Ontogenetic consequences of dysgenic crosses in *Drosophila virilis*

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ABSTRACT Hybrid dysgenesis (HD) syndrome in *Drosophila virilis* presumably results from the mobilization of several unrelated mobile genetic elements in dysgenic hybrids. Morphogenetic events during oogenesis and spermatogenesis were investigated in detail in the progeny of *D. virilis* dysgenic crosses. Using germ-cell specific anti-Vasa staining, we monitored the fate of germline cells at different ontogenetic stages in strains of *D. virilis* and their hybrids. Anti-Vasa staining indicated that the major loss of pole cells occurs in dysgenic embryos at stage 11-14 after primordial germ cells (PGC) pass the midgut wall. At later ontogenetic stages, including larvae, pupae and imagoes, we often observed an abnormal development of gonads in dysgenic individuals with a frequent occurrence of unilateral and bilateral gonadal atrophy. Dysgenic females were characterized by the presence of various sterile ovarian phenotypes that predominantly include agametic ovarioles, while other atypical forms such as tumor-like ovarioles and dorsalized ovariolar follicles may also be present. Testis abnormalities were also frequently observed in dysgenic males. The sterility manifestations depended on the strain, the growing temperature and the age of the flies used in crosses. The observed gonadal sterility and other HD manifestations correlated with the absence of maternal piRNAs homologous to *Penelope* and other transposons in the early dysgenic embryos. We speculate that gonadal abnormalities mimicking several known sterility mutations probably result from the disturbance of developmental gene expression machinery due to the activation of unrelated families of transposons in early dysgenic embryos.

KEY WORDS: *Drosophila virilis, anti-Vasa staining, sterility, hybrid dysgenesis, transposon*

Introduction

The deleterious traits that occur in the interstrain hybrids of *Drosophila melanogaster,* described as hybrid dysgenesis (HD) by M. Kidwell (1983), were later also found in *Drosophila virilis* (Lozovskaya *et al.*, 1990; Scheinker*et al.*, 1990). Among the dysgenic traits, such as male recombination, point mutations, chromosomal aberrations and nondisjunction, the most prominent and easily visualized trait in the HD systems is gonadal atrophy in adult flies (Kidwell 1983; Lozovskaya *et al.*, 1990; Evgen'ev*et al.* 1997; Blumenstiel and Hartl, 2005).

Gonadal sterility in *D. melanogaster* HD manifests in two independent systems known as the *I-R* and *P-M* systems, which are controlled by the *I* and *P* elements, respectively (for excellent background material, see Bucheton *et al.*; 2002, Rio, 2002; Kidwell and Lisch 2002; and Khurana *et al.*, 2011).

However, in *D. virilis,* several unrelated transposable elements (TEs) belonging to different classes (*Penelope, Ulysses, Paris, Helena, Telemac,* and likely *Tv1*) are simultaneously activated (Scheinker *et al.*, 1990; Petrov *et al.*, 1995; Evgen'ev *et al.*, 1997; Vieira *et al.*, 1998). It is noteworthy that recently mobilization of several resident TEs besides *P*-elements has been also demonstrated in the *P-M* dysgenic hybrids (Khurana *et al.*, 2011).

It was suggested that *Penelope* and probably *Paris* and *Helena*

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play important roles in HD manifestations because the *D. virilis* strains used to produce dysgenic phenotypes differ in the presence of these TEs (Petrov *et al.*, 1995; Evgen'ev *et al.*, 1997; Vieira *et al.*, 1998). In general, gonadal sterility and other HD manifestations observed in *D. virilis* dysgenic males and females are reminiscent of those observed in the *D. melanogaster P-M* HD system. In the adult hybrids resulting from crosses between females lacking functional *P*-elements, which encode active transposase from certain laboratory strains and males carrying complete *P*-elements from wild populations (or the equivalent), gonadal sterility manifests as rudimentary ovaries with partially or completely sterile sets of ovarioles and rudimentary testes depending on the temperature conditions. Sterility in this system is more frequent in females than in males (Kidwell *et al.*, 1983). The *I*-factor in *I-R* HD system is responsible for another type of fly sterility resulting from embryo mortality, and the transposition of the *I*-element is sex specific because it never occurs in males (Finnegan, 1989; Bucheton *et al.*, 2002). Reciprocal crosses in either system result in essentially fertile progeny.

In the *P-M* dysgenic embryos, germline cells degenerate autonomously and independently of the surrounding somatic tissue, including ovarian mesodermal tissue. This degeneration results in agametic sterile ovarioles in adult females (Niki, 1986).

It has also been shown that the third intron of the *P* element is spliced normally only in the primordial embryonic germ cells (pole cells) and adult germline cells (Laski and Rubin, 1989). This mechanism is crucial for encoding an active transposase, which functions to excise or insert *P*-elements in *D. melanogaster* germline cells. Notably, the *Penelope* retroelement in *D. virilis* also contains an intron and may produce spliced and unspliced mRNAs; however, the role of this intron splicing in *Penelope* function and HD is unknown (Arkhipova *et al.*, 2003; Shostak *et al.*, 2008).

The strains used in the *D. virilis* dysgenic crosses by analogy to the previously described *D. melanogaster P-M* system are designated as "*P*-like" (such strains contain multiple full-length *Penelope* copies) and "*M*-like" (such strains lack intact *Penelope* copies in the genome). All dysgenic traits are the result of crosses between the males from an old *D. virilis* laboratory marked strain160 (strong *P*-like strain) possessing multiple copies of *Penelope* and the females of various wild-type strains lacking active *Penelope* elements (Lozovskaya *et al.*, 1990; Vieira *et al.*, 1998; Blumenstiel and Hartl, 2005; Rozhkov *et al.*, 2010; Rozhkov *et al.*, 2011).

The parental *D. virilis P*-like strain itself may exhibit a significant level of sterility as observed, for example, in the *D. melanogaster* Harwich *P*-like strain (Lozovskaya *et al.*, 1990). In both HD systems, bilateral and unilateral ovary and testis sterility are often observed. Visible chromosomal mutations and other features in the progeny of *D. virilis* dysgenic crosses are also partially shared with the *P-M* HD system described in *D. melanogaster* (Lozovskaya *et al.*, 1990; Evgen'ev *et al.*, 1997; Vieira *et al.*, 1998). When we previously discovered HD in *D. virilis*, we estimated the total frequency of gonadal sterility in the adult progeny from dysgenic and reciprocal crosses (Lozovskaya *et al.*, 1990). However, at that time, we did not describe the

pattern of the observed gonad abnormalities or the ontogenetic stages of occurrence.

Subsequently, Blumenstiel and Hartl (2005) attempted to find a correlation between maternally transmitted *Penelope*-derived small RNAs and the sterility phenotypes observed in the adult *D. virilis* dysgenic hybrids, similar to what was clearly demonstrated for the *P-M* and *I-R* dysgenesis systems in *D. melanogaster* (Brennecke *et al.*, 2008; Chambeyron *et al.*, 2008; Khurana *et al.*, 2011). Here, we continue our studies on *D. virilis* HD and provide a detailed analysis of the gonad abnormalities observed in the ontogenesis of dysgenic hybrids. Using a germ cell-specific Vasa-antibody, we determined the stages where primordial germ cell death begins in dysgenic embryos and monitored the consequences of abnormal primary gonad formation in different stages of morphogenesis, including imagoes.

The observed dysgenic phenotypes correlated with the expression of *Penelope* and several other TEs in the dysgenic embryos and were inversely correlated with the levels of maternally supplied primary piRNAs homologous to these TEs.

Results

Frequency and spectrum of gonadal abnormalities observed in dysgenic females

Each pair of the *D. virilis* ovaries consists of 63-65 ovarioles with an anterior structure called germarium. Similar to *D. melanogaster*, the germarium harbors 2-3 germ stem cells (GSC) in its anteriormost region (GSC niche), two follicle stem cells (FSC)
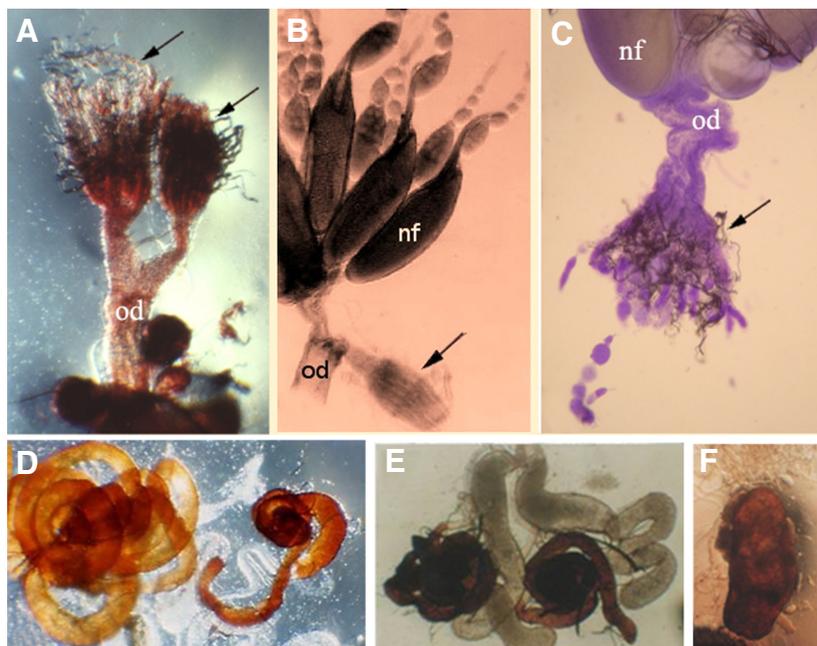

Fig. 1. Ovaries and testes of dysgenic progeny of *D. virilis* (A) and (B) (strain 9 female x strain 160 male) and *D. melanogaster* (C) (female *Df(1), yw$^{67c23(2)}$* x male Harwich) grown at 25°C. (A) *Bilateral sterile ovaries;* **(B)** *left normal ovary and right sterile atrophied ovary;* **(C)** *unilateral sterile ovary; od-oviduct; nf-normal follicle developed to an egg; arrows indicate atrophied ovaries;* **(A,C)** *neutral red,* **(B)** *unstained. 20x.* **(D)** *The right testis is reduced; the left testis has a normal size.* **(E)** *Bilateral sterile darkly pigmented testes are shown.* **(F)** *A cistern-like abnormal testis surrounded by developed spermatozoids released from the damaged testis.*

TABLE 1

THE FREQUENCY (IN %) OF DIFFERENT TYPES OF OVARIES IN THE FEMALES OF STRAINS AND F1 CROSSES (FEMALES X MALES)

Strains and crosses	No. of females	N/N	N/SN	N/ST	SN/SN	SN/ST	ST/ST
9	106	100 (106)	0	0	0	0	0
9 x 160	442	49 (219)	10 (42)	12(55)	7 (30)	8 (36)	14 (62)
160 x 9	100	98 (98)	0	1 (1)	0	0	1 (1)
160	112	97 (109)	0	1 (1)	1 (1)	0	1 (1)
13	181	100 (181)	0	0	0	0	0
13 x 160	271	23 (62)	10 (26)	11(31)	11 (30)	22(22)	23 (62)
160 x 13	139	97 (135)	0	1 (2)	0	1 (1)	1 (1)
1	32	97 (31)	0	3 (1)	0	0	0
1 x 160	60	84 (50)	2 (1)	5 (3)	2 (1)	2 (1)	5 (3)
142	24	42 (10)	4 (1)	4 (1)	50 (12)	0	0
142 x 160	48	52 (25)	19 (9)	13 (6)	8 (4)	2 (1)	6 (3)
160 x 142	69	98 (67)	0	1 (1)	0	0	1 (1)
110	36	100 (36)	0	0	0	0	0
110 x 160	112	97 (109)	0	0	0	0	3 (3)
160 x 110	91	95 (86)	0	0	1 (1)	0	4 (4)
142 x 110	78	94 (73)	0	1 (1)	2 (2)	0	1 (1)
110 x 142	58	100 (58)	0	0	0	0	0

N/N - both normal ovaries, N/SN* - one normal and another semi-sterile ovary, N/ST - normal/ sterile, SN/SN - both semi-sterile, SN/ST - semi-sterile/sterile, ST/ST - both sterile. The numbers in brackets indicate the number of females with the certain type of ovaries.

The normal ovarian phenotype was indicated as "N", indicating that all or more than 3/4 of ovarioles in the ovary did not deviate from the normal developmental pattern.

*A quite frequent ovarian abnormal phenotype observed in certain *D. virilis* strains and hybrids, designated by us as semi-normal (SN), manifested as a set of different types of ovarioles coexisting in the same ovary, including agametic and tumor-like ovarioles.

and their differentiated daughters, cystoblasts and prefollicle cells, respectively (Spradling, 1993).

To estimate the total frequency of sterility, we counted flies (both males and females) with complete unilateral or bilateral gonadal atrophy in a sample of *D. virilis* strains and their hybrids produced in bidirectional crosses with the "classical" *P*-like strain 160 (Table 1). Besides, we estimated the frequency of various abnormal ovarioles within each individual. It was evident, as has been previously demonstrated (Lozovskaya *et. al*, 1990; Evgen'ev *et al.*, 1997; Blumenstiel and Hartl, 2005), that significant levels of gonadal sterility with complete bilateral or unilateral gonadal atrophy were observed in the dysgenic progeny of crosses between females from typical *M*-like strains lacking *Penelope* (e.g., strain 9) and males of strain 160 (Figs. 1A,B). It is noteworthy that "agametic" rudimentary ovarioles with no obvious separation between the egg chambers, that represent the major manifestation of gonadal sterility in *D. virilis* dysgenic females, phenotypically resembled those observed in *P-M* hybrid dysgenesis in *D. melanogaster* (Fig. 1C).

Ovaries of the wild-type M-like strain 9 have approximately 63-65 ovarioles, none of which deviate from the standard, whereas ovaries from other *M*-like and *P*-like strains had sets of ovarioles of different numbers and phenotypes (Table 2), for example, 36-37 ovarioles in the progeny of the cross between females of strain 13 and males of strain 160. Moreover, a bilaterally normal phenotype (N/N) in 100% of females was found in strains 9, 13 and 110 (Table 1), but different abnormal ovariolar phenotypes (predominant "agametic" and "tumor-like") leading to complete or partial ovarian sterility were detected in dysgenic hybrids (Table 2 and Supp. Fig. S1).

To further characterize the developmental abnormalities of

dysgenic ovaries we took advantage of antibodies that specifically label germline cells and fusomes within the germ cells, anti-Vasa and anti-1B1 respectively (see Huynh, 2005 for excellent review). Agametic ovarioles often produced in dysgenic hybrids lack normal germ cells as revealed by staining with anti-Vasa and 1B1 antibodies and appeared as empty tubes either lacking stained material or filled with small cells or degraded cell conglomerates (Supp. Fig. S1). In rare cases DAPI staining revealed the presence of mesodermal cells, follicle epithelial cells along the tube and, occasionally, a few large, presumably cap cells adjacent to the filaments (data not shown). "Tumor-like" ovarioles lacked the true germarium zone as well as true follicles (Supp. Fig. S1 C,F). This type of abnormality was rather frequent in the dysgenic progeny between strains 13 and 160 (Table 2). DAPI staining of "tumor-like" ovarioles revealed small follicle epithelium cells, a band of stem prefollicular cells, and a cavity in which a few large, presumably undifferentiated germline cells were sometimes observed in an unattached state (Supp. Fig. S1 G). The sterile phenotype, in its most extreme bilateral or uni-lateral manifestation with atrophied ovarioles or with the addition of a few tumor-like ovarioles, reached high levels in the crosses between M-like strains and strain 160 (Table 1 and 2).

In addition to the severe defects in oogenesis, the dysgenic hybrids showed a range of other phenotypes that occurred at lower penetrance. These phenotypes included additional spermathecae and follicles with disturbed polarity. The swallowed, inverted (turned 180 degrees with respect to each other) mature follicles were sometimes observed with abnormal appendages. The frequency of females with such abnormal follicles was roughly similar to the frequency of females with tumor-like ovarioles, comprising 2-4%, depending on the strain (Table 2).

Importantly, the levels of gonadal atrophy observed in the progeny of dysgenic crosses significantly decreased with the age of parents used in crosses and increased in the flies grown at low (18°C) temperatures (Tables S2 and S3). On the other hand the

TABLE 2

THE FREQUENCY (IN %) OF FEMALES THAT CONTAIN DIFFERENT OVARIOLAR PHENOTYPES IN THE STRAINS, DYSGENIC AND RECIPROCAL HYBRIDS OF *DROSOPHILA VIRILIS*

Strains and crosses	Agameticovarioles	Tumor-like ovarioles	Abnormal polarity	Abnormal appendages
9	1	0	0	0
160	9	0	1	2
9X160	98*	4	2	1
160x9	95*	0	0	0
13	91	2	13	0
13 x 160	97	53	2	0
160 x 13	98	3	0	0
1x160	1	3	3	0
142	1	0	12	4
142x160	1	8	4	0
160x 142	0	0	0	0
110	1	0	0	0
160x110	1	0	0	0
9x110	1	2	0	0

The numbers of ovaries with various types of ovariolar abnormalities were determined (n=100 females were included for each entity; three replicates were performed with n= 30-35 females).

*We counted all females that have at least one or several agameticovarioles in otherwise normal ovary and, hence, we detected similar numbers of females with agameticovarioles in dysgenic and reciprocal hybrids although the hybrids from such crosses differ drastically in the levels of gonadal atrophy (sterility) as seen in Table 1.

aged dysgenic males and females are not becoming less sterile (Table S2), as was shown for HD in *D. melanogaster* (Bucheton *et al.*, 2002; Khurana *et al.*, 2011).

High levels of sterility and gross abnormalities of dysgenic testes

The more extreme phenotype of testis atrophy was often accompanied by heavy pigmentation and was manifested in a low number or a complete lack of spermatocysts (Fig. 1 D,E and Table 3). Cistern-like testes filled with spermatocytes represent another type of abnormal gonad development, and such abnormal testes were sometimes found unconnected to ectodermal ejaculatory ducts (Fig. 1 F). When studying testis phenotypes, we distinguished, similar to the ovarian phenotypes, normal (N), sterile (ST) with severely reduced testis, and semi-normal (SN) types of testes. All of these phenotypes had unilateral or bilateral manifestations (Table 3). As in the case of ovaries, there were different frequencies of testis phenotypes in different experiments, and the maximal level of male sterility was observed in dysgenic hybrids under low temperature conditions (18°C; Table S3). Overall, the frequency of female gonad abnormalities in dysgenic hybrids was significantly higher than that of the males (Tables S2 and S3), however, this value greatly depends on the strains used in dysgenic crosses (Tables 1 and 3).

Severely reduced number of pole cells constituting the primary gonads of dysgenic embryos

The *vasa* gene (*vas*) is essential for germline development in *Drosophila melanogaster* and other *Drosophila* species, including *D. virilis* (Lasko and Ashburner, 1990). Zygotic *vasa* is expressed in pole cells earlier than any other pole cell-expressing genes that have thus far been identified. Moreover, the Vasa protein is highly conserved and is continuously present in *Drosophila* germline cells

throughout development (Lasko and Ashburner, 1990; Styhler *et al.*, 1998; Spradling *et al.*, 2012). To determine the time of germline cell loss in *D. virilis* hybrids from dysgenic crosses, we compared the localization and the number of pole cells in embryos of parental strains and their hybrids at various stages. In our work, we explored conservative nature of the Vasa protein and using antibodies against *D. melanogaster* Vasa protein demonstrated that pole cell formation at the posterior pole of embryos from dysgenic crosses appeared normal. Thus, we usually observed 16-19 phenotypically normal round pole cells at the cellular blastoderm stage in embryos of the parental strains and dysgenic hybrids (Fig. 2 A), and the large processes/filopodial extensions usually produced by *Drosophila* pole cells at the early gastrula (Jaglarz and Howard, 1995) were seen at this stage in *D. virilis* strains and dysgenic hybrids (Supp. Fig. S2 A-C).

Next we monitored pole cell migration from the posterior pole through the midgut to the final location where germ cells form the primordial gonads (Fig. 2 A-E). Our observations indicate that in dysgenic embryos, although most pole cells did migrate into the gonads (Fig. 2C), some pole cells cross the midgut epithelium but failed to reach the gonadal mesoderm (Fig. 2 D and E). Ultimately, these pole cells became mislocalized, lost their round shape, remain spread throughout the embryo and eventually died after migration through the posterior midgut wall (Fig. 2 F and G).

It has been previously demonstrated that apoptosis occurs in the primordial germ cells (PGCs) that leave the germline fate in response to genetic and environmental perturbations (Sano *et al.*, 2002; Sato *et al.*, 2007).

Overall, the migration defects in dysgenic embryos were observed starting from stage 12.

Later, at the time of gonadal coalescence at stage 14, some pole cells had arrived at the gonad area, but the number of the cells forming the primary gonad was often reduced and varied from 3 to 15 in dysgenic embryos (Fig. 2C, Table 4). Sometimes, only one gonad in the dysgenic embryo was visible. The data summarized in Table 4 and Fig. 2 illustrate a variable penetrance of this phenotype.

Manifestations of gonadal dysgenesis (GD) at the larval and pupal stages

To determine whether germline cells die at postembryonic stages of development in dysgenic hybrids, we scored the germ cell number not only in the dysgenic embryos but also in the third instar larvae gonads. The data summarized in Table 4 indicate that the ratio of classes with different germline cells
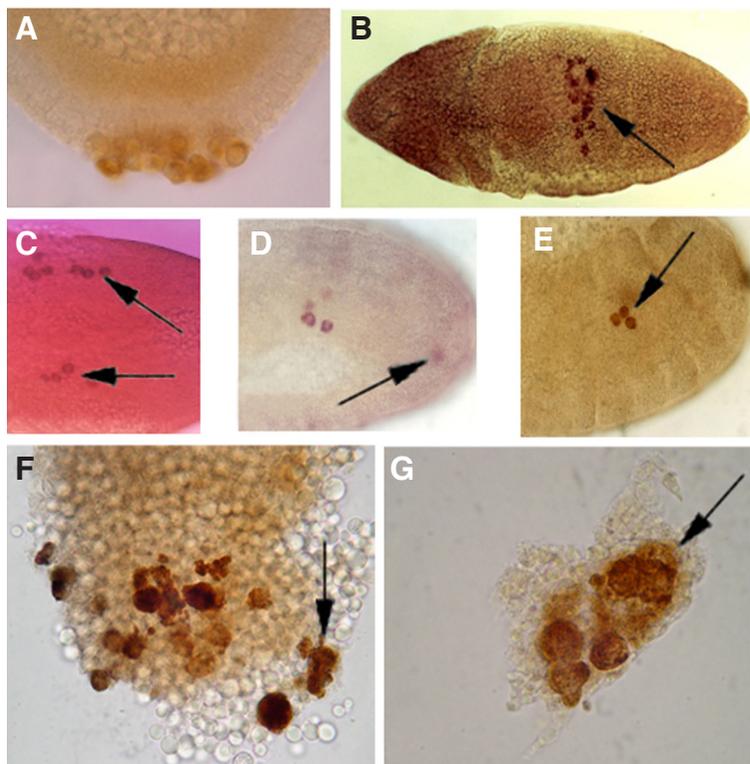
Fig. 2. *D. virilis* dysgenic embryos stained with anti-Vasa antibodies.(A) *Normal pole cells in strain 9 embryos at the stage of syncytial blastoderm;* **(B)** *Germline cells at the dorsal side of the embryo after they left midgut area (indicated by an arrow);* **(C)** *The final location of the germline cells forming severely reduced bilateral gonads indicated by arrows.* **(D,E)** *Dysgenic embryos at the various stages of pole cell migration to the location of primordial gonads possessing 3 to 15 pole cells. The arrows in D and E mark the mislocalized pole cells.* **(F)** *The dissected midgut area of dysgenic embryo at stage 10 at the moment, when pole cells are moving through the area.* **(G)** *The area of dissected dysgenic embryo (stage 14) at the moment of primordial gonad formation. The arrows indicate individual pole cells. 100x oil immersion. The embryos in this figure are representative of >50 samples.*

quantity is similar in both stages. Therefore, the major germ cell loss likely occurs in the hybrid embryos at the stages indicated above, and the abnormal gonad development observed at the later stages is of a secondary nature. However, our analysis does not exclude a certain level of germline cell loss at later stages of dysgenic hybrid metamorphosis.

Thus, while complete unilateral atrophy was often observed in the dysgenic imagoes (Table 1) the formation of only one primordial gonad in dysgenic embryos represents an extremely rare phenomenon.

To further investigate the fate of germline cells in dysgenic hybrids, we stained isolated *D. virilis* dysgenic ovaries of third instar larvae with anti-Vasa antibodies. Sometimes dysgenic gonads exhibited apparently normal process of ovariole formation from germ cell proliferation to the formation of filaments of primordial ovarioles at the top of a gonad (Supp. Fig. S3 A,B). In contrast, in most dysgenic larvae, the number of germ cells was strongly reduced (Supp. Fig. S3 C,D) and we often observed local concentration of germ cells at only one side of the primordial gonad (e.g. Supp. Fig. S3C). The same was true for the pupal ovaries (Supp. Fig. S4). Furthermore, in the ovaries of dysgenic larvae and late pupae stained with anti-Vasa antibodies, typical agametic and tumor-like ovarioles were often observed in the same ovary among other phenotypically normal ovarioles (Supp. Fig. S4).

Similarly, analysis of the male gonads demonstrated that in the testes of dysgenic larvae and pupae, the spermatid clusters frequently had abnormal structure (Supp. Fig. S5 A-C) and were often severely reduced in number or altogether lacking (Supp. Fig. S5 D-F).

Developmental expression and biogenesis of transposons in D. virilis *strains and interstrain hybrids*

Previously, it has been demonstrated that several unrelated TEs, namely, *Penelope*, *Ulysses*, *Paris*, *Telemac* and *Helena,* are mobilized in *D. virilis* dysgenic hybrids (Scheinker *et al.,* 1990; Petrov *et al.*, 1995; Evgen'ev *et al.*, 1997), and this mobilization likely leads to the multiple abnormalities observed in the progeny of dysgenic crosses. We also demonstrated using Northern hybridization that *Penelope* transcription is significantly induced in the ovaries of the hybrids from dysgenic crosses (Evgen'ev *et al.,* 1997). Here, we performed whole-mount hybridization experiments exploring probes homologous to *Penelope* and *Ulysses*, which are activated in HD, and demonstrated that the detected levels of transcripts of both of these TEs were significantly higher in the ovaries of dysgenic females compared with the reciprocal hybrids (Fig. 3). These observations corroborated our previous conclusions based on Northern hybridization (Evgen'ev *et al.*, 1997). Interestingly, the signal in both cases was clearly observed in the cytoplasm of nurse cells (Fig. 3 B,D). We failed to observe any induction of *gypsy* transcription in the dysgenic ovaries (data not shown) and according to various data, *gypsy* in *D. virilis* is not activated by dysgenic crosses and does not transpose in *D. virilis* strains (Petrov *et al.*, 1995, Rozhkov *et al.*, 2011).

Characteristically, we did not detect *Penelope* transcripts in agametic ovarioles that probably resulted from the death of germline cells at the previous stages of development (Fig. 3B).

Next, we performed RT-PCR experiments to determine the stages when the transcription of several TEs including the ones
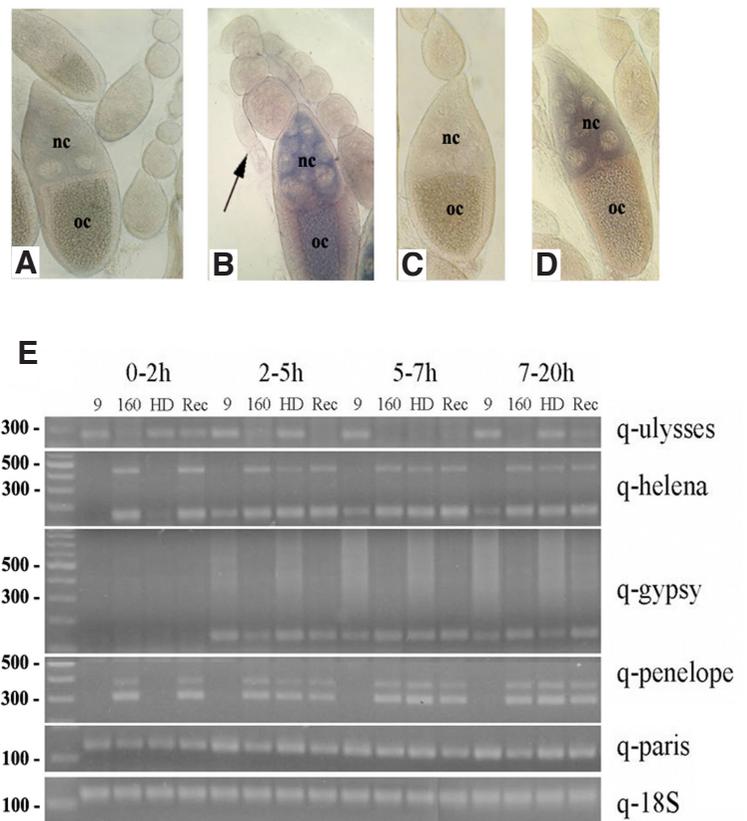

Fig. 3. Transcription levels of different transposons in the embryos of *D. virilis* strains and hybrids from reciprocal and dysgenic crosses. (A,B) *Whole mount hybridization with* Penelope *probe was performed to detect transcripts in the ovaries of* D. virilis *reciprocal* **(A)** *and dysgenic* **(B)** *hybrids. An arrow indicates "agametic" ovariole from the same ovary with the normal-looking ones, which shows only background staining with* Penelope *probe.* **(C,D)** *Whole-mount hybridization was performed with the* Ulysses *DNA probe;* **(C)** *reciprocal hybrids;* **(D)** *dysgenic hybrids; oc-oocyte, nc-nurse cells; 40x.* **(E)** *Semiquantitative RT-PCR data for the transcription of five transposons at different embryonic stages of* D. virilis *parental strains and interstrain hybrids. The two bands seen in* Penelope *gels apparently represent intronless and intronic* Penelope-*derived RNAs.*

activated by dysgenic crosses begins. Fig. 3E shows that transcription of *Penelope, Helena* and *Gypsy,* had already begun in 2-5-hour embryos at the stage of the syncytial blastoderm. At least this statement can be safely made for the above three TEs that apparently do not have maternally transmitted transcripts (Fig. 3E).

The analysis performed indicated that RNAs homologous to all five TEs studied are present at all stages of embryogenesis following syncytial blastoderm in dysgenic and reciprocal hybrids.

It is known that full-length *Penelope* and *Helena* (Evgen'ev *et al.*, 1997; Vieira *et al.*, 1998) are not present in the genome of *D. virilis* M-like strain 9, and hence, the piRNAs homologous to these TEs should not be maternally transmitted to the dysgenic embryos. The results depicted in Fig. 3E corroborated this notion because we failed to detect *Penelope* and *Helena*-derived transcripts in the early embryos (0-2h) of strain 9 and in dysgenic embryos at this stage. As for *Paris* we detected one euchromatic site (60B) of this TE in the microchromosome (chromosome 6) of *D. virilis* strain 9 which coincides with the previously obtained results where besides

<div style="text-align:center">TABLE 3</div>

THE FREQUENCY (%) OF DIFFERENT TYPES OF TESTES IN THE MALES OF STRAINS AND F1 CROSSES (FEMALES X MALES)

Strains and crosses	No. of males	N/N	N/SN	N/ST	SN/SN	SN/ST	ST/ST
9	125	100 (125)	0	0	0	0	0
9 x 160	357	49 (173)	11 (38)	16 (57)	3 (11)	5 (17)	16 (58)
160 x 9	100	99 (99)	0	0	0	0	1 (1)
160	100 10	93 (93)	0	5 (5)	1 (1)	0	1 (1)
13	166	89 (148)	0	6 (10)	1 (2)	0	4 (6)
13 x 160	211	54 (114)	7 (15)	21 (44)	3 (7)	1 (1)	14 (30)
160 x 13	139	95 (132)	0	3 (4)	2 (3)	0	0
1	32	100 (32)	0	0	0	0	0
1 x 160	54	100 (54)	0	0	0	0	0
142	30	90 (27)	0	0	0	0	10 (3)
142 x 160	63	91 (57)	0	6 (4)	0	0	3 (2)
160 x 142	68	99 (67)	1 (1)	0	0	0	0
110	39	97 (38)	0	3 (1)	0	0	0
110 x 160	129	96 (124)	0	2 (3)	0	0	2 (2)
160 x 110	77	94 (72)	1 (1)	0	4 (3)	0	1 (1)
142 x 110	45	100 (45)	0	0	0	0	0
110 x 142	56	98 (55)	0	0	0	0	2 (1)

N/N - both normal testis, N/SN - one normal and another semi-sterile (see text) testis, N/ST - normal/sterile, SN/SN - both semi-sterile, SN/ST - semi-sterile/sterile, ST/ST - both sterile. The numbers in brackets indicate the number of males with different levels of sterility.

a few euchromatic sites of *Paris* and *Helena* several heterochromatic copies of these TEs were also detected (Petrov *et al.*, 1995). Among these transposons, only *gypsy*Dv showed somatic nature, since its transcripts were not detected in 0-2h zygotic embryos of both parents and their hybrids.

It was demonstrated (Blumenstiel and Hartl, 2005) that there is a correlation between maternally inherited *Penelope*-derived small RNAs and the sterility phenotypes observed in the adult *D. virilis* dysgenic hybrids. However, in this work the authors did not discriminate between *Penelope*-derived si- and piRNAs and besides small RNAs homologous to other TEs mobilized in HD were not monitored. Here we extended our analysis to other transposons and demonstrated (Fig. 4) that there is drastic difference between the levels of piRNAs homologous to *Penelope*, *Helena* and *Paris* determined in the embryos from reciprocal and dysgenic crosses. Dysgenic embryos were characterized by very low quantity of correspondent piRNAs (Fig. 4).

The trace amounts of *Penelope* and *Paris*-derived piRNAs observed in dysgenic embryos probably represent primary piRNAs, that may be produced by highly diverged heterochromatic copies of these TEs present in the maternal genome of strain 9 and representing the remnants of ancient invasions of *D. virilis* by these TEs (Petrov *et al.*, 1996; Lyozin *et al.*, 2001). It is of note that in general the levels of maternally transmitted piRNAs homologous to the analyzed TEs in early embryos differ by hundreds fold with *Ulysses* and *gypsy Dv* retroelements exhibiting the highest level

<div style="text-align:center">TABLE 4</div>

THE FREQUENCY (%) OF EMBRYONIC GONADS AND LARVAL OVARIES IS SHOWN WITH DIFFERENT NUMBERS OF GERM CELLS IN DYSGENIC HYBRIDS

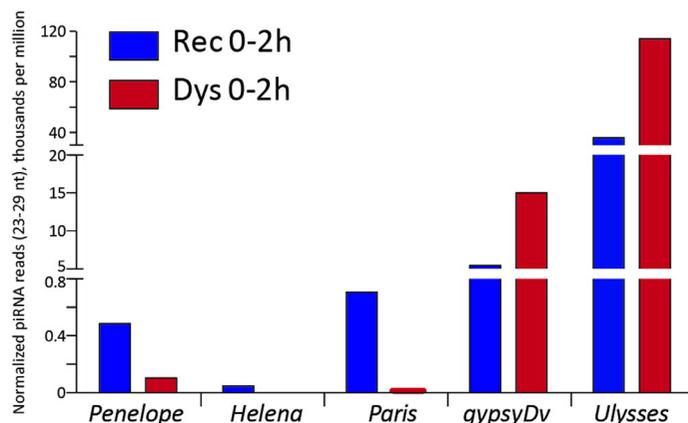| Stage	Germ cells number and % from the primordial gonads studied			
Embryos (n=67)	<5 cells 25 (37%)	6-8 cells 32 (46%)	9-12 cells 9 (13%)	>15 cells 3 (4.5%)
Third instar larvae (n=44)	2-6 cells 12 (27%)	9-20 cells 19 (42%)	30-50 cells 10 (23%)	100 cells 3 (7%)

Fig. 4. Abundance of piRNA homologous to the five studied transposable elements (TEs) in early embryo libraries (0-2 h) of *D. virilis* reciprocal (in blue) and dysgenic (in red) hybrids. *The characteristics of piRNAs homologous to the TEs were described previously (Rozhkov* et al.*, 2010).*

while *Penelope* and *Helena* showing comparatively low concentration of correspondent piRNAs (Fig. 4). However, the high level of maternal *gypsyDv* in the 0-2h embryos does not match its presumable somatic transcription and, therefore, remains to be further investigated. Overall, these dramatic differences resemble different expression levels and/or biogenesis of TE-derived RNAs in the ovaries.

Discussion

During the period of HD investigation in *D. virilis* (1989-2012), the parental strains and particularly strain 160 underwent evident modifications. Thus, the number of *Penelope* copies in this strain as determined through *in situ* hybridization increased from 37 in 1989 to 54-55 in 2010 (Rozhkov *et al.*, 2011). Furthermore, while we failed to observe any increase in gonadal sterility frequency under cold or high temperature conditions in 1989 (Lozovskaya *et al.*, 1990), in 2010, the level of GS in dysgenic males and females, in contrast to such in *P-M* HD, decreased with age of the parents involved in the crosses and increased when flies were grown under low temperature conditions (Tables S2 and S3). On the other hand in contrast to *P-M* system in *D. melanogaster* we did not observe any restoration of fertility in the aged dysgenic hybrids with time (Khurana *et al.*, 2011) and 7 day-old dysgenic flies exhibit the same level of sterility as 35 days hybrids (Table S2).

Phenotypic analysis of ovarioles in dysgenic hybrids allows to suggest the possible nature of the defects observed. Thus, agametic ovarioles in newly hatched females are the result of severe pole cell disturbances in embryos, while tumor-like ovarioles probably resulted from uncontrolled division of germ stem cells. The most frequent ovariole and/or follicle phenotypic abnormalities observed in *D. virilis* dysgenic hybrids and occasionally in individual strains may be summarized as the following: 1. "agametic"; 2. "tumor-like"; 3. "beads-on-a-string" ovarioles with the chain of "dilatations" resulting from follicle degeneration, 4. small oocyte enclosed by a chorion in an egg chamber; and 5. egg chamber with abnormal, reversed polarity. Notably, there are multiple well-known genes required for germ cell migration, that can mutate to induce various abnormalities in pole cell behavior or induce programmed death (Lasko, 1994; Deshpande *et al.*, 2001; Coffman *et al.*, 2002; Yatsu

et al., 2008; Spradling *et al.,* 2012). The disturbances in germ cell behavior observed in *D. virilis* dysgenic hybrids as well as in the *P-M* syndrome described in *D. melanogaster* are phenotypically but not necessary molecularly similar to abnormalities exhibited by the many known mutations that disrupt germ cell migration and development but never exhibit reciprocal effect in crosses.

In *D. melanogaster P-M* and *D. virilis* HD systems, the germ cell abnormalities are observed when females lacking specific TEs are crossed with males carrying multiple copies of these TEs. Furthermore, in both systems, maximal levels of F1 sterility are temperature-dependent.

Characteristically, the most extreme sterile phenotype is agametic in both systems, while the less extreme phenotypes comprise a set of different classes, which were not quantitatively investigated in the *P-M* system yet. While in *D. melanogaster* the activation of a single transposon, *P*-element, is responsible for the dysgenic phenotype, it has been suggested that in the case of *D. virilis,* the mobilization of several unrelated TEs is responsible for HD with *Penelope* possibly playing the pivotal role in the HD (Evgen'ev *et al.,* 1997; Vieira *et al.,* 1998; Blumenstiel and Hartl, 2005). However, it has recently been shown that several strains containing multiple intact *Penelope* copies behave as neutral and, hence, do not produce dysgenic phenotypes when crossed with *M*-like or *P*-like strains. Furthermore, there are a few atypical strains (e.g., 142) that do not contain full-length euchromatic *Penelope* copies but behave similar to weak M- or neutral strains when crossed with strain 160 males (Tables 1 and 3). These results suggest that previously used strain classifications (*P*-like, *M*-like and neutral) based on the presence of full-size *Penelope* copies (Evgen'ev *et al.,* 1997; Vieira *et al.,* 1998; Bluminstiel and Hartl, 2005) represent an oversimplification, and it is likely that other TEs, in addition to *Penelope* and unknown host genome factors, are also involved in determining the strain cytotype.

In the vast majority of HD studies, the analysis involved female and male gonad development and function in the dysgenic adult hybrids (Kidwell and Lisch, 2002; Bucheton *et al.,* 2002). In contrast, here, we monitored the initial manifestations of the HD syndrome starting from early dysgenic embryos through the larval and pupal stages up to adults.

It is noteworthy that the analysis of the most extreme mutant sterile phenotypes in the *P-M* system suggested that the defects predominantly arise at mid- to late embryonic and early larval development of F1 dysgenic hybrids, likely resulting from the *P* element transposase activity leading to chromosome breakage "pulverization" and "hops" into multiple sites within a genome in PGCs (Kidwell and Novy, 1979; Rio, 2002; Kidwell and Lisch, 2002). It has also been demonstrated that retrotransposon mobilization induces DNA breaks and apoptosis in human cancer cells (Belgnaoui *et al.,* 2006). Moreover, there are multiple mutations in *D. melanogaster* that lead to programmed death and/or various abnormalities of pole cell behavior (Lasko and Ashburner, 1990; Coffman *et al.,* 2002; Spradling *et al.,* 2012). In a few cases, the molecular mechanism of certain mutation action was determined. Thus, it was shown in *D. melanogaster* that pole cells lacking maternal NOS enter the apoptotic pathway and are unable to migrate properly into the embryonic gonads. It is believed that the failure to establish/maintain transcriptional quiescence in germ cells, which normally occurs in the precellular blastoderm, is likely to be the major cause of the migration defects in *nos* and

pum embryos (Deshpande *et al.,* 2001).

It has also been demonstrated that the apoptosis occurs in PGCs that leave the germline fate in response to genetic and environmental perturbations (Sato *et al.,* 2007). Although we observe similar abnormalities in the behavior of pole cells, including their loss in the process of migration, in the early *D. virilis* dysgenic embryos, the molecular defects underlying this phenotype are unknown. It is clear, however, that the self-renewal of germline cells is disturbed in dysgenic hybrids because we observed high levels of gonad atrophy at all stages of fly development. We speculate that the abnormalities including germ cells death observed in early *D. virilis* dysgenic embryos may result from the expression of a few TEs (i.e., *Penelope* and *Helena*) at these stages in the absence of homologous maternally inherited primary piRNAs, which should provide silencing of TEs and, hence, inhibit their deleterious effects such as breakage of DNA in the early stages of ontogenesis (Brennecke *et al.,* 2008; Rozhkov *et al.,* 2010; Khurana *et al.,* 2011).

Consistent with this idea, the defects in migration first become evident around the time the pole cells begin to exit the midgut, and at this stage, the transcription of all studied TEs, including *Penelope* and *Helena,* that are absent in the maternal genome (strain 9) already begins in dysgenic embryos (Fig. 4).

The expression of *Penelope* and other TEs at the absence of homologous silencing piRNA at early stages of embryogenesis in dysgenic hybrids might lead to the observed abnormalities in pole cell migration and death of a significant part of germline cells. The consequences of these germline disturbances may also be realized epigenetically at the later stages of ontogenesis, including adult dysgenic hybrids.

Materials and Methods

Fly strains and crosses

Three *D. virilis* wild strains, 9 (Batumi, Georgia), 13 (Krasnodar), and 1 (Erevan), and three marker strains (160, 142, 110) were used in this study. In addition, *D. melanogaster* M-like strains (*Df(1), y w^{67c23(2)}*), the *Harwich* strong *P*-like strain and the reciprocal hybrids between these strains were analyzed. All *D. virilis* strains used here have been previously described (Lozovskaya *et al.,* 1990; Evgen'ev *et al.,* 1997; Vieira *et al.,* 1998; Zelentsova *et al.,* 1999). The flies were raised on standard media containing propionic acid as a mold inhibitor, with the addition of live yeast. The basic crosses (dysgenic crosses) involved males of the strain 160 (carrying approximately 53-54 *Penelope* copies) and females of wild-type or marker laboratory strains. As a control, reciprocal crosses and parental strains were used. For an estimation of initial fecundity (the number of all ovarioles in both ovaries), samples of 30 females from various strains and crosses were prepared. The counting included all phenotypes. To monitor gonadal atrophy at the level of individual ovarioles, we divided all flies into the following categories: "normal", when the gonad had less than a 25% reduction in size; "sterile", when the gonads were severely atrophied and apparently completely sterile; and "semi-normal", when the gonads were reduced in size by at least 25%. We also distinguished between bilateral and unilateral atrophy.

Gonad preparation and staining

The flies were dissected in physiological solution (0,9% NaCl) or PBS. For certain experiments, neutral red (0.1%) was added into the saline solution to determine the levels of cell death. The ovaries and testes were extracted under a 2 x 10 magnification and dissected with fine needles. To protect the preparations from dehydration, they were typically covered with a drop of neutral paraffin oil. For ovariole counting and investigation,

the preparations were used as "hanged-drops" and analyzed under the microscope under 20x, 40x, and 60x magnifications with phase contrast and 90x oil immersion. DAPI staining of the embryos was performed as previously described (Rothwell and Sullivan, 2007).

Whole mount hybridization with **Penelope** *and* **Ulysses** *probes*

DIG labeling and detection of the transposon DNA probes used in the *in situ* hybridization experiments were performed as previously described (Roche 2006; Rozhkov *et al*., 2011).

Ovaries of 5-7-day-old dysgenic and reciprocal females were dissected in PBS and fixed with a 4% paraformaldehyde/PBS solution for 20 min at RT. The treatment of ovaries with a 20 μg/ml proteinase K/PBS solution for 15 min was followed by fixation in a 4% paraformaldehyde/PBS solution for 20 min at RT. During these steps, PBS with 0.1% Tween-20 (PBT) was used as a rinsing solution. Pre-hybridization and hybridization steps were performed at 60°C in hybridization buffer (50% formamide, 5x SSC, 0.1% Tween 20, 1 mg/ml tRNA and 50 μg/ml heparin). The anti-DIG-AP antibody (Roche) was used at a 1:2000 dilution. The color typically developed within one hour.

Vasa- and 1B1-antibody staining

We utilized a previously described method (Styhler *et al*., 1998) with minor modifications. Briefly, the embryos of the *D. virilis* parental strains (9 and 160) and hybrids were collected on agarose plates for 24 hours, washed in H_2O and dechlorinated in 5% hypochloride. Then, the embryos were washed with H_2O and fixed in heptane saturated with 4% paraformaldehyde for 20 min on a rolling platform. The embryos were devitellinized by shaking in absolute methanol and stored at -20°C. The embryos and the fixed gonads dissected from the larvae and pupae were rehydrated in a methanol/PBS series, and, after PBS washes and preincubation in 1% BSA, incubated overnight with rabbit anti-Vasa (1:1000) and mouse anti-1B1(1:50). The washes were performed with PBS for 1-2 hours with at least three solution changes. The secondary antibodies were biotinylated goat anti-rabbit IgG (1:2000) and goat anti-mouse IgG (1:2000). The signal was amplified by incubation with preformed avidin-biotin complex according to the ABC method (Vector Laboratories). The signal was detected by DAB/hydrogen peroxidase reaction. The Vasa-antibodies were kind gift of Dr. S. Styler from P.Lasko laboratory (McGill University, Canada) and 1B1 antibodies (against hu-li-tai-shao protein) were generously provided by Dr. N. Tulina from Carnegie Institute of Washington.

Semiquantitative reverse transcription analysis (RT-PCR)

The analyses were performed using 1 μg of DNase I (Fermentas)-treated total RNA from different stage embryos. cDNA was prepared using the Strand cDNA Synthesis Kit and random hexamer primers (Fermentas). A volume of 2 μl of the 5-fold diluted cDNA was used in the 30 μl Taq-polymerase PCR mix (SibEnzyme) with 35 amplification cycles. All primers used in the investigation are given in Table S1.

Small RNA library development from early embryos and analysis of transposon-derived RNAs

The small RNA libraries from early embryos were cloned and analysed as described (Rozhkov *et al*., 2010, Rozhkov *et al*., 2011). Small RNAs were mapped to the latest releases of *D. virilis* genome. Transposon-derived small RNAs with up to three mismatches were mapped onto Repbase. For the transposon analysis, only small RNAs matching the bodies of selected five TEs were considered as the true TE-derived sequences. Small RNA counts were normalized to 1 million small RNAs after subtraction of small RNA reads corresponding to abundant noncoding RNAs.

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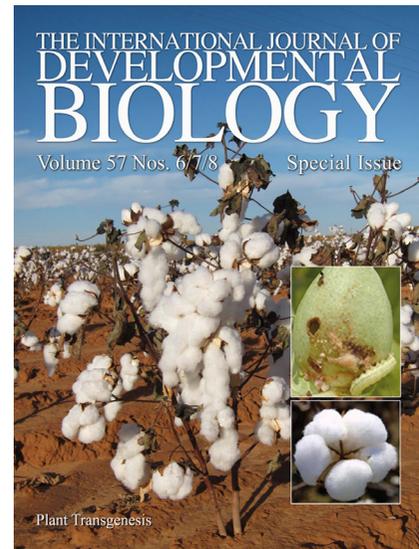
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