplementary Table 1S. To denote the alleles inherited from a specific parent, for example, WL1238 or VIR320, we use a marker name followed by the underscore and parent designation, for example, *PhlC_1238* and *PhlC_320*. In all crosses mentioned, the maternal parent is indicated first.

The lines used for the genetic analysis were derived from crosses of individual lines from the RIL population obtained as F6 progeny of the cross WL1238 × VIR 320 described in (Bogdanova et al. 2009). The st320 tester carried the visible *st* marker originating from WL1072 and molecular markers *PhlC* and *AJ832139*, as well as the cytoplasm from VIR320. The scheme used to obtain the st320 tester is given as Supplementary Material. The tester line gp37 was obtained as F5 progeny ascending to a single F3 plant from the cross VIR320 × RIL-37 homozygous for *gp_1238 scs2_320*. This crossover combination occurred in the line RIL-37 (Bogdanova et al. 2009). The cytoplasm was inherited from the wild pea.

Plant growing and pollen counts

Seeds were sown in a greenhouse in hydroponic beds filled with a claydite/vermiculite mixture and watered thrice a day with a standard Knop nutrient solution (0.8 g/l calcium nitrate, 0.2 g/l magnesium sulphate, 0.2 g/l acid potassium phosphate, 0.2 g/l potassium nitrate, and traces of ferric phosphate). Plants were illuminated by 8 h daylight/16 h

Table 1 Allelic state of the markers in the lines used in current study

Line	Plastid marker	Genes of nuclear–cytoplasmic compatibility		Markers on LGIII		
	rbcL	*Scs1*	*Scs2*	*PhlC*	*AJ832139*	*st*
RIL-32, 33, 65	1238	320	1238	320	320	*St*
RIL-37	1238	1238	320	1238	1238	*St*
RIL-78, 106	1238	320	1238	320	320	*St*
gp37	320	320	320	320	320	*St*
st320	320	320	320	320	320	*st*

Line	Markers on LGV						Visible markers	
	Met2	*gp*	*Nca*	*scr*	*pnp*	*Apy*	*k*	*tl*
RIL-32, 33, 65	1238	*gp*	1238	1238	1238	1238	*K*	*tl-w*
RIL-37	1238	*gp*	1238	1238	1238	1238	*k*	*Tl*
RIL-78, 106	1238	*gp*	1238	1238	1238	1238	*k*	*tl-w*
gp37	1238	*gp*	1238	320	320	320	*K*	*Tl*
st320	–	*Gp*	–	–	–	–	*K*	*Tl*

All lines were homozygous for the alleles indicated

– not determined

incandescent light of 10,000–12,000 lux intensity. To estimate pollen fertility, acetocarmine staining was used (Singh 2003). 100–300 pollen grains per flower in 3–4 flowers per plant were counted with the use of a light microscope. Pollen grains with unstained cytoplasm were regarded as sterile; pollen fertility was estimated as the proportion of fertile pollen grains to the total pollen grains counted.

Genomic DNA extraction, PCR analysis, and endonuclease digestion

About 100 mg of leaves were harvested and rubbed with a Teflon pestle through a stainless steel grid (1 × 1 mm^2) into a vessel containing 1.5 ml of 0.15 M NaCl. After centrifugation at 4,000*g* for 5 min in a 1.5 ml Eppendorf tube, the pellet was resuspended in 200 µl of buffer containing 100 mM Tris–HCl (pH 8.0), 100 mM NaCl, 5 mM EDTA, and 0.5% SDS (w/v), then stirred and left for 30 min at room temperature for extraction. After centrifugation at 8,000*g* for 5 min, the supernatant was collected and, for DNA purification, combined with an equal volume of 5 M LiCl solution, stirred and left for 15 min on ice. The mixture was centrifuged at 8,000*g* for 5 min, the supernatant collected, and 1 ml of 96% ethanol was added to precipitate the DNA. The precipitate was collected by centrifugation at 8,000*g* for 5 min, washed with 100 µl of 75% ethanol and centrifuged. The supernatant was discarded; the pellet dried at 50° for 5 min and dissolved in 50 µl of deionised water. The insoluble contaminants were removed by centrifugation at 8,000*g* for 5 min, and the supernatant was transferred to fresh tubes. About 10 ng of genomic DNA was used for PCR amplification under the following conditions initial denaturation at 94°C for 2 min 30 s followed by five cycles of 30 s at 93°, 1 min at 58°, 1 min 30 s at 70°, then by 35 cycles of 20 s at 93°, 1 min at 56°, 1 min at 72°, and final extension for 3 min at 72°. PCR products were analyzed in an 0.8% agarose gel in TAE buffer. To distinguish alleles inherited from a specific parent, the CAPS approach was used (Konieczny and Ausubel 1993). Five to twelve microliters of PCR reaction was digested with an appropriate endonuclease (Supplementary Material, Table 1S) in a total volume of 16 µl according to the manufacturer's instructions. All enzymes were purchased from Sibenzyme (Novosibirsk, Russia). Products of endonuclease digestion were analyzed in a 1.5% agarose gel in TAE or in a 10% polyacrylamide gel in TBE.

Results

Phenotypic expression of *Scs1* and *Scs2*

Earlier we found that nuclear–cytoplasmic conflict, manifested as strong chlorophyll deficiency, chlorophyll variegation, and reduction of blade organs, developed when the cytoplasm of the wild parent VIR320, was combined with the alleles of two unlinked nuclear genes *Scs1* and *Scs2*, from WL1238, both present in the heterozygous state. That is alleles of both genes originating from WL1238 were dominant and complementary. If an allele of only one of these genes came from the cultivated parent and the other originated from the wild parent, the nuclear–cytoplasmic conflict in its typical form did not develop. However, chlorophyll pigmentation of the plants that avoided the typical

Table 2 Pollen fertility of F1s from the crosses of VIR320 × RILs with indicated allelic state of *Scs1* and *Scs2*

Scs1	*Scs2*	Number of RILs	Range	Mean ± SE	SD
320	320	5	0.94–0.96	0.950 ± 0.003	0.007
1238	320	20	0.42–0.59	0.509 ± 0.010	0.043
320	1238	28	0.44–0.91	0.691 ± 0.022	0.115
1238	1238	7	0.21–0.41	0.329 ± 0.033	0.087

conflict was not as fully developed as in their reciprocals (Bogdanova et al. 2009). We studied pollen fertility in F1 hybrids resulting from crosses of VIR320 as the donor of cytoplasm with 60 RILs of the mapping population. Special attention was paid to the allelic state of the *Scs1* and *Scs2* genes as judged from the allelic state of the closely linked markers on LGIII and LGV, *PhlC* and *gp*, respectively. The pollen counts from 2 to 25 flowers (12 on average) from 1 to 11 F1 plants (four on average) per RIL were combined and used to calculate pollen fertility corresponding to the RIL studied.

Table 2 shows that F1s homozygous for both *scs1_320* and *scs2_320* (line 1) had high pollen fertility, while F1s homozygous for one of the compatibility genes and heterozygous for the other (lines 2 and 3) had reduced pollen fertility, although the appearance of plants was quite normal (Bogdanova et al. 2009). Heterozygotes for *Scs1* (line 2) generally had lower pollen fertility of 0.509 than heterozygotes for *Scs2* (line 3) with the mean value of 0.691. At the same time, the range of pollen fertility of the latter heterozygotes was wider and the standard deviation of the character was higher. The F1 plants heterozygous for both compatibility genes manifested the signs of the typical nuclear–cytoplasmic conflict and had significantly lower pollen fertility than heterozygotes of either of the compatibility genes. The numeric value of pollen fertility of double heterozygotes 0.329 was roughly equal to the product of the values of pollen fertility in the classes of heterozygotes for one compatibility gene ($0.691 \times 0.509 \approx 0.352$). We conclude that each of the *Scs1* and *Scs2* genes in the heterozygote reduces pollen fertility, and the combined action of the two genes brings about a substantial reduction of pollen fertility, as well as chlorophyll deficiency and reduction of blade organs, if the cytoplasm is inherited from the wild parent.

Inheritance of *Scs1* and *Scs2*

Previously (Bogdanova et al. 2009) we failed to find homozygotes for *Scs1_1238* and *Scs2_1238* in the background of VIR320 cytoplasm. Similar results were observed in crosses of *Pisum sativum* with *P. fulvum* where some genotypes were absent, presumably due to sporophyte lethality

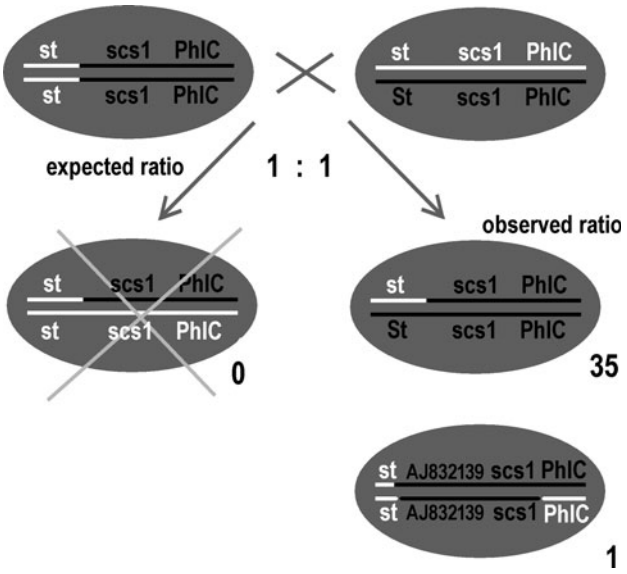

Fig. 1 A scheme of the test-cross to assess viability of gametophytes carrying *Scs1* from cultivated parent on the wild pea VIR320 cytoplasm background. Markers designated in *black* derive from the wild pea VIR320, those in *white* from cultivated pea WL1072. *Crossed lines* symbolize death of male gametophytes

(De Martino et al. 2000). In the present study, we performed genetic analysis to establish whether *Scs1* and *Scs2* caused sporophyte or/and gametophyte lethality, using linked visible markers *st* (reduced stipules) on LGIII and *gp* (yellow pod) on LGV.

Test for male gametophyte lethality of *Scs1*

First, a tester st320 was established which carried the visible *st* marker originating from WL1072, while the molecular markers *PhlC* and *AJ832139*, compatibility gene *scs2*, as well as the cytoplasm, were from VIR320 (Table 1; Supplementary Material Fig. 1S). Then, in the F2 of the cross WL1072 × VIR320, one plant was chosen which had reduced stipules (*st*) and other markers on LGIII, as inherited from WL1072, and homozygous for *scs2*_320. This plant was used as the pollen parent in a cross with VIR320 to produce heterozygotes for *st/St Scs1_1072/scs1_320 PhlC_1072/PhlC_320*. The resulting F1 plants had reduced pollen fertility (see Table 2), while their chlorophyll pigmentation was rather intense, although underdeveloped if compared with F1s of the reciprocal cross, since *scs2_320* was homozygous and did not contribute to the conflict. The pollen of these heterozygous plants was used to pollinate the st320 tester plants (Fig. 1).

If all male gametophytes were viable, the expected segregation in the progeny of the test-cross would be 1 St:1 st. Of 36 plants obtained, 35 had normal stipules and one plant had reduced stipules. High pollen fertility of the latter plant (>90%) indicated that was homozygous for *scs1_320*.

The LGIII markers used are arranged in the order *st–AJ832139–PhlC* (Bogdanova and Yadrikhinskiy 2010) with *Scs1* between *AJ832139* and *PhlC* (see below in "Mapping the *Scs1* gene"; Fig. 7). The analysis of the molecular markers showed that it was heterozygous for *PhlC* and homozygous for *AJ832139_320*, that is, this st plant arose by virtue of the crossover gametophyte of the genotype *st_1072 AJ832139_320 scs1_320 PhlC_1072*, indicating the lack of *Scs1_1072* gametophyte input in the progeny (Fig. 1). Similar results were obtained in a test for viability of *Scs1_1072* male gametophytes in the case where the cytoplasm originated from another wild pea L100. A test-cross performed of the *st* homozygous tester line WL851 with heterozygotes for *st* and *Scs1_1072/scs1_L100* produced 25 plants all of which were St, indicating lack of the *st_1072 Scs1_1072* gametophytes in the progeny. Based on these results, we conclude that *Scs1* from cultivated pea is a male gametophyte lethal gene in certain cytoplasmic backgrounds from *P. elatius*.

Test for sporophyte lethality of *Scs1*

To assess *Scs1* sporophyte lethality, two similar test-crosses were performed. First, the heterozygotes were obtained as described above by pollinating VIR320 as a donor of the cytoplasm with pollen of an F2 plant originating from the cross WL1072 × VIR320 with reduced stipules (*st_1072/st_1072*) and *scs2_320/scs2_320*. Then, the resulting plants heterozygous for *St_320/st_1072*, *scs1_320/Scs1_1072*, and *PhlC_320/PhlC_1072* were crossed as seed parents with tester lines WL1072 or WL851 homozygous for *st*. If all sporophytes were viable, the expected segregation in the progeny of the test-cross would be 1 St:1 st, but if the homozygotes *st*/*st* died, all the resulting plants would carry normal (non-reduced) stipules (Fig. 2).

Of 62 plants obtained, 50 had normal stipules (St) and manifested strong chlorophyll deficiency and reduction of blade organs, the phenotype consistent with nuclear–cytoplasmic conflict in its typical form. This occurred since the plants obtained were heterozygous for both *Scs1* and *Scs2*, receiving the alleles incompatible with the VIR320 cytoplasm from the tester lines, while the seed parent provided compatible allele of *scs2* for which it was homozygous and the compatible allele of *scs1* came due to linkage with *St*. The remaining 12 plants (five St and seven st) had bright-green coloration. Analysis of the *rbcL* gene showed that all of them carried paternal plastid DNA, presumably due to non-canonical biparental inheritance as previously observed by Bogdanova (2007). Although the numbers are small, this segregation is consistent with the expected 1:1 segregation of St versus st. The cytoplasm of the above mentioned 50 plants was not tested for the wild pea descent, but it was implied by the manifestation of the

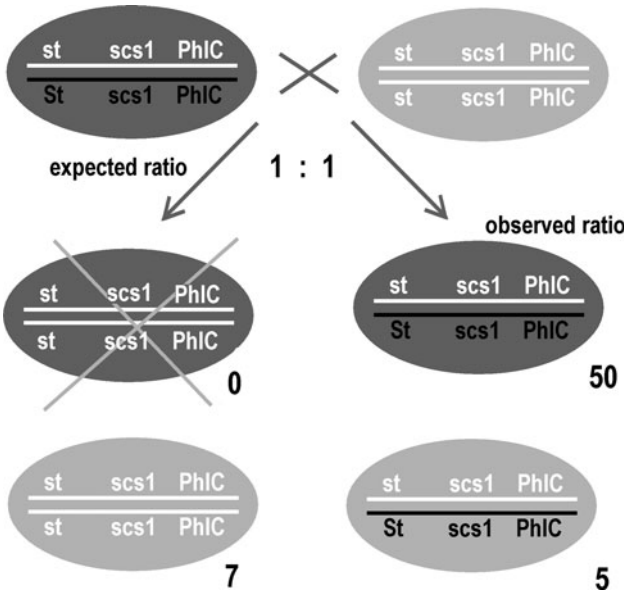

Fig. 2 A scheme of the test-cross to assess viability of sporophytes homozygous for *Scs1* from cultivated parent on the wild pea cytoplasm background. To denote the origin of cytoplasm *light grey color* is used for cultivated pea descent, *dark grey* for wild pea descent; markers designated in *black* derive from the wild pea VIR320, those in *white* from cultivated pea WL1072 or WL851. *Crossed lines* symbolize death of sporophytes

nuclear–cytoplasmic conflict. Based on this result, we conclude that the *Scs1* allele from the cultivated pea is associated with sporophyte lethality in the background of cytoplasm from the wild pea VIR320. In this case, we do not regard the allelic state of *Scs2* which did not segregate in the crosses made. Similar results were obtained in a study of sporophyte viability in the cytoplasm of wild pea L100 from Israel employing molecular markers *PhlC* and *AJ832139*, where 20 progeny obtained were heterozygous for *Scs1*. Eight progeny homozygous for Scs*1_1238* carried biparentally inherited plastids and therefore were not informative.

Test for male gametophyte lethality of *Scs2*

To assess *Scs2* male gametophyte lethality, we followed inheritance of a closely linked visible marker *gp* (yellow pod) in a test-cross. The tester line was obtained as the progeny of a single F3 plant from the cross VIR320 × RIL-37 homozygous for *gp_1238 scs2_320*, *scs1_320* and carrying the morphologic marker *k* (reduced alae of the flower) to control for occasional self-pollination. The tester line was pollinated with F1 plants heterozygous for *gp* and *Scs2* (*Gp_320/gp_1238*, *scs2_320/Scs2_1238*) resulting from the cross VIR320 × RIL-65 (Fig. 3). The RIL-65 line was homozygous for *scs1_320*, so that this gene did not segregate in the following crosses.

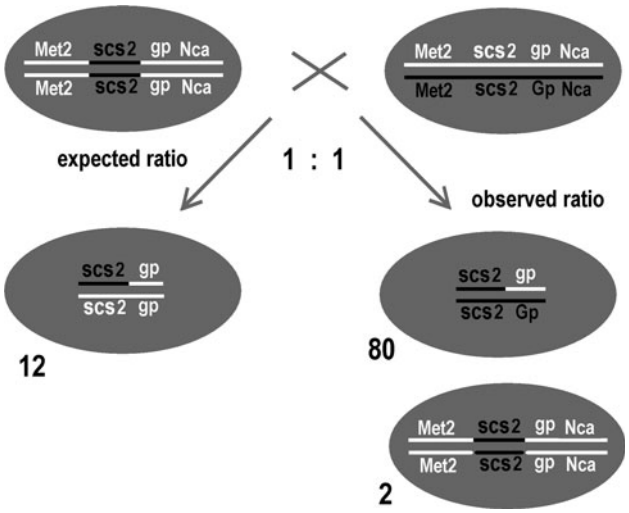

Fig. 3 A scheme of the test-cross to assess viability of gametophytes carrying *Scs2* from cultivated parent on the wild pea VIR320 cytoplasm background. Markers designated in *black* derive from the wild pea VIR320, those in *white* from cultivated pea WL1238. Allelic states of the markers that have been analyzed are indicated

The tester line and F1 heterozygotes employed had the cytoplasm from VIR320 that was checked by the analysis of *rbcL*. The occurrence of homozygous *gp* plants with yellow pods in the progeny may serve as evidence of the transfer of *gp_1238 Scs2_1238* gametes via the male gametophyte. Of 94 plants obtained, 14 were homozygous *gp*. The allelic state of *scs2* in these plants was scored by pollen fertility counts; 12 plants had pollen fertility of about 0.7 typical of the heterozygotes *scs2_320/ Scs2_1238* (Table 2) and two plants had fully fertile pollen. To uncover the nature of these two plants we analyzed the allelic state of the nearest known bordering markers, *Met2* and *Nca* (according to the genetic map constructed as described below in "Mapping the *Scs2* gene"; Fig. 8). According to the analysis of the markers *Met2* and *Nca* these two plants resulted from the input of *Met2_1238 scs2_320 Nca_1238* crossover gametophytes (Fig. 3). The data obtained evidence that the gametophytes carrying *Scs2_1238* in the background of VIR320 cytoplasm are viable, although their viability is reduced to approximately 12/80, or about 1/7.

Test for sporophyte lethality of *Scs2*

To test for *Scs2* sporophyte lethality, a test-cross was performed of the heterozygotes for *Scs2* with a homozygous *Scs2_1238* tester. First, in the F2 progeny of the above mentioned cross employed to test for viability of gametophytes carrying *Scs2* (Fig. 3) [F3(VIR320 × RIL-37) × F1(VIR320 × RIL-65)], the Gp plants were chosen and tested to carry the LGV markers originating from VIR320. They also carried the morphologic marker *k* (reduced alae) (Fig. 4) to control for occasional self-pollination. Then, to obtain heterozygotes for LGV markers including *Scs2*, they were crossed as seed parents with RIL-106 carrying the LGV markers originating from WL1238: *Met2*, *gp*, *Scs2*, *Nca*, *scr*, *Apy*, and *pnp*, as well as morphologic markers *k* and *tl-w* (tendrils converted to leaflets). The resulting progeny were heterozygous for *tl* (flat tendrils) substantiating the cross-pollination, and had reduced alae (Fig. 4a). The employed lines had the *scs1* allele from VIR320 (Table 1). All heterozygous plants were checked for *rbcL* to assure the wild-pea origin of the cytoplasm. RIL-32 and 33 were used as tester lines homozygous for the markers of LGV originating from WL1238 and *scs1* from VIR320 (Fig. 4a). If *Scs2* is associated with sporophyte lethality, the class of homozygotes for closely linked markers of LGV, namely, *gp*, would be absent, otherwise the expected segregation is 1:1. Of the 82 plants obtained, 45 were Gp and 37 plants were gp, in good correspondence with a 1:1 ratio. This led to the conclusion that homozygotes for the *Scs2_1238* allele in background of wild pea cytoplasm were viable. Their phenotype was very similar to that of plants subject to the typical syndrome of nuclear–cytoplasmic incompatibility described in earlier studies (Bogdanova and Kosterin 2006). They had reduced blade organs and aberrant chlorophyll pigmentation (Fig. 5); pollen fertility of these plants was about 20–30%.

Mapping the *Scs1* gene

To map the *Scs1* gene, the following scheme was used. Plants of the RIL-52 and 46 from the mapping RIL population homozygous for *Scs1_1238* and *scs2_320* were crossed as pollen parents with VIR320. The resulting F1 plants carried cytoplasm from the wild pea and were heterozygous for *Scs1* (Fig. 6) so that fertility of their pollen was about 50% (Table 2). In the F2 progeny, we analyzed segregation for the markers *PhlC*, *AJ832139* and *Scs1*. The allelic state of the molecular markers was determined by the CAPS method and that of *scs1* by pollen counts (Fig. 6).

The segregation ratio of the markers studied in 123 F2 plants differed significantly from the expected Mendelian 1:2:1 ratio due to gametophyte/sporophyte lethality of *Scs1_1238*. The distribution of pollen fertility in the F2 plants (Supplementary Material, Fig. 2S) was clearly bimodal. Therefore, we excluded from the analysis the intermediate classes (pollen fertility of 0.55–0.70) with relatively low abundance, which we found difficult to classify unambiguously as semisterile or fertile. We regarded the plants with pollen fertility less than 0.55 as semisterile and heterozygous for *Scs1* (48 plants) while the plants with pollen fertility higher or equal to 0.90 were regarded as fertile and

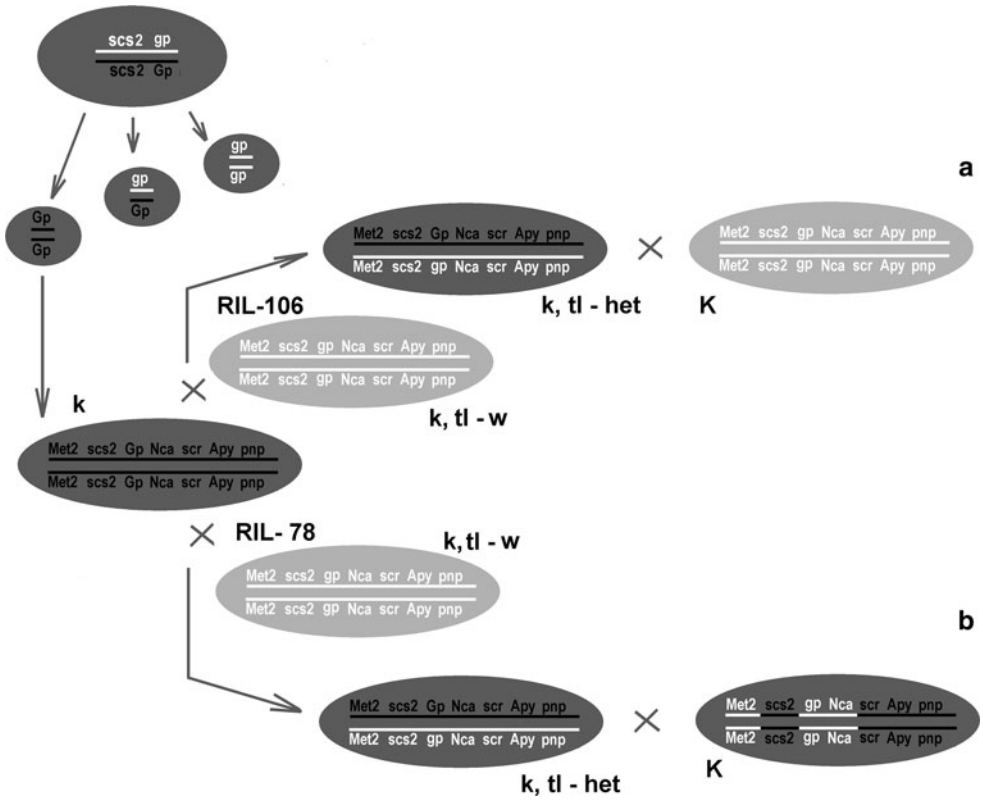

Fig. 4 A scheme of the test-cross to assess viability of sporophytes homozygous for *Scs2* from cultivated parent on the wild pea cytoplasm background (**a**) and to estimate map distances (**b**). To denote the origin of cytoplasm *light grey color* is used for cultivated pea descent, *dark grey* for wild pea descent; markers designated in *black* derive from the wild pea VIR320, those in *white* from cultivated pea WL1238. "*het*" indicates heterozygotes. The original plants in the *upper-left corner* are *Gp/gp* heterozygotes shown in Fig. 3

homozygous for *scs1_320* (55 plants). Among these 103 plants, five crossovers were found with fertile pollen. Three of them were heterozygous for *PhlC* and homozygous for *scs1_320 AJ832139_320*, and two crossover plants were homozygous *PhlC_320 scs1_320* and heterozygous for *AJ832139* (Fig. 6). The genetic map of the LGIII segment including the genes analyzed as constructed with Mapmaker 3.0 software (Lander et al. 1987) places the *Scs1* gene in a rather narrow interval of about 2.5 cM between the adjacent markers *PhlC* and *AJ832139* (Fig. 7).

Mapping the *Scs2* gene

To map the *Scs2* gene, a test-cross was employed of the heterozygotes for a number of LGV markers including *Scs2*, with a homozygous tester line, gp37. The gp37 tester line used had the following LGV marker alleles (Table 1) *Met2_1238*, *gp_1238*, *scs2_320*, *Nca_1238*, *scr_320*, *Apy_320*, *pnp_320.* The plants of the tester line were homozygous for *K* (normal alae). The heterozygotes marked by a visible recessive marker were obtained as follows. First, plants homozygous for the LGV markers originating from VIR320 were chosen as described above in "Test for sporophytic lethality of *Scs2*" (Fig. 4). The plants were selected to carry the morphologic marker *k* in homozygote. Then they were crossed as seed parents with RIL-78 which carried LGV markers *Met2*, *gp*, *Scs2*, *Nca*, *scr*, *Apy*, *pnp* originating from WL1238, as well as morphologic markers *k* and *tl-w* in homozygote (Fig. 4b). The resulting hybrids were heterozygous for the LGV markers, had reduced alae and flat tendrils (heterozygotes for *tl* assuring successful hybridization). All plants were checked for *rbcL* to assure that they had the wild pea cytoplasm. Then, a test-cross was performed (Fig. 4b), which produced 102 progeny in which the allelic state of molecular markers was determined by the CAPS method, *gp* was scored visually, and the allelic state of *Scs2* was determined by pollen count. Plants with pollen fertility of 60–80% were regarded as heterozygotes and those with pollen fertility 90–100% as homozygotes for *scs2_320*. The genetic map of the LGV segment including the genes analyzed as constructed with Mapmaker 3.0 software is shown in Fig. 8a. It should be noted that in this experiment, no crossovers were observed between *gp* and *Scs2*.

The above-described experiment to assess viability of the homozygotes for *Scs2_1238* was performed as a test-cross and also allowed construction of a genetic map of the LGV segment involving the *Scs2* gene. In the mentioned cross, two genotypic classes for *Scs2* were observed: homozygotes for *Scs2_1238* with evident signs of nuclear–cytoplasmic conflict (Fig. 5) and heterozygotes with normal appearance and pollen fertility of 0.6–0.8; again, *gp* was scored visually and molecular markers *Met2*, *Nca*, *scr*, *Apy*, *pnp* were scored by the CAPS method. One gp plant and one Gp plant looked more vigorous than the rest of the progeny and had fully fertile pollen. The analysis of the *rbcL* marker showed that they inherited their plastids

Fig. 5 Phenotype of a plant homozygous for *Scs2* from WL1238 in the background of the cytoplasm from the wild parent VIR320 (*white arrowheads*), leaves on the foreground are from neighboring normal plants (*black arrowheads*) (color figure online)

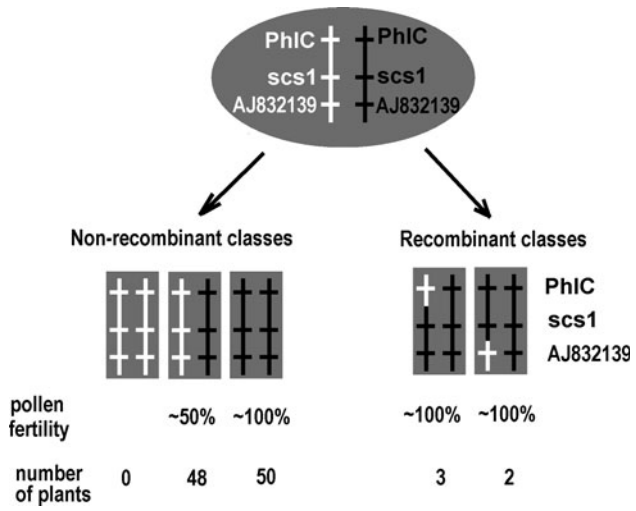

Fig. 6 A scheme used to map the *Scs1* gene in relation to LGIII markers. Markers designated in *black* derive from the wild parent VIR320, those in *white* from cultivated pea WL1238

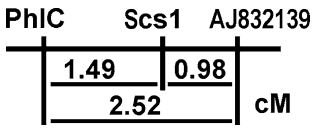

Fig. 7 Genetic map of LGIII segment containing *Scs1*

biparentally; hence, they were excluded from the analysis. The genetic map of the LGV segment, including the analyzed genes constructed with Mapmaker 3.0 software, is shown in Fig. 8b. Unlike the first mapping experiment, in this experiment, we observed 7 crossovers between *Scs2* and *gp*, and all of them carried the same combination of the *Scs2_1238* and *Gp_320* alleles, while the reciprocal combination s*cs2_320 gp_1238* was not observed. The two maps shown in Fig. 8 differ substantially. In the map obtained in the first experiment (Fig. 8a), the region adjacent to the *Scs2* gene, *Met2-gp-Nca*, is sharply contracted while a more distant segment *pnp-tl* is expanded comprising 81.5 cM as calculated with Mapmaker 3.0 or 54.4 cM as calculated with the Kosambi formula (Kosambi 1944).

Discussion

In the genetic analysis carried out earlier (Bogdanova et al. 2009) we failed to observe the homozygotes for the markers linked to the genes of nuclear–cytoplasmic compatibility *PhlC* (LGIII) linked to *Scs1*, and *gp* (LGV) linked to *Scs2*. This led us to the assumption that both the genes *Scs1* and *Scs2* could be sporophyte and/or gametophyte lethals. In an interspecies cross *Pisum sativum* × *P. fulvum*, sporophyte lethality was implied for segregating gene(s) (De Martino et al. 2000). We show in the present study that *Scs1* originating from cultivated pea is sporophyte as well as gametophyte lethal if the cytoplasm comes from the wild pea VIR320 or L100. A pollen fertility of heterozygotes *Scs1_1238/scs1_320* of about 50% (Table 2) corresponds well to lethality of gametophytes carrying *Scs1_1238*.

The viability of *Scs2_1238* gametophytes as well as sporophytes is somewhat unexpected taking into account the lack of homozygotes for *Scs2* in our earlier studies. We suppose that viability of *Scs2_1238* carriers might depend on the presence of some unlinked gene(s) in the VIR320 genome. Indeed, the frequency of homozygous *Scs2_1238* F2 segregants as judged by occurrence of homozygous *gp* plants and their phenotypes, varies substantially from RIL to RIL. The extreme cases are RIL-97 and RIL-116 which produced no *gp* homozygotes among 77 F2 progeny (in sum) in the cross with VIR320 (Bogdanova et al. 2009), and RIL-65 which, when crossed with VIR320, gave rise to five *gp*/*gp* segregants among 38 plants. The latter ratio, although not differing significantly from 1/4 ($\chi^2 = 2.84$,

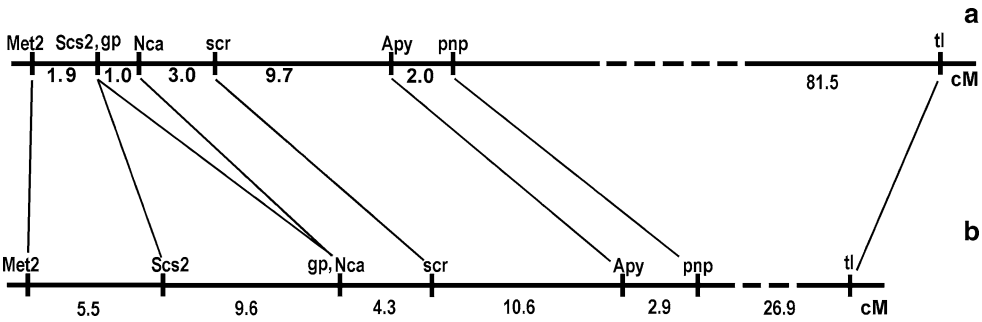

Fig. 8 Genetic map of LGV segment including *Scs2* as constructed with Mapmaker 3.0, in two mapping experiments involving **a** RIL-78 (see Fig. 4b), **b** RIL-106 (see Fig. 4a)

$0.05 < p < 0.1$), corresponds better to 3/15 which assumes the death of 1/4 of the *gp*/*gp* carriers, or 1/16 of all progeny ($\chi^2 = 1.11$). We suppose that RIL-97 and RIL-116 carry a recessive gene from VIR320 which, when homozygous, kills the homozygotes for *Scs2_1238*. RIL-65 presumably does not carry this gene and in the F2, only 1/4 of *Scs2_1238* homozygotes die. However, there are intermediate cases like RIL-39 and RIL-78, which gave rise to one *gp*/*gp* segregant each among 46 and 66 plants, respectively. These segregation ratios do not correspond to any Mendelian ratio. We suppose that there segregate other (also hypothetical) genes conditioning that *Scs2_1238* homozygotes occasionally survive.

We mapped the nuclear–cytoplasmic compatibility gene, *Scs1*, on the pea genetic map with rather good accuracy. In the mapping experiment, the genetic distance between the closest bordering markers, *PhlC* and *AJ832139*, comprised about 2.5 cM. Based on the mapping RIL population (Bogdanova et al. 2009), this distance as estimated with the Haldane's formula (Haldane and Waddington 1931) comprised 3.0 cM (unpublished), and in F2 progeny of the cross WL1072 × L100 it comprised 3.5 cM (Bogdanova and Yadrikhinskiy 2010). This allows us to search for candidate genes at the molecular level making use of synteny between the genomes of pea and *Medicago truncatula* (Kalo et al. 2004). The gene of *M. truncatula* homologous to *PhlC* is encoded as NC_016409 (Medicago truncatula chromosome 3), position 22,386,715–22,391,032 and the homolog of *AJ832139* is in the position c23,538,014–23,539,540, that is physical distance between the closest bordering markers comprises 1,146,982 bp of which 150,000 bp refer to a gap of unknown length. This represents the genome segment to search for candidate genes of nuclear–cytoplasmic compatibility.

The genetic map distances of the LGV segment including *Scs2* were surprisingly variable in the two experiments described. The reasons for such variability are obscure; this might be attributed to some structural differences in chromosomes of the wild and cultivated pea subspecies, like in the case observed by Hall et al. (1997). In our case, the lines employed in genetic analysis might have inherited from VIR320 and WL1238 chromosome segments with different level of homology. It is also difficult to explain why all seven crossovers between *gp* and *Scs2* in the experiment designed to test for sporophyte viability of *Scs2* (Fig. 4a), were of the same type, combining *Scs2_1238* and *Gp_320* while reciprocal crossovers were not observed ($\chi^2 = 7.0$, $p < 0.01$). It is not excluded that the reciprocal combination *scs2_320 gp_1238* is viable in the background of the wild cytoplasm, only if some gene(s) on the other side of *scs2* also originate(s) from WL1238. This is supported by a number of observations regarding crossover events in this region. The mentioned combination of alleles *scs2_320 gp_1238* was observed only in double-crossovers, namely, in the experiment designed to test viability of the *Scs2* male gametophytes (Fig. 3) as two plants homozygous for *Met2_1238 scs2_320 gp_1238* and in the gp37 tester line which fixed the *Met2_1238 scs2_320 gp_1238* combination of alleles inherited from RIL-37 (Table 1). In fact, the order of markers on the genetic map might be influenced by possible chromosome rearrangements differing in the parental lines. The chromosome region close to *gp* was shown to be inverted in certain pea germplasm (Hall et al. 1997); however, this does not explain why in the single mapping experiment we observed the crossover combination of alleles *Scs2_1238 Gp_320*, while reciprocal crossovers were absent. Curiously, the crossover plant observed in the test for viability of *Scs1* gametophytes (Fig. 1) was also a double-crossover although not in the nearest neighborhood. A similar tendency for an overrepresentation of double-crossovers in the proximity of the *scs* gene was observed in wheat (Simons et al. 2003). This suggests the possibility that correct chromatin assembly requires that some chromosome regions of the cultivated pea near the nuclear–cytoplasmic compatibility genes retain their *cis*-position in the background of alien cytoplasm of wild descent. Perhaps, the incompatibility genes themselves have some relation to chromatin assembly.

Ethical standards: the experiments comply with the current laws of the Russian Federation.

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Conflict of interest The authors declare that they have no conflict of interest.

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