BIOLOGIA PLANTARUM 61 (2): 342-348, 2017 DOI: 10.1007/s10535-016-0691-6

Embryo lethality in wheat-rye hybrids: dosage effect and deletion bin mapping of the responsible wheat locus

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Abstract

The speciation allele at *Eml-A1* of hexaploid wheat, which causes embryo lethality in wheat-rye hybrids, was investigated using cytologically modified genetic stocks. It was demonstrated that an extra dose of this allele had no effect on embryo development in these hybrids. There was no positive effect on embryo development and, therefore, no overcoming of the postzygotic barrier. An abortion of the hybrid embryos at an earlier stage of development was also not observed. Physical mapping was performed using chromosome 6A deletion lines. This study revealed the location of *Eml-A1* on the most distal part of the long arm of chromosome 6A. To identify possible candidate genes responsible for embryo lethality, *in silico* sequence homology analysis was performed. Two candidate genes for *Eml-A1* that are involved in shoot apical meristem maintenance were identified on chromosome 6AL. However, functional validation assays need to be designed and performed.

Additional key words: interspecific hybrids, physical mapping, postzygotic incompatibility, *Secale cereale*, shoot apical meristem, *Triticum aestivum*.

Introduction

The process of speciation relies on the restriction of gene flow between populations. One mechanism of specification is postzygotic reproductive isolation which, after successful fertilization, leads to the elimination of hybrids. The Bateson-Dobzhansky-Muller model explains the incompatibilities between parental genotypes on the basis of complementing or the synergistic interaction of genes, which can be involved in the same pathways (Dobzhansky 1937, Müller 1942, Bomblies and Weigel 2007). This interaction could provide an explanation for divergent evolution occurring among paralogues of an essential duplicated gene, for which the functional copy can be located at different loci in different accessions or species (Lynch and Conery 2000, Bikard *et al.* 2009).

In distant hybridisation, reproductive barriers between the parents may be based on the existence of two or more complementary homoeologous genes, each having at least two different alleles: compatible, non-complementing and incompatible, complementing. The combination of incompatible alleles of both complementary genes leads to an abnormal phenotype in intra- or inter-specific F_1 or F_2 hybrids. Incompatible alleles in either of the two genes may be fixed in different taxa or forms of autogamous species, or be present in all possible combinations in plants of allogamous species. In the latter, segregation studies can be performed. According to this model, any abnormal (novel) trait in a wide hybridisation F_1 may be the result of complementary interaction of at least two parental genes.

Beside hybrid dwarfness described recently (Tikhenko *et al.* 2015), embryo lethality is a very common event in wide crosses. Structural changes in the cells of hybrid embryos have been studied in detail for interspecific crosses in *Nicotiana* (Kostoff 1930, McCray 1932), *Datura* (McLean 1946) and in intergeneric hybridisation between *Triticum monococcum* and *Aegilops umbellulata* (Sears 1944). In addition, when tetraploid wheat is crossed with rye, only a few seeds develop normally whereas the vast majority show early

Submitted 18 April 2016, *last revision* 19 July 2016, *accepted* 15 August 2016.
Abbreviations: CSDT6AS - Chinese Spring ditelosomic 6AS; CSDT6AL - Chinese Spring ditelosomic 6AL; CSN6D/T6A - Chinese Spring nullisomic 6D/tetrasomic 6A; CSm-t6BS/T6A - CS mono-telosomic 6BS/tetrasomic 6A; CSS - chromosome survey sequence; CST6A - Chinese Spring tetrasomic 6A; DAP - days after pollination; DT - ditelosomic; Eml - embryo lethality; L - inbred line; SAM - shoot apical meristem.
Acknowledgements: We thank Corinna Trautewig for excellent technical support. This study was funded by the German Research Foundation (No. BO 1423/17-1/603175). The first two authors contributed equally to this work
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stage endosperm abortion (Raina 1984). These problems can be overcome by embryo rescue (Shao and Taira 1990). In contrast, when hexaploid bread wheat is crossed with rye, the vast majority of seeds develop normally (Müntzing 1979). The percentage of non-germinating seeds was found to depend on the genotype of the rye parent but never exceeded 42 % (Oettler 1983, Tikhenko *et al.* 2003).

Previously by crossing 101 inbred lines from the rye genetic collection of St. Petersburg State University, Russia, with wheat cv. Chinese Spring, we identified four lines that gave abnormal seeds only (Tikhenko *et al.* 2005). The seeds contained a normal endosperm but displayed a premature abortion of embryo development, and sometimes even developed no embryo at all. Histological analysis revealed that, although abnormal embryos contained apparently normal radicles, they lacked a shoot apical meristem (SAM), coleoptile and epiblast. The differentiation of these organs may therefore be linked with the development of the SAM. This abnormality was named embryo lethality (*Eml*). Classical segregation analysis (Voylokov and Tikhenko 2002) of crosses between common wheat with certain rye inbred lines showed that *Eml* is the result of an interaction of two incompatible alleles: dominant rye *Eml-R1b* located on the 6RL chromosome (Tikhenko *et al.* 2011) and wheat *Eml-A1*, located on the 6A chromosome (Tikhenko *et al.* 2010). So far, it has not been possible to assign a dominant or recessive status to the wheat incompatible *Eml-A1* allele (Tikhenko *et al.* 2005).

In the present study, we determined the dosage effect of the *Eml-A1* gene of common wheat on embryo development in wheat-rye crosses and performed deletion mapping of this gene on the 6A wheat chromosome. In addition, sequence analysis of the region carrying the *Eml-A1* gene was performed. Such work may help to identify genes involved in postzygotic barrier mechanisms and the regulation of apical meristem formation during embryo development in monocots.

Materials and methods

Plants: For dosage effect studies hexaploid wheat (*Triticum aestivum* L.) cv. Chinese Spring (CS) and three lines carrying a double dose of chromosome 6A: tetrasomic line 6A (2n = 44; CST6A), CS nullisomic 6D - tetrasomic 6A (CSN6D/T6A), and CS mono-telosomic 6BS/tetrasomic 6A (CSm-t6BS/T6A), supplied by the John Innes Centre, Norwich, UK, were crossed with rye (*Secale cereale* L.) line L2, carrying the *Eml-R1b* allele (incompatible with wheat genome) and rye lines L6 and L7, carrying the *Eml-R1a* allele (control). Rye lines were kindly provided by Dr. A. Voylokov from the rye collection of the St. Petersburg State University, Russia.

For physical mapping *Eml-A1* gene, two ditelosomic lines (CSDT6AS and CSDT6AL) and seven deletion lines for chromosome 6A were used. These wheat lines were kindly provided by Dr. W. Jon Raupp, Wheat Genetic and Genomic Resources Center, Kansas State University, Manhattan, USA. Plant material included three partial deletion lines for chromosome 6AS and four partial deletion lines for 6AL. All lines were crossed with rye lines L6, L7, and L2. Hybrid caryopses were rescued 16 d after pollination (DAP) and embryos classified as normal (completely differentiated, Fig. 1*A*) or abnormal (without apical meristem, undifferentiated or without embryos, Fig. 1*B*).

Histological analysis: Embryos of wheat-rye crosses were fixed with 2 % (m/v) glutaraldehyde and 2 % (m/v) formaldehyde in phosphate buffer (50 mM, pH 7.0) at 8 °C for 16 h. After three 15 min washes with buffer (1×) and distilled water (2×), plant materials were dehydrated in a graded ethanol series followed by embedding in Spurr's low viscosity resin. Longitudinal median sections of 2 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).

Scanning electron microscopy: After overnight fixation with 4 % formaldehyde in 50 mM phosphate buffer (pH 7.0), isolated wheat-rye embryos were washed with buffer and dehydrated in an ethanol series. Samples were critical point dried in a *Bal-Tec* critical point dryer (*Bal-Tec AG*, Balzers, Switzerland), attached onto aluminium sample blocks and gold coated in an *Edwards S150B* sputter coater (*Edwards High Vacuum*, Crawley, UK). Shoot apices were examined with a *Hitachi S4100* scanning electron microscope (*Hisco Europe*, Ratingen, Germany) at 5 kV acceleration voltage.

Detection of possible candidate genes using an *in silico* sequence homology search: *In silico* sequence homology analysis was performed between the wheat chromosome 6AL survey sequence; CSS (chromosome survey sequence) contigs (Consortium, T.I.W.G.S. 2014) and a set of candidate genes. Published reports were surveyed for genes that might regulate a similar trait in other species, *i.e.*, maize, rice or *Arabidopsis thaliana* (Table S1). Corresponding gene sequences were obtained from the *EnsemblPlants* database (http://plants.ensembl.org/index.html) or the available genome database of the respective species. All *BLASTN* sequence homology searches between CSSs and candidate gene sequences were performed at a threshold of E-value ≤ 1E-10 using the *blastall* command at a *linux* platform. Outputs were parsed using an in-house *Perl* script under the parameters of identity ≥ 75 % and alignment length ≥ 50 bp.

Results

Abnormal embryos (Fig. 1) without shoot apical meristems were found in crosses of rye line L2 with wheat cv. Chinese Spring and all nullisomic-tetrasomic lines except N6AT6B and N6AT6D (Tikhenko *et al.* 2005, 2010). This abnormal development was the result of an interaction between incompatible alleles from each parent. Since common wheat is a natural amphidiploid, the effect of additional doses of *Eml-A1* alleles on embryo development in hybrid embryos can be studied. To study a possible dosage effect of *Eml-A1*, three wheat lines (CST6A, CSN6D/T6A, and CSm-t6BS/T6A) carrying a double dose of this allele were crossed with rye lines L2, L6, and L7. Analysis of hybrid embryos revealed that crosses between wheat lines carrying double dosage of chromosome 6A with rye inbred lines L6 or L7 (*Eml-R1a*) resulted in 85.7 - 100.0 % normal embryo formation (Table 1, Fig. 2*A*,*B*,*C*). When crossed with rye line L2 (*Eml-1Rb*) hybrid seeds developed invariably abnormal embryos (Fig. 2*D*,*E*,*F*). This shows that double dosage of the *Eml-A1* wheat allele cannot overcome the postzygotic barrier imposed by a single *Eml-R1b* rye allele.

Two ditelosomic (DT) and seven homozygous deletion lines of wheat for chromosome 6A, generated in the cv. Chinese Spring, were tested in crosses with inbred rye lines to confirm the presence of *Eml-A1* causing embryo lethality in wheat-rye hybrids. The crosses between DT lines CSDT 6AS and CSDT 6AL with rye line L2 gave opposite results. When CSDT 6AS was crossed with rye L2, 88.6 % of the seeds contained normal embryos, which was almost identical to the 92.6 % of seeds with normal embryos obtained when CSDT6AS was crossed with rye L6 (Table 2). In contrast, crosses of CSDT6AL with rye L2 always resulted in abnormal embryo formation. Of 172 tested hybrid seeds, 150 had abnormal embryos and 22 were without embryos altogether. When CSDT6AL was crossed with rye L6, 84.1 % of the caryopses contained normal embryos (Table 2).

Crosses between the three deletion lines CS del 6AS-1, CS del 6AS-3, and CS del 6AS-5, which contained a full size long arm of chromosome 6A, with rye line L2 produced caryopses with abnormal embryos, or without embryos just as was observed after crossing CSDT 6AL × L2. This result confirms that the wheat incompatible *Eml-A1* locus is located on the long arm of chromosome 6A.

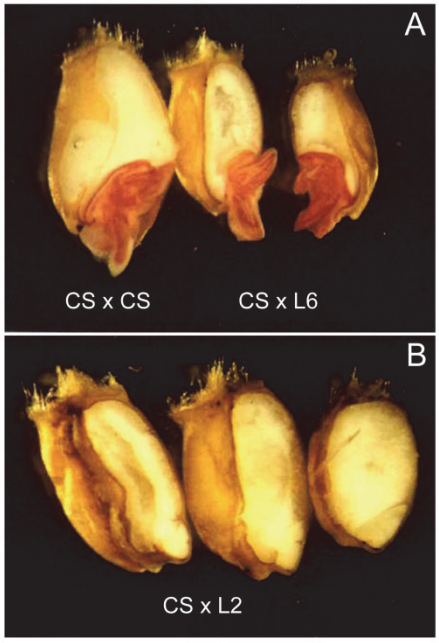

Fig. 1. Embryo development in crosses of wheat cv. Chinese Spring with rye lines after tetrazolium staining: seeds have normal differentiated embryos in crosses (CS × CS and CS × L6) (*A*); seeds from CS × L2 cross have abnormal embryos (*B*).

Table 1. Effect of incompatible *Eml-A1* allele (gene) on embryo development in crosses of wheat lines carrying double dosage of 6A chromosomes with rye inbred lines L2 (*Eml-R1b*), L6 and L7 (*Eml-R1a*).

Parental wheat lines		Parental rye lines	Number of tested seeds	Number of seeds with embryos normal	abnormal	Seeds with normal embryos [%]
1	CST6A	L2	263	0	263	0.0
		L6	14	14	0	100.0
		L7	105	102	3	97.1
2	CS N6D/T6A	L2	199	0	199	0.0
		L6	33	33	0	100.0
		L7	21	18	3	85.7
3	CSmt6BS/T6A	L2	245	0	245	0.0
		L6	16	15	1	93.8
		L7	122	112	10	91.8
4	CS (euploid)	L2	81	0	81	0.0
		L6	21	21	0	100.0
		L7	97	93	4	95.9

Table 2. Development of the hybrid embryos in crosses of Chinese Spring ditelosomic and deletion lines with rye inbred lines L2 (*Eml-R1b*), L6, and L7 (*Eml-R1a*).

Parental wheat lines			Parental line	Number of tested seeds	Number of seeds with embryo		Seeds with normal embryos [%]
					normal	abnormal	
6AS	1	CSDT 6AS	L2	70	62	8	88.6
			L6	27	25	2	92.6
	2	CS del 6AS-1	L2	22	0	22	0.0
			L6	24	21	3	87.5
	3	CS del 6AS-3	L2	35	0	35	0.0
			L6	18	17	1	94.4
	4	CS del 6AS-5	L2	17	0	17	0.0
			L6	16	16	0	100.0
6AL	5	CSDT 6AL	L2	172	0	172	0.0
			L6	44	37	7	84.1
	6	CS del 6AL-1	L2	105	85	20	81.0
			L6	55	52	3	94.5
			L7	9	9	0	100.0
	7	CS del 6AL-4	L2	103	90	13	87.4
			L6	38	37	1	97.4
	8	CS del 6AL-5	L2	201	175	26	87.1
			L6	54	50	4	92.6
			L7	12	9	3	75.0
	9	CS del 6AL-8	L2	140	109	31	77.9
			L6	51	47	4	92.2
	10	CS (euploid)	L2	81	0	81	0.0
			L6	21	21	0	100.0

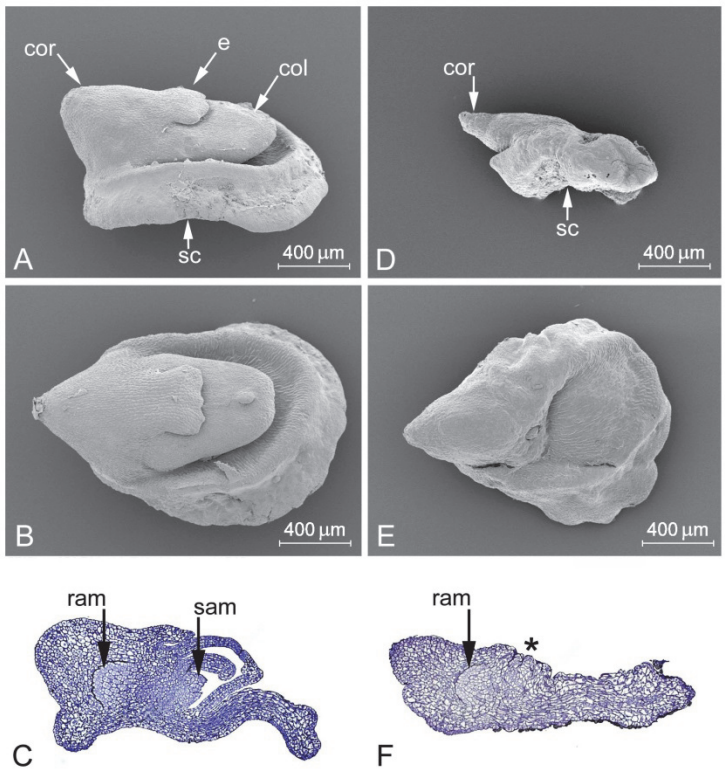

Fig. 2. Scanning electron micrographs and histology of wheat × rye hybrid embryos at 14 DAP. Crosses between wheat line CSm-t6BS/T6A with rye line L6 result in normal embryos with well-developed shoot and root meristems (*A* - *C*). Crosses between wheat line CSm-t6BS/T6A with rye inbred line L2 (*Eml-1Rb*) always result in abnormal embryo development characterized by the absence of a shoot apical meristem (*arrows* in *D* - *F*). col - coleoptile, cor - coleorhiza, e - epiblast, ram - root apical meristem, sam - shoot apical meristem, sc - scutellum.

Four deletion lines, missing the long arm of chromosome 6A from the centromere to position 0.9, were also crossed with rye L2. The hybrid seeds of these crosses yielded 75 - 100 % normal embryos. This suggests that the incompatible wheat allele, which is complementing *Eml-1Rb*, is located on the distal part of the long arm of chromosome 6AL in a position between 0.90 - 1.00. This part of chromosome 6A contains three DNA markers (*Xgwm427*, *Xgwm570* and *Xgwm617*) (Fig. 3) which served as reference points for subsequent analysis *via* sequence homology searches. Information about these SSR markers was obtained from *Genoplante* database (http://wheat.pw.usda.gov/ggpages/SSRclub/GeneticPhysical).

In a bid to identify the *Eml* locus, we used an *in silico* mapping approach *via* sequence homology searches. These methods were applied to check whether putative candidate genes can be identified on chromosome 6AL. The *in silico* approach was first performed with 189 putative candidate genes from *Arabidopsis thaliana* (Leibfried *et al.* 2005). All of these genes have been described as being differentially regulated in *Arabidopsis* plants upon ethanol-induced overexpression of the *WUSCHEL* gene, a positive regulator of stem cell fate in the SAM. Additionally, another set of genes (Table 1 Suppl.) involved in SAM development in maize, rice, and *Arabidopsis thaliana* was included in the analysis. Of all the sequences tested, only two showed significant homology to the 6AL CSS information (identity ≥ 75 % and alignment length ≥ 50 bp). The first was *ADAXIALIZED LEAF1*, which regulates leaf and embryonic pattern formation in rice (Hibara *et al.* 2009) and is the orthologue of *DEFECTIVE KERNEL1* (DEK1) in maize and *Arabidopsis* (Johnson *et al.* 2005, Takada and Iida 2014). The second gene was a protodermal miR394 involved in stem cell competence in the SAM of *Arabidopsis* (Knauer *et al.* 2013). Further experiments need to be performed to validate if any of the two genes is a true candidate gene for the development of SAM.

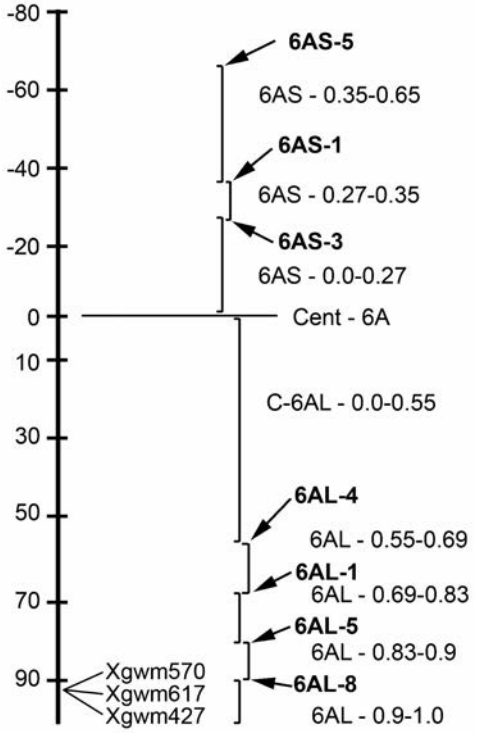

Fig. 3. Design of chromosome 6A of Chinese Spring with bins of tested deletion lines and intervals.

Discussion

Nullisomic-tetrasomic compensation lines of cv. Chinese Spring (NTCS), established by Sears (1966), possess four doses of a particular chromosome while missing a pair of another chromosome of the same homoeologous group. By compensating duplication/deficiencies of specific chromosomes, the balance of the regulation of different traits may be shifted in hexaploid wheat. Considerable phenotypic effects on the expression of the individual genes was found in the NTCS lines themselves (Goncharov and Watanabe 2005) and in hybrids between NTCS and rye (Lelley 1976, Tikhenko *et al.* 2010). The incompatible wheat *Eml-A1* gene, complementing to the *Eml-R1b* allele, is located on chromosome 6A (Tikhenko *et al.* 2010). The remaining two homoeologous chromosomes 6B and 6D probably lost this gene or carry an inactive *Eml* allele. When wheat CS lines that have 6B or 6D in a nullisomic state are crossed with rye L2, all hybrid embryos develop without a SAM. In the present paper, we tested whether the negative effects on embryo development exerted by the *Eml-R1b* allele might be the result of a gene dosage effect. However, duplication of the *Eml-A1* wheat allele (gene) was found to have no positive effect on embryo development in incompatible crosses and did not overcome this postzygotic barrier.

According to genetic analysis, an F_2 population or sets of doubled haploids (DH) or recombinant inbred lines (RILs) should be produced for mapping the *Eml-A1* wheat gene. Thereby, crosses between hexaploid wheat lines and the rye line L2, carrying the incompatible *Eml-R1b* allele, have failed to identify a compatible gene in the wheat genome (Tikhenko *et al.* 2005). Hybrid seeds produced in these crosses typically contained abnormal embryos. So far we have been unable to detect and accession of common wheat with compatible allele to *Eml-R1b* which could be used for the development of a mapping population.

Deletion stocks of common wheat give a unique opportunity for genetic analysis in amphidiploids and have been used extensively in mapping different loci (Endo and Mukai 1988, Endo *et al.* 1991, Sutka *et al.* 1999). We used two DT CS lines and a set of partial deletion lines of wheat chromosome 6A for crosses with

rye line L2 (Table 2). The incompatible *Eml-A1* wheat allele, which complements the *Eml-1Rb* allele, is located on the distal part of the long arm of chromosome 6AL from 0.90 - 1.00 (Fig. 3).

In certain crosses between common wheat and rye, abortion of SAM during the differentiation of the hybrid embryo leads to embryo lethality. Development of the hybrid embryos with lethal genotypes progresses until the transition stage (Tikhenko *et al.* 2008). There are no visible differences between embryos from incompatible (CS × L2) and compatible (CS × L6) crosses until 10 DAP. The shoot apical meristem is visible as a dimple on the flattened face of the embryo (Tikhenko *et al.* 2008). This primary meristematic region later forms the shoot-root axis of the embryo. Following this stage, differences become apparent. Since the root meristem is not affected (Fig. 2), degradation of the SAM apparently starts after the shoot-root axis has been formed. This suggests that the interaction between the incompatible wheat and rye alleles leads to the malfunction of genes involved in maintaining the SAM. To identify candidate genes, we focused on known genes in both *Arabidopsis* and monocots (maize and rice) that are involved in SAM maintenance, such as the *KNOX* and *WUS* genes, specific and independent of meristem regulators, and other genes or gene interactions that lead to embryo lethality. From these genes, only two showed significant homology to the 6AL CSS information (identity ≥ 75 % and alignment length ≥ 50 bp), and thus were mapped to wheat chromosome 6AL. These included *ADAXIALIZED LEAF1* (*ADL1*), which regulates leaf and embryonic pattern formation in rice (Hibara *et al.* 2009), and a protodermal miR394 involved in stem cell competence in the SAM of *Arabidopsis* (Knauer *et al.* 2013). However, which of the two genes is *Eml-A1* remains to be clarified; furthermore, relevant functional validation assays for the true candidate need to be designed and performed.

T. aestivum is a natural amphidiploid in which three semi-independent genomes are transformed into one. The ancestral function of the duplicated genes is split between the original copies of the A, B and D genomes. A mutation in one of the original copies can alter the interaction with incompatible alleles, like *Eml-R1b*, without having a negative effect on the wheat genetic background alone. Incompatibility between the wheat genome and the *Eml-R1b* allele of rye can also be the result of a single gene duplication event causing a dispersed duplicated pair, as shown in *A. thaliana* (Bikard *et al.* 2009) and *O. sativa* (Chen *et al.* 2008, Mizuta *et al.* 2010, Yamagata *et al.* 2010, Ouyang and Zhang 2013). The interaction between two parental incompatible genes is in most cases unknown. However, it is highly likely that the *Eml* loci in common wheat and rye are not only involved in reproductive barriers but also play a role in SAM maintenance. The genome location of the wheat and rye *Eml* loci indicates that embryo lethality in wheat-rye hybrids is the result of an interaction between wheat and rye homoeo-alleles. Furthermore, our results show that the search for the gene underlying the *Eml* locus has to be extended to genes involved in SAM maintenance.

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