Theor Appl Genet (2009) 118:801–809
DOI 10.1007/s00122-008-0940-y

ORIGINAL PAPER

Genetic analysis of nuclear-cytoplasmic incompatibility in pea associated with cytoplasm of an accession of wild subspecies *Pisum sativum* subsp. *elatius* (Bieb.) Schmahl.

Vera S. Bogdanova · Elvira R. Galieva · Oleg E. Kosterin

Received: 5 August 2008 / Accepted: 21 November 2008 / Published online: 20 December 2008

Abstract The genetic basis of nuclear-cytoplasmic incompatibility was examined using the wild pea (*Pisum sativum* subsp. *elatius*) accession VIR320. When this accession is used as the female parent in crosses with domesticated peas (*Pisum sativum* subsp. *sativum*) the F_1 is highly sterile and displays chlorophyll deficiency, chlorophyll variegation, reduction of leaflets and stipulae while the reciprocal cross produces hybrids that appear normal. A mapping recombinant inbred line (RIL) population was established based on a cross in a compatible direction of a tester line WL1238 with VIR320. The ability to cause nuclear-cytoplasmic conflict was analysed by crossing individual RIL plants as pollen parents with VIR320 as donor of cytoplasm and scoring each F_1 for major signs of the conflict. It is concluded that two unlinked nuclear genes are involved in the genetic control of the observed incompatibility. One of the genes, denoted as *Scs1*, is closely linked to the *PhlC* gene on linkage group III and the other, denoted as *Scs2*, is closely linked to the *gp* gene on linkage group V. Alleles of both genes in WL1238 are dominant and appear to be lethal in the homozygous condition in the VIR320 cytoplasm background.

Communicated by F. J. Muehlbauer.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-008-0940-y) contains supplementary material, which is available to authorized users.

V. S. Bogdanova · E. R. Galieva · O. E. Kosterin (✉)
Institute of Cytology and Genetics,
Siberian Department of Russian Academy of Sciences,
acad. Lavrentiev av., 10, 630090 Novosibirsk, Russia
e-mail: kosterin@bionet.nsc.ru

V. S. Bogdanova
e-mail: vera@bionet.nsc.ru

Introduction

The plant cell comprises the nuclear, mitochondrial and plastid genomes which are coadapted for proper functioning (Maroof et al. 1992). However, if nucleus and cytoplasm from different sources are brought together by cross-hybridization or in vitro manipulation, nuclear-cytoplasm incompatibility may occur. In *Oenothera* species, nuclear and organellar genomes may be categorised into several classes that can be combined via cross-pollination into compatible or incompatible combinations (Stubbe 1964). The consequences of genome-plastome incompatibility include anomalies in leaf pigmentation, plastid ultrastructure, chlorophyll a/chlorophyll b ratio (Glick and Sears 1994), and pollen inactivation (Stubbe and Steiner 1999). Leaf variegation, as a consequence of nuclear-cytoplasmic incompatibility was observed in crosses between races of *Medicago truncatula* (Lilienfeld 1962), and segregation analysis indicated that one nuclear gene was involved in this interaction (Lilienfeld 1965).

Conflict between the nuclear genome and alien cytoplasm may arise in cybrids, that is, in vitro regenerated somatic cell hybrids which can be rather easily obtained in the Solanaceae (Zubko et al. 2001). When the nuclear genome of *Atropa beladonna* and cytoplasmic genomes of tobacco are combined in such hybrids, chlorophyll deficiency develops due to the inability of the nightshade nuclear genome to support effective editing of the tobacco *atpA* (ATP synthase CF1 alpha chain) transcript in plastids (Schmitz-Linneweber et al. 2005).

Of special interest is the nuclear-cytoplasmic conflict observed in interspecific and intergeneric crosses in cereals because it has been subject to genetic analysis. The most commonly observed effect of alien cytoplasms is male sterility. In the case of CMS (cytoplasmic male sterility),

abnormal nuclear-mitochondrial interaction brings about non-functional pollen in otherwise normally looking plants (Hanson 1991). A large number of different alloplasmic combinations were thoroughly studied in hybrids of *Triticum* and *Aegilops* species. Pollen sterility in different nucleus-cytoplasm combinations varied to a large extent (Tsunewaki 1993), while female fertility, as estimated from the backcrossed seed fertility, was more tolerant to genetic stress caused by the alien plasmon transfer (Tsunewaki et al. 1996). Other effects of nuclear-cytoplasmic interaction included leaf variegation (Mukai and Tsunewaki 1976; Tsunewaki 1993), pistillody, germless grain formation, premature sprouting, haploid and twin seedling formation, depressed growth vigor, delayed heading (Tsunewaki 1993), and decreased plant size (Ohtsuka 1991; Simons et al. 2003). A systematic study of plant phenotypes in a large number of different nucleus-cytoplasm combinations in wheats showed that effects of genotype, plasmon, and their interaction had substantial effect also on quantitative traits with high statistical significance (Tsunewaki et al. 2002). Similar results were obtained for alloplasmic barley collection (Goloenko et al. 2002). In crosses of *Zea* species and subspecies, significant differences were observed in 56 of the 58 characters studied (Allen 2005).

Genetic basis of nuclear-cytoplasmic conflict has been studied in cases of incompatibility of certain *Triticum* species with the cytoplasms of *Aegilops squarrosa* (Ohtsuka 1991) and *Ae. longissimum* (Anderson and Maan 1995). An analysis of alloplasmic mono-trisomic lines on the basis of Chinese Spring wheat with *Ae. squarrosa* cytoplasm led to a conclusion that a factor(s) responsible for nucleus-cytoplasm compatibility resided on chromosome 1D (Ohtsuka 1991). Gene analysis suggested that two kinds of nuclear genes were responsible for the compatibility. One is related to the appearance of shriveled seeds due to incomplete development of the endosperm and appearance of chlorophyll variegation (incomplete development of chloroplasts). The other is related to vigor of F_1 plants (Ohtsuka 1991). Unlike tetraploid wheats with genomic composition AABB, *Triticum timopheevii* with genome composition AAGG is compatible with cytoplasm of *Ae. squarrosa*. The corresponding factor, *Ncc-tmp*, providing this compatibility was introgressed into *Triticum durum* cv. Langdon by crossing. Southern blot analysis showed its location on the 1A chromosome (Asakura et al. 1997a). Additional test-crosses showed that *T. timopheevii* also possessed the functional homoeoallelic *Ncc-tmp* gene in the G-genome (Asakura et al. 2000). Although the *Ncc-tmp1A* and *Ncc-tmp1G* genes conferred compatibility with the *Aegilops* cytoplasm, they were not essential for compatibility of the durum wheat with the cytoplasm of *T. timopheevii* (Asakura et al. 2000).

Another series of genetic analyses mapped genes conferring nuclear-cytoplasmic compatibility with *Aegilops longissimum* cytoplasm. An alloplasmic line with the cytoplasm of *Ae. longissimum* and nucleus of *T. turgidum* was obtained which produced viable seeds due to introgression via repeated back-crosses of the compatibility gene, denoted as *scs*, 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 for the independently obtained introgressed line of *T. durum* (Asakura et al. 1997a). These results suggest that the *T. timopheevii*-derived *Ncc* gene conferring compatibility with *Ae. squarrosa* cytoplasm and *scs* conferring compatibility with *Ae. longissimum* cytoplasm represent the same locus. Detailed mapping of the *timopheevii*-derived compatibility gene using microsatellite markers placed scs^{ti} in the centromeric region of chromosome 1A (Simons et al. 2003). An unusual line of *T. durum* was identified which carried an scs^{d} allele being weaker in that it did not restore plant vigor (Gehlhar et al. 2005). It is known that the presence of the 1D chromosome either from *Aegilops* or from hexaploid *T. aestivum* is essential for maintenance of alloplasmic lines combining tetraploid AABB genome with the *Aegilops* cytoplasm (Ohtsuka 1991). The gene conferring the compatibility and residing in chromosome 1D of *T. aestivum*, scs^{ae}, was mapped to 1DL with the use of the radiation hybrid (RH) method (Hossain et al. 2004). Although scs^{ti} gene improved embryo-endosperm compatibility, resulting in plump viable seeds, another gene, *Vi* supposedly of spontaneous origin, was required to restore male fertility (Maan 1992b). This gene was mapped to chromosome 1BS (Anderson and Maan 1995).

Earlier, we reported a case of nuclear-cytoplasmic incompatibility in crosses of a wild pea accession VIR320 originating from Palestine and belonging to the subspecies *Pisum sativum* subsp. *elatius* (Bieb.) Schmahl. (Bogdanova and Berdnikov 2001). When this accession is used as a maternal parent in crosses with vast majority of cultivated *P. sativum* representatives, the resulting F_1 s are nearly sterile plants that also display chlorophyll deficiency, chlorophyll variegation, and reduced leaflets and stipules. The signs of incompatibility disappear in case of incidental biparental plastid inheritance indicating that incompatibility is mainly due to improper functioning of plastids rather than mitochondria (Bogdanova 2007). In the present work we perform genetic analysis of the nuclear-cytoplasmic incompatibility in pea manifested as chlorophyll deficiency and reduction of blade organs with the use of recombinant inbred line (RIL) population based on a cross between a standard line, WL1238, and VIR320 in a compatible direction. We show that two unlinked nuclear genes are involved in the genetic control of the studied character.

Materials and methods

Plant material

The accession VIR320, originating from Palestine, was received from the collection of the Vavilov All-Russian Institute of Plant Breeding, St. Petersburg (Russia). The line is classified as *Pisum sativum* subsp. *elatius* (Bieb.) Schmahl. The other parental line, WL1238, was received from the Weibullsholm collection, Landscrona (Sweden). This line is fully compatible with other *P. sativum* subsp. *sativum* germplasm and was selected because it is homozygous for a number of classical morphological mutations.

Seeds were sown in a greenhouse in hydroponic beds filled with 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 sulfate; 0.2 g/l acid potassium phosphate; 0.2 g/l potassium nitrate and traces ferric phosphate). Plants were illuminated by 8 h daylight/16 h incandescent light of 10,000–12,000 lux intensity.

Mapping population and linkage analysis

To perform linkage analysis, a RIL population was established based on a cross WL1238 × VIR320, that is, made in the direction known to produce phenotypically normal plants (crosses are given with the maternal parent indicated first). About 100 F_2 plants were chosen as progenitors for the mapping RIL population and reproduced by self-pollination, randomly choosing one seed from a plant each generation, up to the F_6. F_6 lines were characterised for compatibility with the cytoplasm of VIR320. Commonly, two F_7 plants of each RIL were used as pollen parents in the analysis, and the combined data were used to characterise the F_6 line. Phenotyping of RIL lines in respect of nuclear-cytoplasmic compatibility is schematically represented in Fig. 1.

To estimate genetic distances between the markers scored, Haldane's formula was used: $r = R/(2 - 2R) \times 100$, where r stands for the map distance in centimorgans and R for the proportion of recombinant lines (Haldane and Waddington 1931). A linkage map of linkage group III was constructed using the Mapmaker 3.0 software (Lander et al. 1987), available for free download at http://iubio.bio.indiana.edu/soft/molbio/qtl/mapmaker/.

Genetic markers

Description of morphologic markers in pea can be found at http://data.jic.bbsrc.ac.uk/cgi-bin/pgene. Visible markers scored in the present analysis and linkage groups (LGs) to which they refer were as follows: *wb*, stipules and the underside of leaflets nearly waxless, LGII; *k*, wings reduced and adpressed to keel, LGII; *le,* short internodes, LGIII; *b*, pink flowers, LGIII; *m*, absence of brown marbling of testa, LGIII; *tl*, tendrils converted to leaflets, LGV; *gp*, yellow pods, LGV; *fl*, flecking on leaflets and stipules, LGVI; *pl*, light hilum colour, LGVI. All recessive alleles came from WL1238.

To distinguish alleles of the DNA markers studied, cleaved amplified polymorphic sequence (CAPS) approach (Konieczny and Ausubel 1993) was used: PCR-products obtained from gene-specific primers with the use of WL1238 and VIR320 template were digested with several endonucleases, and those enzymes were chosen which produced restriction spectra differing between parental forms. If no restrictase produced distinguishable pattern, dCAPS (derived CAPS) approach was used: PCR-products were sequenced and checked for single-nucleotide polymorphism, then mismatched gene-specific primers were

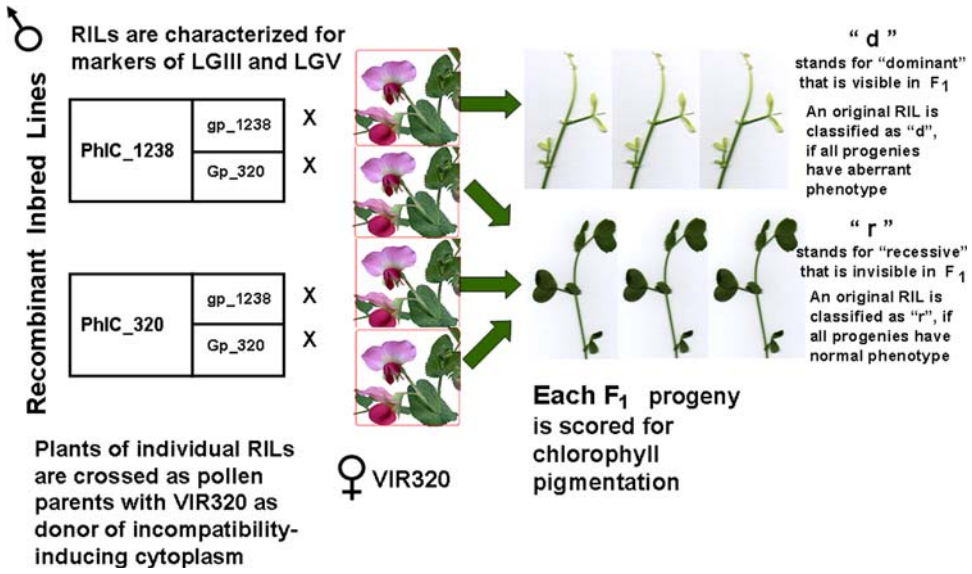

Fig. 1 Schematic representation of crosses performed to analyse segregation for nuclear-cytoplasmic compatibility in individual RIL lines from the mapping population

Table 1 Molecular markers used for genetic analysis, accession numbers of public databases used to design gene-specific primers, sequence of forward and reverse primers and restriction endonucleases used to distinguish the alleles inherited from VIR320 or WL1238

Marker	Name of gene product	Linkage group	Accession number	Forward and reverse primers	Restriction endonuclease
aatC	*Medicago sativa* aspartate aminotransferase (AAT1)	III	L25334	5′AATTGAGTGCTAAGCTTATTTTT 5′gatccagatggggcagaacca	*Bst*NSI
uni	*Pisum sativum* UNIFOLIATA (UNI)	III	AF035163	5′GGAGGCTTGGAGGAACTTTTTCA 5′caatgcaatgcgtaacagtgaac	*Taq*I
Rnp33[a]	*Pisum sativum* 33 kDa ribonucleoprotein	III	AF255058	5′ATGTCTGTAACTTCCACCACT 5′ctgtcttcagcaacacttact	*Tru*9I
Gsn	*Pisum sativum* putative glutamine synthetase	III	AM238618	5′CATCATCCAACGATCCACAG 5′CTGAATGGATCCTTAAAAATGG	*Taq*I
PhlC[a]	*Pisum sativum* phospholipase C	III	AF280748	5′CACAGAGAATGAAGCACAATC 5′ttccataccctgtcaaggaaac	*Hpa*II
Cbl	*Pisum sativum* calcineurin B-like protein	III	AY883569	5′CCTCTCAACCCTCAAAATCATAC 5′acattgacacgttgacaccaat	*Acu*I
Gpt	*Pisum sativum* glucose-6-phosphate/ phosphate-translocator precursor (GPT)	III	AF020814	5′AAGGTTTTGGTCGGAAAGTT 5′gtagctgaattagggttgcatgact	*Bst*4CI
Vc-3	Pea vicilin	III	X14076	5′AAAGTTGACAATTCCTAAAA 5′cactgagtacaacaagga	–[b]
cri	*Pisum sativum* MYB-related transcription factor PHAN1	V	AF299140	5′CCCATTTCAACTTCCTCTCTTC 5′gcattgcatcaacccctaat	*Taq*I
rbcL	Pea chloroplast gene for ribulose 1,5-bisphosphate carboxylase	Plastid	X03853	5′TTATTATACTCCTGACTATCAAACC 5′tacagaatcatctccaaatatctcg	*Asp*LEI

[a] Primers kindly provided by Dr. F. A. Konovalov

[b] Absence of PCR product in WL1238

designed to introduce cleavage site for some restriction endonuclease in one of the parental forms (Neff et al. 1998). Molecular markers used for genetic mapping are listed in Table 1. An allelic state of these markers was determined in each RIL by presence/absence of PCR-product (for *Vc-3*), by dCAPS (for *Gpt*) or CAPS (remaining markers) approaches by PCR-amplification with gene-specific primers listed in Table 1. To denote the alleles inherited from the cultivated parent, WL1238 and wild parent, VIR320, we use marker name followed by underscore and parent designation, for example, *PhlC_1238* and *PhlC_320*.

Genomic DNA extraction, PCR analysis and endonuclease digestion

About 100 mg of leaves was 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 a buffer containing 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM EDTA, 0.5% SDS (w/v), 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 solution of LiCl, stirred and left for 15 min on ice. Then the mixture was centrifuged at 8,000 g for 5 min, the supernatant collected, and 1 ml of 96% ethanol was added to precipate 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°C 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; the supernatant was transferred to fresh tubes. About 10 ng of genomic DNA was used for PCR amplification with following conditions applied: initial denaturation at 94°C 2 min 30 s, followed by 5 cycles: 30 s at 93°C, 1 min at 58°C, 1 min 30 s at 70°C, then 35 cycles including 20 s at 93°C, 1 min at 56°C, 1 min at 72°C, final extension 3 min at 72°C. PCR products were analysed in 0.8% agarose gel in TAE buffer. Five to twelve microliters of PCR reaction was digested with an appropriate endonuclease (Table 1) in total volume of 16 µl according to manufacturer's instructions. All enzymes were purchased from Sibenzyme (Novosibirsk, Russia). Products of endonuclease digestion were analysed on 1.5% agarose gel in TAE except for PCR-amplified *Gpt* gene fragment which was analysed on 10% polyacrylamide gel in TBE.

Results

The F_6 RIL population (WL1238 × VIR320) was analysed for nuclear-cytoplasmic compatibility with the cytoplasm

of the VIR320 accession of *Pisum sativum* subsp. *elatius* (Fig. 1). We expected that the lines that inherited allele(s) of compatibility factor(s) from cultivated parent, WL1238, would produce progeny with typical signs of nuclear-cytoplasmic conflict manifested as a chlorophyll deficiency, often of variegating appearance, ranging from pale green (mostly at veins), to yellowish and whitish in colour, and strong reduction of leaflets and stipulae. In the following discussion we will refer to such lines as "deficient". Those RIL plants that inherited allele(s) of the compatibility factor(s) from the wild parent, VIR320, were expected to manifest no signs of nuclear-cytoplasmic conflict. Indeed, the resulting progenies of RIL plants could be assigned to one of the clear-cut classes. Some RILs gave F_1 of typical "deficient" phenotype (Fig. 2a, b), while others produced F1 without major signs of nuclear-cytoplasmic conflict, with chlorophyll pigmentation normal to pale green (especially under conditions of low intensity illumination), but never yellowish or whitish. Sometimes pale green leaves had intense green sectors (Fig. 2c), associated with paternally inherited chloroplasts as judged from the restriction pattern of PCR-amplified *rbcL* gene (data not shown). This is similar to the appearance of green sectors in the "deficient" background (Fig. 2b), caused by paternal chloroplast inheritance in the situation of the nuclear-cytoplasmic conflict (Bogdanova and Kosterin 2006; Bogdanova 2007). No distinct classes among "non-deficient" progenies could be distinguished.

Pollen parents were classified as referring to one of phenotypic classes: "d" standing for "dominant", that is, visible in F_1, if all progenies were "deficient"; and "r" class standing for "recessive", that is, not manifested in F_1, if all progenies lacked typical "deficient" phenotype (Fig. 1); "h" standing for "heterozygous", if both "deficient" and "non-deficient" plants were observed in the progeny of a particular RIL. Of 95 RILs analysed, six were heterozygotes and were excluded from further analysis. The remaining lines were classified as 32 "d" and 57 "r". This ratio differs significantly from both 1:1 (expected for monogenic inheritance) and 1:3 (expected for two-hybrid inheritance). In our analysis we initially adopted the monogenic model and provisionally designated the gene responsible for nuclear-cytoplasmic incompatibility as *Scs* by analogy with the wheat *species cytoplasm specific* gene with similar effect (Maan 1992b). The estimated map distances between *Scs* and visible markers segregating in the RIL population are given in Supplementary Table s1.

The markers *wb, k* (LGII); *le* (LGIII); *tl* (LGV); *fl, pl* (LGVI) did not display linkage with *Scs*. Although pairwise analysis of LOD scores in Mapmaker 3.0 did not produce significant linkages, the loci *m, b* and *gp,* mapping at 36.3, 29.5, 26.7 cM from *Scs*, respectively, attracted our attention. As two of these markers, *m* and *b* are located on

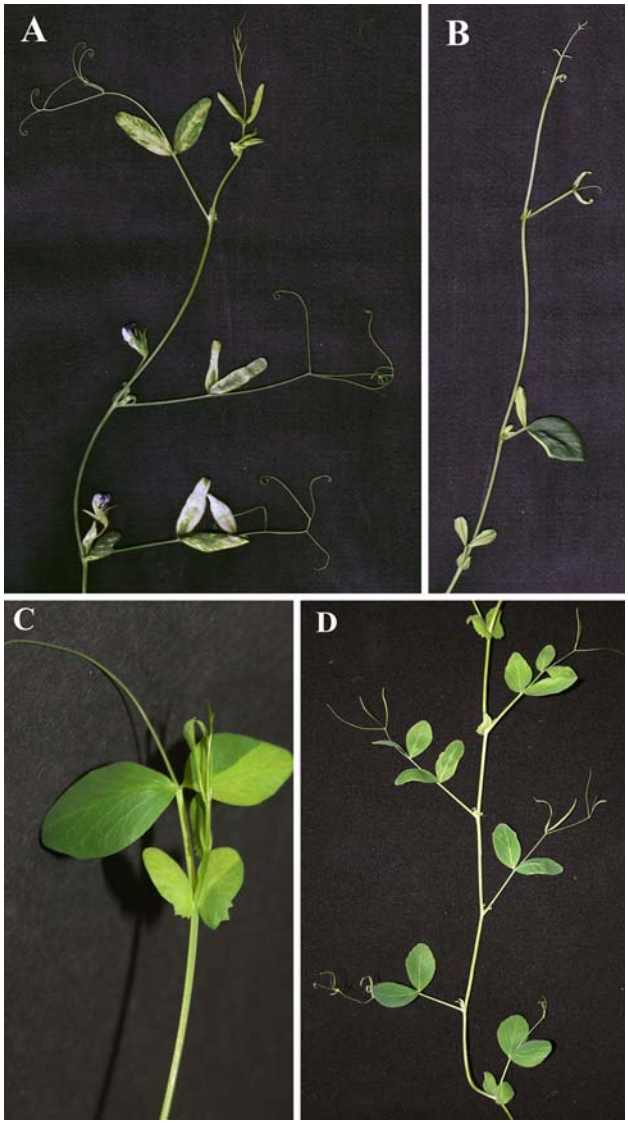

Fig. 2 F1 plants resulting from the crosses VIR320 × RIL. **a** Plant of the "deficient" phenotype with strongly reduced leaflets and stipulae and displaying chlorophyll variegation. **b** Plant with a patch of green tissue. **c** F1 plant without major signs of nuclear-cytoplasmic conflict with dark green sector in pale green background. **d** F1 plant without major signs of nuclear-cytoplasmic conflict with reduced stipulae at some of the higher nodes of the main stem

LGIII, we expanded our analysis to include other, molecular markers known to be located on this linkage group: *aatC*, *uni* (Weeden et al. 1998), *Rnp33*, *PhlC, Cbl* (Konovalov et al. 2005), *Gsn, Gpt, Vc-3* (Weeden and Moffett 2007). The linkage map constructed using segregation data for the markers on LGIII (Fig. 3a) was in good agreement with the recently compiled map of linkage group III (Weeden and Moffett 2007), except for *b* which could not be mapped unambiguously.

Then, using the "try" command of the Mapmaker 3.0 the *Scs* gene was placed between *PhlC* and *Cbl*. The map distances calculated with Haldane's formula was estimated as

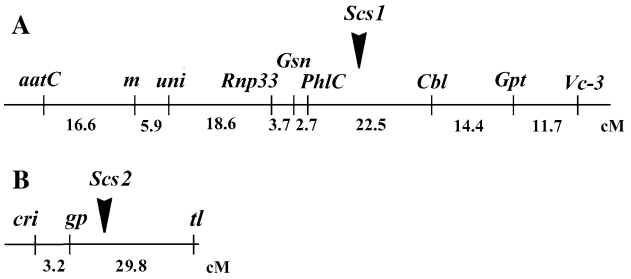

Fig. 3 Linkage map of the genetic markers analysed on LGIII (**a**) and LGV (**b**)

16.2 cM between *Scs* and *PhlC* and 22.0 cM between *Scs* and *Cbl,* thus defining *PhlC* as the nearest genetic marker to *Scs*. The estimates of map distances obtained were inconsistent in that their sum greatly exceeded the distance between the border markers *PhlC* and *Cbl* (22.5 cM). However, another discrepancy was more challenging. The map distance between *Scs* and *PhlC* calculated separately for the "d" class, that is, RILs manifesting typical nuclear-cytoplasmic incompatibility syndrome, was 1.6 cM, strikingly different from the same distance estimated for the "r" class (32.4 cM). The RILs carrying the *PhlC_320* allele, in their majority belong to the "r" class without major signs of nuclear-cytoplasmic conflict, while those with the *PhlC_1238* allele include significant numbers of representatives of both "r" and "d" classes. Therefore, we concluded that (1) *Scs* was closely linked to *PhlC* and (2) an additional factor was segregating in the RIL population that was necessary to produce the typical syndrome of nuclear-cytoplasmic incompatibility when combined with *Scs*. We reanalysed the *gp*—*Scs* linkage as two populations, one with lines homozygous for the *PhlC_1238* allele and one with lines homozygous for the *PhlC_320* allele. We found a strong correlation between the compatibility phenotype and variation at the *Gp* locus in line with the *PhlC_1238* allele (Fig. 1; Table 2). The *gp* gene is known to be located on LGV and conditions yellow colouration of young pods. Map distances calculated with Haldane's formula for the markers on LGV are shown in Fig. 3b.

We conclude from the data of Table 2 that the typical syndrome of nuclear-cytoplasmic incompatibility is conditioned by two nuclear genes one of which is linked to *PhlC* on linkage group III and the other is linked to *gp* on linkage group V. We designated the former gene as *Scs1* (in place of the above used provisional designation *Scs*) and the latter gene as *Scs2*. The nuclear-cytoplasmic conflict, manifested as strong chlorophyll deficiency with colouration varying from pale green to yellowish and whitish, chlorophyll variegation and reduction of blade organs, is developed provided that cytoplasm is inherited from the wild parent, VIR 320, and both alleles *Scs1* and *Scs2* from WL1238 are present in heterozygous state, that is both *Scs1_1238* and *Scs2_1238* are dominant. If only one of *Scs* genes comes from the cultivated parent and the other originates from the wild parent, the nuclear-cytoplasmic conflict in its typical form does not develop so that the F_1 plants are green and have normal leaflet morphology. Of 89 RILs studied, three do not fit this scheme and may be regarded as recombinants (Table 2). Chlorophyll pigmentation of the plants that avoid typical conflict is not fully developed as compared to their reciprocals that can be seen under conditions of low-intensity illumination (Fig. 2c). Nevertheless, in these cases the colouration would not be interpreted as yellowish or whitish, and cannot be confused with the "deficient" phenotype. An impression is that the pale green plants, heterozygous for *Scs1* (as judged from heterozygous state of *PhlC*) are paler in colour than the plants heterozygous for *Scs2* (as judged from heterozygosity for *gp*). In addition, plants heterozygous for *Scs1* and homozygous for *scs2_320* often have reduced stipulae (Fig. 2d) at nodes 7–10, but normal stipulae at other nodes. This phenotypic manifestation also depends on some environmental factor(s).

The data of Table 2 do not allow an estimation of the genetic distance between *Scs1* and *PhlC* and between *Scs2* and *gp* because the recombinant class carrying *PhlC* and *gp* markers from cultivated parent, but not producing a typical nuclear-cytoplasmic conflict (line 1 column 3 in Table 2), may result either from recombination between *PhlC* and *Scs1* or between *Scs2* and *gp*. Furthermore, if either of the genes s*cs1* or s*cs2* comes from the wild parent, the recombination events involving the other gene cannot be registered. Therefore, we can make only an approximate

Table 2 Number of RILs producing and not producing the nuclear-cytoplasmic incompatibility syndrome when crossed with VIR320 as pollen parents in relation to allelic state of *PhlC* and *gp* genes

Allele of *PhlC* (LGIII) as inherited from designated parent	Allele of *gp* (LGV) as inherited from designated parent	Number of RILs compatible with VIR320 cytoplasm, "r"	Number of RILs non-compatible with VIR320 cytoplasm, "d"
WL1238	WL1238	2[a]	31
WL1238	VIR320	20	0
VIR320	WL1238	29	1[a]
VIR320	VIR320	6	0

[a] Recombinant classes

estimate of the map distances. Considering the class with *gp_1238* and *PhlC_1238*, we obtain two recombinant RILs among 33 (line one in Table 2), giving 3.2 cM as the calculated distance using Haldane's formula. This estimate reflects the sum of map distances between *Scs1* and *PhlC,* and between *Scs2* and *gp*.

We studied inheritance of *Scs1* and *Scs2* genes in F_2 progenies obtained from several F_1 plants that were used to score nuclear-cytoplasmic compatibility as described above. We scored the allelic state of *PhlC* (linked to *Scs1*) in 45 F_2 progenies resulting from a cross VIR320xRIL-52, where RIL-52 plants carried *PhlC_1238* and *Gp_320*. All progenies from this cross carried cytoplasm from VIR320. This F_2 population segregated for the *PhlC* and *Scs1* genes while the other compatibility factor, *scs2*, did not segregate and originated from the wild parent, so that no plants with a typical nuclear-cytoplasmic conflict were observed. Among 45 progenies tested, 17 were heterozygotes at *PhlC*, and 28 plants were homozygous for *PhlC_320*. The absence of homozygotes for the *PhlC_1238* allele indicated the elimination of homozygotes for the closely linked *Scs1*. We suggest that in the background of the VIR320 cytoplasm, *Scs1* is either gametophyte or recessive sporophyte lethal.

Segregation for the *Scs2* gene in the F_2 was estimated based on the segregation for the closely linked marker *cri* located on LGV (3.2 cM from *gp*, according to the analysis of the RIL population). The *gp* gene is the closest marker to *Scs2,* but it is not convenient for segregation analysis because it is completely recessive and heterozygotes cannot be scored. Therefore, we used the *cri* gene as the closest DNA marker available to distinguish all three genotypes. The F_2 of the cross VIR320xRIL-116 was analysed, where RIL-116 was homozygous for *cri_1238* and *PhlC_320*. As only one incompatibility gene was segregating and the other was inherited from the wild parent, no plants with typical nuclear-cytoplasmic conflict were observed. Among 50 F_2 plants scored for the allelic state of *cri*, 26 were heterozygotes and 24 were homozygotes for *cri_320*. In accordance with the lack of homozygotes for *cri_1238*, homozygotes for *gp* were also missing, indicating the lack of homozygotes for the linked gene *Scs2*. Gametophyte or sporophyte lethality of *Scs2* in the background of the VIR320 cytoplasm is the probable reason for the lack of homozygotes, as in the case of *Scs1*.

One of the recombinants indicated in line one of Table 2, RIL-37, carried *PhlC_1238* and *gp_1238*, but the cross VIR320xRIL-37 produced F_1 plants without major signs of nuclear-cytoplasmic conflict. These plants had reduced stipulae at some nodes (Fig. 2d), suggesting heterozygosity for *Scs1* linked to *PhlC*. Therefore, we supposed that it was a crossover between *gp* and *Scs2*. In such a case we expected the appearance of *gp_1238* homozygotes in the F_2 of this cross since *gp* was separated from the lethal *Scs2* by the recombination event. Indeed, among the 41 F_2 progenies of the cross VIR320 × RIL-37, seven plants, roughly one-fourth, were homozygous for *gp_1238*.

Another observation made in the analysis of F_2 segregating populations is the shortage of tl^w homozygotes. Two of the studied F_2 populations, VIR320 × RIL-116 and VIR320 × RIL-97, comprising a total of 77, plants segregated for *Tl*. This gene is semidominant and allows the identification of heterozygotes, which have flattened tendrils, while tl^w homozygotes carry leaflets in place of tendrils. Segregation observed was as follows: 33 *Tl*:41 *Tl*/tl^w:3 tl^w. This ratio significantly differs from the expected ratio of 1:2:1, $\chi^2 = 93.8$, $\alpha \ll 0.001$. Probably, these F_2s segregate for a conditional recessive lethal or detrimental allele linked to tl^w and manifested in the background of VIR320 cytoplasm.

The expected segregation of genetic markers inherited from each of the parents in the RIL population self-pollinated for six generations is 1:1, with 1/64 of the lines still retaining heterozygosity at a locus. Noteworthy in this respect is segregation of markers linked to the genes involved in nuclear-cytoplasmic incompatibility in the case where cytoplasm comes from the cultivated parent WL1238. Among 95 RILs analysed that underwent unconscious selection during six generations, segregation for the *gp* marker was 65 *gp_1238*:29 *Gp_320*:1 heterozygote. The latter heterozygote (RIL-4) was identified due to segregation in the following generation; also it was heterozygous for the "deficient" phenotype. The ratio 65:29 significantly differs from expected 1:1 ($\chi^2 = 13.79$, $\alpha < 0.001$). At the same time, segregation at the *tl* locus mapped at about 30 cM from *gp* is not distorted: 42 tl^w*_1238*:41 *Tl_320*:6 heterozygotes. We suggest that some gene linked to *gp*, probably, *Scs2*, improves compatibility of nuclear genomes with cytoplasm originating from the cultivated parent. Segregation for the *PhlC* marker was 53 *PhlC_1238*:36 *PhlC_320*. We did not observe heterozygotes for *PhlC*, and we suspect they fell into the class of heterozygotes for the incompatibility character which were not analysed for DNA markers. The ratio 53:36 deviates somewhat from 1:1; however, the value $\chi^2 = 3.2$ is not significant ($\alpha > 0.05$) indicating that the linked gene *Scs1* has less impact on compatibility with WL1238 cytoplasm than *Scs2*.

Discussion

We performed genetic analysis of nuclear-cytoplasmic incompatibility manifested as chlorophyll deficiency, chlorophyll variegation and reduction of leaflets and stipulae, a suite of characters associated with the cytoplasm of the wild pea line VIR320 when crossed with domesticated pea

lines. We concluded that the typical phenotype of an incompatible nuclear-cytoplasmic combination is conditioned by two unlinked nuclear genes, which we denote as *Scs1* and *Scs2*. The former is located on LGIII and is closely linked to the gene *PhlC* coding for phospholipase C, while the latter is located on LGV and is closely linked to the gene *gp* (*luteo-legumina*) that determines yellow colouration of young pods. 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 nuclear and plastid genomes (Bogdanova 2007) while in cereals, the mitochondrial genomes are primarily involved (Aksyonova et al. 2005). We failed to observe homozygotes for the *PhlC_1238* allele in segregating F_2s of the crosses VIR320 × RIL. A number of homozygotes for *gp_1238* most probably result from a crossover event between *Scs2* and *gp*. We conclude that *Scs1* and *Scs2* are either gametophyte or sporophyte lethals, eliminating their carriers when the cytoplasm comes from the wild accession VIR320. It is not excluded that lethality is due to some linked loci but it seems unlikely that each of the compatibility genes neighbours a conditional lethality factor. In addition to *Scs1* and *Scs2*, which confer visible phenotypic effect in a heterozygous state, there probably are other deleterious factors, including a putative recessive lethal, manifested in the background of the VIR320 cytoplasm, which is linked to tl^w. This gene, however, has no relation to the striking phenotypic syndrome of nuclear-cytoplasmic conflict we analyse.

Our data do not allow the precise estimate of genetic distance between the genes involved in nuclear-cytoplasmic conflict and their nearest neighbours, but these distances are rather small comprising in sum about 3.2 cM. Genetic mapping of the gene conferring compatibility of *Triticum timopheevii* nucleus with the cytoplasm of *Aegilops longissimum* revealed tightly linked microsatellite markers at both sides of the relevant gene scs^{ti} at distances of 8.2 and 5.8 cM, which were transformed to 2.3 and 0.6 cM after removal of apparently double crossovers (Simons et al. 2003). Also, RAPD markers closely linked to the nuclear gene from *T. timopheevii* conferring compatibility with the *Ae. squarrosa* cytoplasm which is, probably, the same gene as above mentioned scs^{ti}, were identified (Asakura et al. 1997b). The advantage of the linkage analysis performed in the present work is that the map data are gene-based and allow a search for candidate genes utilising collinearity of pea genome with that of *Medicago* (Kalo et al. 2004) as well as synteny of plant genomes within the Fabaceae family (Choi et al. 2004). The nearest registered neighbours of the *Scs1* gene on LGIII are *PhlC* and *Cbl*. BLAST search of the sequenced portion of *Medicago truncatula* genome using protein sequences of pea *PhlC* and *Cbl* gene products (encoded in AF280748 and AY883569, respectively) reveals their homologs on chromosome three of *Medicago truncatula* encoded in AC145219 and AC147712, respectively, which are separated by about 2,200,000 bp on the genome sequence assembly map (*Medicago* Genome Sequence Consortium 2007) and about 22 cM on the barrel medic genetic map (Mun et al. 2006), the maps being available for comparison at http://www.comparative-legumes.org/lis. These findings indicate rather good correspondence of the pea and barrel medic genomes in this region. In chromosome seven of *Medicago truncatula,* homologous to LGV of pea (Choi et al. 2004), probable candidate genes for *Scs2* are expected to fall between an orhtolog of *cri* (*crispa*, MYB-related transcription factor PHAN1) encoded in AF299140 in pea and AC165219 in *M. truncatula* and ortholog of *Bpm* (2,3-biphosphoglycerate-independent phosphoglycerate mutase) encoded in AC124215 of *M. truncatula,* which are separated by about 2,700,000 bp on genome sequence assembly map (*Medicago* Genome Sequence Consortium, 2007) and about 12 cM on the genetic map (Mun et al. 2006). Unlike the above-mentioned segment of chromosome three, physical map of the chromosome seven segment of *M. truncatula* contains an inversion or transposition as compared to the genetic map of this chromosome, so that an estimate of the physical distance between genes of interest is somewhat controversial.

Acknowledgments The authors are grateful to Dr. N·F.Weeden for helpful comments on the manuscript and revising English language, to the anonymous referees for valuable suggestions and to Mrs. L.P. Romkina for excellent technical assistance. Sequencing reactions were performed at DNA Sequencing Center (ICG-ICBFM SD RAS). This work was supported by Russian Foundation for Fundamental Research, grant number 07-04-00111-a.

References

Aksyonova E, Sinyavskaya M, Danilenko N, Pershina L, Nakamura C, Davydenko O (2005) Heteroplasmy and paternally oriented shift of the organellar DNA composition in barley-wheat hybrids during backcrosses with wheat parents. Genome 48:761–769

Allen JO (2005) Effect of teosinte cytoplasmic genomes on maize phenotype. Genetics 169:863–880

Anderson JA, Maan SS (1995) Interspecific nuclear-cytoplasmic compatibility controlled by genes on group 1 chromosomes in *durum* wheat. Genome 38:803–808

Asakura N, Nakamura C, Ohtsuka I (1997a) A nuclear compatibility gene, *Ncc-tmp,* of *Triticum timopheevi* for the cytoplasm of *Aegilops squarrosa*. Genes Genet Syst 72:71–78

Asakura N, Nakamura C, Ohtsuka I (1997b) RAPD markers linked to the nuclear gene from *Triticum timopheevii* that confers compatibility with *Aegoilops squarrosa* cytoplasm on alloplasmic durum wheat. Genome 40:201–210

Asakura N, Nakamura C, Ohtsuka I (2000) Homoeoallelic gene *Ncc-tmp* of *Triticum timopheevii* conferring compatibility with the

cytoplasm of *Aegilops squarrosa* in the tetraploid wheat nuclear background. Genome 43:503–511

Bogdanova VS (2007) Inheritance of organelle DNA markers in a pea cross associated with nuclear-cytoplasmic incompatibility. Theor Appl Genet 114:333–339

Bogdanova VS, Berdnikov VA (2001) Observation of a phenomenon resembling hybrid dysgenesis, in a wild pea subspecies *Pisum sativum* ssp *elatius*. Pisum Genet 33:5–8

Bogdanova VS, Kosterin OE (2006) A case of anomalous chloroplast inheritance in crosses of garden pea involving an accession of wild subspecies. Dokl Biol Sci 406:44–46 Russian

Choi HK, Mun JH, Kim DJ, Zhu H, Baek JM, Mudge J, Roe B, Ellis N, Doyle J, Kiss GB, Young ND, Cook DR (2004) Estimating genome conservation between crop and model legume species. Proc Natl Acad Sci USA 101:15289–15294

Gehlhar SB, Simons KJ, Maan SS, Kianian SF (2005) Genetic analysis of the species cytoplasm specific gene (scs^d) derived from durum wheat. J Hered 96:404–409

Glick R, Sears BB (1994) Genetically-programmed chloroplast dedifferentiation as a consequence of plastome-genome incompatibility in *Oenothera*. Plant Physiol 106:367–373

Goloenko IM, Lukhanina NV, Shimkevich AM, Aksyonova EA, Danilenko NG, Davydenko OG (2002) The productivity characteristics of substituted barley lines with marked chloroplast and mitochondrial genomes. Cell Mol Biol Lett 7:483–491

Haldane JBS, Waddington CH (1931) Inbreeding and linkage. Genetics 16:357–374

Hanson MR (1991) Plant mitochondrial mutations and male sterility. Annu Rev Genet 25:461–486

Hossain KG, Riera-Lizarazu O, Kalavacharla V, Vales MI, Maan SS, Kianian SF (2004) Radiation Hybrid mapping of the species cytoplasm-specific (scs^{ae}) gene in wheat. Genetics 168:415–423

Kalo P, Seres A, Taylor SA, Jakab J, Kevei Z, Kereszt A, Endre G, Ellis TH, Kiss GB (2004) Comparative mapping between *Medicago sativa* and *Pisum sativum*. Mol Genet Genomics 272:235–346

Konieczny A, Ausubel FM (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J 4:403–410

Konovalov F, Toshchakova E, Gostimsky S (2005) A CAPS marker set for mapping in linkage group III of pea (*Pisum sativum* L). Cell Mol Biol Lett 10:163–171

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Lilienfeld FA (1962) Plastid behavior in reciprocally different crosses between two races of *Medicago truncatula* Gaertn. Seiken Ziho 13:3–38

Lilienfeld FA (1965) A case of malfunctioning plastids in *Medicago truncatula* Gaertn. Jan J Genet 40:261–274

Maan S (1992a) Transfer of a species cytoplasm specific (*scs*) gene of *Triticum timopheevii* Zhuk to *T. turgidum*. Genome 35:238–243

Maan S (1992b) The *scs* and *Vi* genes correct a syndrome of cytoplasmic effects in alloplasmic durum wheat. Genome 35:780–787

Maroof MA, Zhang Q, Neale DB, Allard RW (1992) Associations between nuclear loci and chloroplast DNA genotypes in wild barley. Genetics 131:225–231

Medicago Genome Sequence Consortium (2007) *Medicago truncatula* genome sequence: release 1.0 at http://www.medicago.org

Mukai Y, Tsunewaki K (1976) Genetic diversity of the cytoplasm in *Triticum* and *Aegilops* IV distribution of the cytoplasm inducing variegation in common wheat. Theor Appl Genet 4S:9–16

Mun J-H, Kim D-J, Choi H-K, Gish J, Debellé F, Mudge J, Denny R, Endré G, Saurat O, Dudez A-M, Kiss GB, Roe BA, Young ND, Cook DR (2006) Distribution of microsatellites in the genome of *Medicago truncatula*: a resource of genetic markers that integrate genetic and physical maps. Genetics 172:2541–2555

Neff MM, Neff JD, Chory J, Pepper AE (1998) dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. Plant J 14:387–392

Ohtsuka I (1991) Genetic differentiation in wheat genomes in relation to compatibility with *Aegilops squarrosa* cytoplasm and application to phylogeny of polyploid wheats. J Fac Agric Hokkaido Univ 65(part 2):127–198

Schmitz-Linneweber C, Kushnir S, Babiychuk E, Poltnigg P, Herrmann RG, Maier RM (2005) Pigment deficiency in nightshade/tobacco cybrids is caused by the failure to edit the plastid ATPase a-subunit mRNA. Plant Cell 17:1815–1828

Simons KJ, Gehlhar SB, Maan SS, Kianian SF (2003) Detailed mapping of the species cytoplasm-specific (scs) gene in durum wheat. Genetics 165:2129–2136

Stubbe W (1964) The role of the plastome in evolution of the genus *Oenothera*. Genetica 35:28–33

Stubbe W, Steiner E (1999) Inactivation of pollen and other effects of genome-plastome incompatibility in *Oenothera*. Plant Syst Evol 217:259–277

Tsunewaki K (1993) Genome-plasmon interactions in wheat. Jpn J Genet 68:1–34

Tsunewaki K, Wang G-Z, Matsuoka Y (1996) Plasmon analysis of *Triticum* (wheat) and *Aegilops*. 1. Production of alloplasmic common wheats and their fertilities. Genes Genet Syst 71:293–311

Tsunewaki K, Wang GZ, Matsuoka Y (2002) Plasmon analysis of *Triticum* (wheat) and *Aegilops*. 2. Characterization and classification of 47 plasmons based on their effects on common wheat phenotype. Genes Genet Syst 77:409–427

Weeden NF, Ellis THN, Timmerman-Vaughan GM, Swiecicki WK, Rozov SM, Berdnikov VA (1998) A consensus linkage map for *Pisum sativum*. Pisum Genet 30:1–4

Weeden NF, Moffett MD (2007) List of genes postulated to be on pea LGIII between *Fed*1 and just distal to *Egl*1. Pisum Genet 39:45–63

Zubko MK, Zubko EI, Ruban AV, Adler K, Mock H-P, Misera S, Gleba YY, Grimm B (2001) Extensive developmental and metabolic alterations in cybrids *Nicotiana tabacum* (+*Hyoscyamus niger*) are caused by complex nucleo-cytoplasmic incompatibility. Plant J 25:627–639