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Analysis of hybrid lethality in F_1 wheat-rye hybrid embryos

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Abstract The effect of the Embryo lethality mutant (*Eml*) of rye was studied in crosses between hexaploid wheat and corresponding inbred rye line (L2). Histological analysis of hybrid embryos revealed morphological differences 16 days after pollination. *Eml* was found to arrest the formation of shoot meristem but had no influence on root meristem formation. The effect of *Eml* cannot be overcome by in vitro embryo rescue via direct regeneration on Kruse medium. The possibility of complementary interactions between wheat and rye genes and of changes in gene expression through increased variation in dosage-regulated gene expression during hybrid formation is discussed.

Keywords Embryo rescue · Histological analysis · Hybrid lethality · Postzygotic barriers · Wheat-rye hybrid

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Introduction

The processes leading to postzygotic barriers in distant hybridizations are poorly known. The prevailing view is that postzygotic isolation is usually caused by extensive negative epistatic interactions of parental genomes in experimentally produced interspecific hybrids (Orr and Presgraves 2000; Burke and Arnold 2001). In flowering plants postzygotic barriers take effect after successful fertilization and have been revealed in different taxons both in intraspecific (Oka 1957; Hermsen 1963, 1967) and interspecific crosses (Hollingshead 1930; Gerstel 1954; Sawant 1956; Lee 1981). These barriers could arise from the first division of hybrid zygotes and during ontogenesis of F1 hybrids and later generations (Stebbins 1957; Rieseberg and Carney 1998). Besides hybrid embryo lethality, which results in seed abortion (Sears 1944; Lee 1981; Comai et al. 2000), distant hybridizations are often accompanied by seedling lethality, death at later developmental stages, and morphological abnormalities such as hybrid dwarfness or hybrid weakness (Hollingshead 1930; Oka 1957; Hermsen 1963, 1967; Takahashi et al. 1970; Shii et al. 1980; Worland and Law 1980). In these publications the description of embryo lethality during seed development relates only to seed viability and the stage at which embryo development was arrested. May and Appels (1984) reported that seedling lethality in *Triticum aestivum* × *Secale cereale* hybrids clearly results from the new combination of rye and wheat chromatin, so this

example could be attributed to hybrid lethality. Wheat plants disomic for a 2RS/2BL translocation chromosome substituting for chromosome 2B show seedling lethality. However, this kind of seedling lethality can be overcome if 2RS/2BL is introduced into a different genetic background (May and Appels 1980, 1984).

Hybrid lethality at the seedling stage has been most thoroughly studied in the genus *Nicotiana* (Gerstel et al. 1979; Inoue et al. 1996; Kobori and Marubashi 2004). The process is characterised by an apoptosis-like programmed cell death (Marubashi et al. 1999; Yamada et al. 2000; Masuda et al. 2003). In the crosses *Nicotiana suaveolens* × *N. tabacum* and *N. suaveolens* × *N. sylvestris* hybrid lethality could be overcome by in vitro culture of embryos on a medium containing cytokinin (Inoue et al. 1994, 1997, 2000). Based on chromosome-specific DNA markers Tezuka and Marubashi (2006) demonstrated that the chromosome Q of *N. tabacum* encodes hybrid lethality in interspecific crosses with *N. suaveolens*.

For cereals three general causes which can limit the viability and fertility of wide hybrids have been postulated: (i) hybrid sterility, (ii) death of a potentially viable embryo due to failure endosperm development, or (iii) selective chromosome elimination of the parental sets of chromosomes during embryo development (Baum et al. 1992). (i) Crosses between relatively closely related species such as *T. aestivum* x *S. cereale* usually lead to sterile F1 hybrids. The sterility can be overcome successfully by chromosome doubling (Larter et al. 1968; Kaltsikes 1974). (ii) In crosses between tetraploid wheat (mainly *T. durum*) and rye (Raina 1984) hybrid lethality is characterized by abnormal development of embryo and endosperm. Most seeds obtained in such crosses fail to germinate. The failure in endosperm development can be overcome by culturing on an artificial medium (Taira and Larter 1978; Raina 1984; Sirkka and Immonen 1993). In contrast to this, seeds from crosses between hexaploid wheat and rye have a high germination capacity (Müntzing 1979). Depending on the rye genotype, the frequency of non-germinating grains does not exceed 42% (Oettler 1983; Tikhenko et al. 2003). (iii) Selective chromosome elimination has been shown in crosses between hexaploid wheat with *Zea mays* (Laurie and Bennett, 1986, 1988a), *Pennisetum glaucum*, *Sorghum bicolor* (Laurie and Bennett, 1988b; Matzk and Mahn 1994; Gernand et al. 2005) or *Hordeum bulbosum* (Snape et al. 1979; Sitch and Snape 1987). Until now, selective chromosome elimination has not been observed in crosses between hexaploid wheat with rye.

After crossing 101 different self-fertile rye inbred lines from the Peterhof genetic collection (Smirnov and Sosnikhina 1984) with hexaploid wheat cultivar 'Chinese Spring' (CS) we found that the germination rate of seeds from most hybrid combinations ranged between 60% and 100%. However, four rye lines yielded only hybrid seeds that failed to germinate. Three of these four rye lines (L2, L3 and L564) are closely related, and one line (L535) has an independent origin. Seeds from crosses of eight common wheat accessions with rye line L2 had an abnormal embryo development. This trait was further analysed by segregation analysis using inter-line F_1 rye hybrids and two wheat accessions (Voylokov and Tikhenko 2002). Rye line L2 carries the mutation *Eml* (Embryo lethality), which terminates the development of the hybrid embryos in amphihaploids. Monogenic control of development of the hybrid embryo by the rye parent was confirmed by crossing CS with a set of rye recombinant inbred lines (Tikhenko et al. 2005). Embryos of non-germinating swelling hybrid seeds from these amphihaploids were not normally differentiated, whereas the endosperms from these seeds looked similar to those from control crosses. The degree of embryo development in hybrid seeds obtained by crossing hexaploid wheat varieties with rye inbred lines L2 and L535 showed that the proportions of undifferentiated embryos of various sizes was affected by parental genotypes (Tikhenko et al. 2005). After crossing CS with rye line L2 44.7% of the hybrid seeds contain a normal sized embryo without visible signs of tissue differentiation. This result suggested in vitro embryo rescue as the method to produce wheat-rye hybrids in this combination.

The aim of this study was to the test whether in vitro embryo rescue could be used to overcome hybrid lethality caused by the rye gene *Eml*. Histological analysis provides clear evidence that rye gene *Eml* arrests the development of shoot apical meristem (SAM) in hybrid embryos.

Materials and methods

Plant material

The wheat cultivar 'Chinese Spring' (CS) (*Triticum aestivum*, 2n = 6× = 42) was used as female parent.

The following self-fertile rye (*Secale cereale*, 2n = 2× = 14) lines were used as pollen donors: (1) line L2, carrying the mutant *Eml* gene; (2) control line L6 **,** with the wild type *eml* allele allowing the development of viable hybrid embryos; (3) inter-line F_1 hybrid L6 × L2. Rye lines L2 and L6 were supplied by the Peterhof genetic collection of the Plant Genetics Laboratory of the Biological Institute of St. Petersburg State University. Wheat spikes were emasculated 1–2 days before anthesis, and pollinated with fresh rye pollen.

In vitro embryo rescue

Embryos were excised 16 and 20 days after pollination (DAP) under a dissecting microscope. Caryopses were surface sterilized by washing in 70% ethanol for 1 min, and soaking for 15 min in 3.0% (v/v) sodium hypochlorite with two drops of Tween 80, followed by three rinses in sterile water for 15 min. Isolated embryos were classified according to the scale of Clark and Sheridan (1988), which includes six developmental stages: stage 1—proembryo, stage 2—lemon-like stage, stage 3—transition stage, stage 4—with first step of the differentiation (coleoptile stage), stage 5—embryonic axis and scutellum are present, stage 6—fully developed embryo with shoot and root meristems. Isolated embryos 16 and 20 DAP were classified visually and ranged from stage 2 to stage 6. Embryos at each stage were cultured scutellum side down for regeneration on Kruse medium (Kruse 1974) and incubated at 24°C in the dark. Regenerative capacity was determined after 20 days.

Histological analysis

Embryos of wheat × rye crosses were fixed with 2% glutaraldehyde and 2% formaldehyde in cacodylate buffer (50 mM, pH 7.0) for 16 h. After three 15 min washes with the same buffer, the embryos were post-fixed with 1% OsO4 for 6 h. The embryos were washed with buffer and distilled water and subsequently dehydrated in a graded ethanol series followed by embedding in Spurr's low viscosity resin. Longitudinal median sections of 1 µm were cut on a Reichert-Jung Ultracut S (Leica, Vienna, Austria) and stained with methylene blue. Digital images were made on a Zeiss Axiovert equipped with an Axiocam (Carl Zeiss, Jena, Germany).

Results

Regenerative capacity of immature embryos was analyzed in four crosses: CS × CS, CS × L6 (Table 1), CS × L2 and CS × F_1 (L6 × L2) (Table 2). Up to 98% of the 16 and 20 DAP old CS × CS embryos were in developmental stage 6. Regenerative capacity of these embryos after 20 days in vitro culture was almost 100% (Table 1). Embryos obtained from the control cross CS × L6 were morphologically similar, but had their regenerative capacity reduced to only 75% after 20 days in vitro culture (Table 1). CS × L2 hybrid embryos showed a delayed development with 34.4% and 64.8% of the 16 DAP old embryos showing features characteristic for developmental stages 2 and 3, respectively. At 20 DAP the embryos were at stages 2 (12.1%), 3 (50.7%) and 4 (37.2%). Even after cultivation on Kruse medium root formation was only sometimes observed and shoot development was completely absent (Table 2). Only hybrid embryos in stages 3 and 4 formed roots when placed on rescue medium. Of the embryos cultured 16 DAP 17.6% of those in stage 3 and 100% of those in stage 4 formed small roots (1–3 mm) before dying without shoot formation. For embryos cultured 20 DAP root formation was observed in 73.2% of the stage 3 and 80.0% of the stage 4. Embryos 16 DAP and 20 DAP occasionally developed primary roots 2.5–3 cm long without lateral roots (data not shown). Amphihaploid embryos 16 and 20 DAP derived from the cross CS × inter-line F_1 rye hybrid (L6 × L2) showed the expected 1:1 segregation behaviour. Half of the embryos ceased development at stages 2 and 3. The remainder continued to stages 5 and 6 and showed a high regenerative capacity from embryos cultured at 16 DAP (84%) and 20 DAP (72.7%) (Table 2). In control crosses, only a few embryos excised at 16 and 20 DAP were at stage 4. However, the comparative analysis of the regenerative capacity of stage 4 hybrid embryos, showed that direct regeneration was possible for embryos from cross CS × CS 16 DAP (33.3%), as well as from cross CS × L6 20 DAP (66.7%) but not for embryos from cross CS × L2 (Table 1, 2). Thus, the effect of rye gene *Eml* on the development of the hybrid embryos becomes evident 16 DAP and cannot be overcome by direct regeneration on Kruse medium.

Histological studies of median longitudinal sections of hybrid and control embryos revealed no

Table 1 Regenerative capacity of the hybrid embryos in control crosses (wheat CS × wheat CS; wheat CS × rye L6)

Male parent	Stage of embryonic development	Embryos dissected at 16 DAP				Embryos dissected at 20 DAP			
		Number of embryos		Regeneration capacity		Number of embryos		Regeneration capacity	
		Number	%	Number	%*	Number	%	Number	%*
Wheat CS	2	0	0	0	0	0	0	0	0
	3	1	1.0	0	0	0	0	0	0
	4	3	3.0	1	33.3	0	0	0	0
	5	99	96.0	97	98.0	1	2.0	1	100
	6	0	0	0	0	61	98.0	61	100
	Total	103	100	98	95.1**	62	100	62	100**
Rye L6	2	0	0	0	0	0	0	0	0
	3	2	1.5	0	0	6	8.3	0	0
	4	7	5.1	0	0	3	4.2	2	66.7
	5	127	93.4	106	83.5	14	19.4	8	57.1
	6	0	0	0	0	49	68.1	44	89.8
	Total	136	100	106	77.9**	72	100.0	54	75.0**

* The percentage was calculated as the ratio of the number of regenerated embryos to the number of plated embryos of the same stage

** The percentage was calculated as the ratio of the number of regenerated embryos at all stages to the number of plated embryos at all stages

Table 2 Regenerative capacity of the hybrid embryos in crosses wheat CS × rye L2; wheat CS × F1 (rye L6 × rye L2)

Male parent	Stage of embryonic development	Embryos dissected at 16 DAP				Embryos dissected at 20 DAP			
		Number of embryos		Regeneration capacity		Number of embryos		Regeneration capacity	
		Number	%	Number	%*	Number	%	Number	%*
Rye L2	2	42	34.4	0	0	26	12.1	0	0
	3	79	64.8	0	0	109	50.7	0	0
	4	1	0.8	0	0	80	37.2	0	0
	5	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0
	Total	122	100	0	0	215	100	0	0
F1 (rye L6 × rye L2)	2	40	8.0	0	0	25	10.2	0	0
	3	113	22.7	0	0	53	21.7	0	0
	4	109	21.8	17	16.0	45	18.5	3	6.7
	5	237	47.5	199	84.0	22	9.0	16	72.7
	6	0	0	0	0	99	40.6	98	99.0
	Total	499	100	216	43.3**	244	100	117	48.0**

* The percentage was calculated as the ratio of the number of regenerated embryos to the number of plated embryos of the same stage

** The percentage was calculated as the ratio of the number of regenerated embryos at all stages to the number of plated embryos at all stages

obvious morphological differences at 10 DAP (Fig. 1a–c). At this developmental stage first signs of differentiation were observed. The epidermis is formed and an internal cone-shaped group of cells marks the position of the apical meristem (Fig. 1a–c). The shoot apical meristem is visible as a dimple on the flattened face of the embryo (arrows in Fig. 1b, c). This primary meristematic region later forms the shoot-root axis of the mature embryo. Throughout stage 4 and 5 the scutellum greatly increases in size through cell division and cell enlargement. The vascular system differentiates simultaneously and links

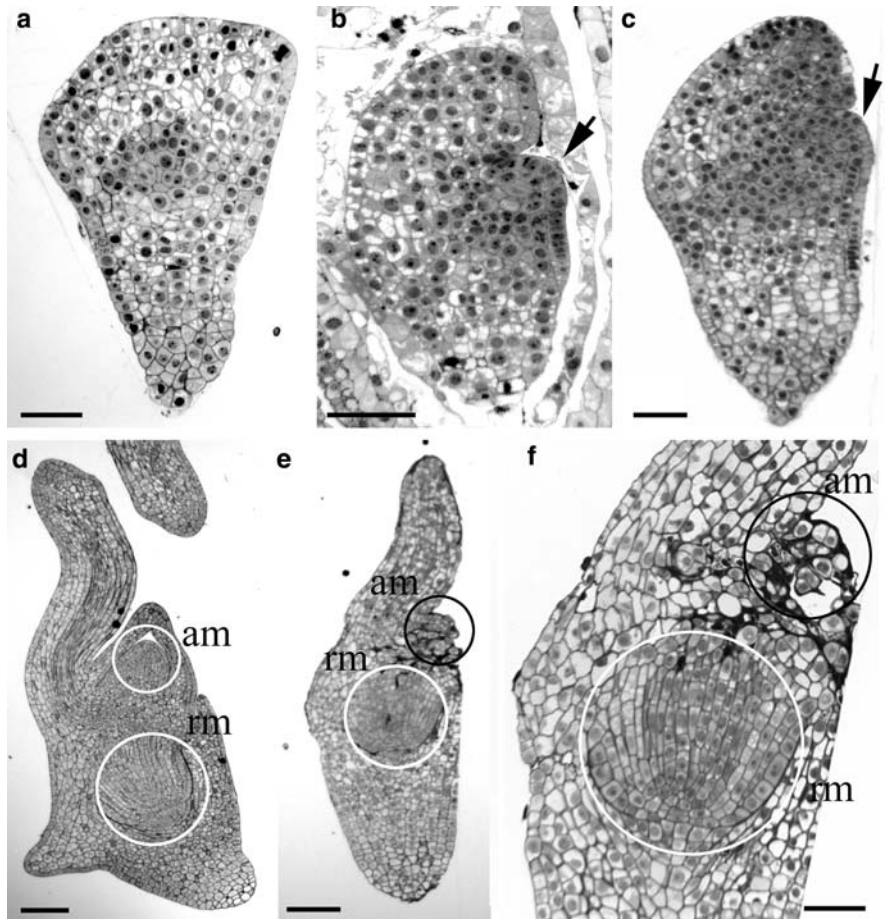

Fig. 1 Histological analysis of embryos 10 DAP (a-c) and 16 DAP (e-f). (**a**) wheat CS × L6, (**b**) wheat CS × wheat CS and (**c**) wheat CS × rye L2 embryos. The shoot apical meristem is visible as a dimple on the flattened face of the embryo (arrowed). (**d**) normal embryo of wheat CS × wheat CS. Embryonic axis constitutes a well-differentiated miniature plant with leaf primordia covering the face of the shoot apex (am) and a strand of vascular procambium extending into the scutellum. (**e**, **f**) abnormal wheat CS × rye L2 embryo. At the site of the apical shoot meristem (am) an area with degenerating cells can be seen. The scutellum is much smaller and without a clear vascular system. Note that the root meristems (rm) appear similar to those in the control lines. In 1a, b, c, f bars = 100 µm and in 1d, e bars = 200 µm

the embryonic axis with the scutellum. In the control, embryo morphogenesis ends 16 DAP. At this time the embryonic axis constitutes a well-differentiated miniature plant with leaf primordia covering the face of the shoot apex and a strand of vascular procambium extending into the scutellum (Fig. 1d). The location of the root apex is evident (Fig. 1d–f). Significant differences between control and CS × L2 hybrid embryos become obvious 16 DAP. At the location where the apical shoot meristem should be, an area with degenerating cells can be discerned in CS × L2 hybrid embryos. The scutellum is also much smaller and without a clear vascular system (Fig. 1e). Only the root meristem is similar to that of the control line embryos (Fig. 1e,f).

At 20 DAP the abnormal embryos had not significantly changed in size when compared to 16 DAP. They showed no signs of further morphogenesis. The root meristematic region was reduced and the suspensor and scutellum regions were less distinct than in earlier stages (data not shown).

Discussion

At the start of the present study, we proposed that the nature of the hybrid lethality in cross common wheat with inbred rye line L2 may resemble the abortion of the embryos in crosses *Triticum durum* with *Secale cereale* (Krowlow 1970). The major problem with these crosses is poor endosperm development: embryos abort early in their development. Recently effective methods for in vitro culture for immature hybrid embryos were developed (Taira and Larter 1978; Raina 1984; Sirkka and Immonen 1993) We applied the embryo rescue method which had shown good results for overcoming the lethality of abnormal embryos for many wide crosses in the *Poaceae*

(Matzk and Mahn 1994). Using Kruse medium we tried to prevent callus formation, which results in a chromosome number mosaicism in both shoot and root apical meristem (Shao and Taira 1990). One of the main factors influencing development of wheat-rye hybrid embryos in vitro is the time for rescuing. Taira and Larter (1978) shown that wheat-rye hybrid embryos respond best to Norstog's modified medium if they are allowed to develop 12–14 days in vivo. Raina (1984) followed these suggestions but realized that 12–14 day old embryos were too young and found that best seedling development was obtained with 20–22 day old embryos. In contrast to this, Sirkka and Immonen (1993) found that embryo rescue 15–17 day after pollination was optimal both for callus and embryo culture of primary triticale. Based on these results we used two time points for rescuing wheat-rye hybrid embryos—16 and 20 DAP. In our experiment, 77.9 – 95.1% of embryos 16 DAP and 75 – 100% (Table 1) of embryos 20 DAP from control crosses CS × CS and CS × L6 formed shoots on the fourth day after start of cultivation on rescue medium. In contrast, none of the abnormal embryos from cross CS × L2 formed shoots even after 20 days of cultivation on Kruse medium (Table 2). Some of these (17.6 –80%) formed a short primary roots 1–3 mm long before dying without shoot formation. In our case, the intraspecific pollination of wheat and self-fertile rye L2 results in normal caryopsis development. However, histological analyses showed that after interspecific crosses between wheat CS with rye L2, which carries the *Eml* mutation, all hybrid embryos have differentiated scutellum and root meristems but no shoot apical meristem and no coleoptile (Fig.1e, f). In control crosses of CS with rye L6, which carries wild type allele of *Eml*, all hybrid embryos have a differentiated scutellum and normal meristem development (Fig.1d). The histological analysis suggests that younger hybrid embryos from cross CS × L2 may also be cultivated on Kruse medium.

The phenotype of embryo lethality caused by the rye *Eml* gene is similar to, segregating mutations of maize and rice (Clark 1996; review Itoh et al. 2006). It also resemble the maize mutants *dek1* and *dek23* which have an atypical development of endosperm and embryo, and the maize mutant *emb12* in which only embryo development is altered (Sheridan and Neuffer 1981). The *dek1* mutant embryos possess a root but no shoot meristem while embryos of *dek23* reach the coleoptilar stage with root meristem and elongated scutellum but never develop a shoot apex. Clark and Sheridan (1986) proposed that the failure of normal function of the *dek23* locus results in a loss of vitality of the cells at that central site. This locus may control the synthesis of a substance in this region that is essential for its differentiation into a shoot apex. Embryos of *emb12* maize mutants, which are blocked primarily during morphogenesis and early maturation, have a distorted embryonic axis and an inverted shoot apical meristem similar to that of *dek23*. A lesion in *ZmPRPL35-1*, probably coding for protein L35 of the large subunit of plastid ribosomes, is responsible for the phenotypic changes observed in *emb12* maize mutants (Magnard et al. 2004). In another case, Pilu et al. (2002) have shown that mutations in two independent genes in maize, *SML* and *DGR*, lead to suppression of the shoot apical meristem (SAM) development during embryogenesis and produce a similar phenotype to *shl* in rice and *Eml* in wheat-rye hybrid. This indicates that the observed defect in wheat-rye hybrid embryos could be connected with meristem development abnormalities. But it is unclear which gene(s) controls SAM development in wheat-rye hybrid embryos. Also in rice, a group of mutants with abnormal SAM development during embryogenesis has been reported (see review Itoh et al. 2006). They carried several shootless mutations and so far four *shootless* loci have been identified (Satoh et al. 1999). Thus, *SHOOTLESS* genes are thought to be directly involved in the formation of the SAM.

Therefore, the abnormal embryo phenotype is a result of the wide hybridization of the rye line L2 with common wheat. Furthermore, the monogenic control of embryo lethality has been confirmed by crossing CS with a set of rye recombinant inbred lines (RIL). Gene *Eml* has been located on rye chromosome 6R applying molecular markers (Tikhenko et al. 2006).

Sears (1940) was the first to report on monofactorially conditioned inviability of an intergeneric hybrid in the *Triticinae*. He identified two alleles in *Triticum monococcum* which act as dominant lethals in hybrids with *Aegilops umbellulata*, but which are without effect in *T. monococcum* itself. The alleles differ in the time, i.e. the developmental stage, at which they cause death. A third, normal allele is present in the closely related *T. aegilopoides*. Multiple hybrids combining the normal allele and one of the inviability factors with the *Ae. umbellulata* genome were viable

(Sears 1944). In his work, Sears suggested two mechanisms by which a factor might cause inviability of a hybrid: (1) by failure to produce some substance essential to the proper development of the hybrid plant, or (2) by some positive action antagonistic to the foreign genome. The viability of the multiple hybrids (*T. monococcum* × *Ae. uniaristata* 4n) × *Ae. umbellulata* and (*T. aegilopoides* x *Ae. uniaristata* 4n) × (*T. monococcum* × *Ae. uniaristata* 4n) prevents the antagonism between an inviability gene and the *Ae. umbellulata* genome. This antagonism might be expected to cause lethality regardless of what additional genomes were present. The absence of an antagonistic action suggests that hybrid death is due to a deficiency of some essential substance. Recent evidence has emerged that the combination of two unrelated genomes may give rise to novel or suppressed gene expression. In general, polyploid species often display new traits and genetic variability (Levin 1983; Feldman et al. 1997; Comai et al. 2000; Liu et al. 1998a, b; Ma et al. 2004; Rapp and Wendel 2005).

Two models could explain the regulatory mechanisms in newly formed hybrids. The first model proposed by Dobzhansky (1937) suggests the existence of complementary genes that determine lethality or sterility in distant hybrids. Each gene has at least two different alleles: 'normal' (non-complementing) and 'abnormal' (as *Eml* rye gene; complementing). Altered phenotypes only occur with a combination of 'abnormal' alleles of both complementary genes in F_1 hybrids. 'Abnormal' alleles in either of the two genes may be fixed in different taxons or forms of autogamous species, or be present in all possible combinations in plants of allogamous species. In the latter case, segregation of abnormal phenotypes in a population may be observed. According to this model, any abnormal (novel) trait revealed in the wide hybrids is the result of interaction of at least two complementary genes.

The second model is based on the changes in gene expression through increased variation in dosage-regulated gene expression, altered regulatory interactions, and rapid genetic and epigenetic changes (Sears 1944, for review Osborn et al. 2003). More studies are necessary to obtain further insights into embryo lethality and a better understanding of genome interaction in polyploids. Consequently, *Eml* appears to be a valuable mutation for investigating the organization of the shoot meristem in monocots as well as a means to study the relationships between wheat and rye genes involved in apical meristem development during embryogenesis.

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