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D. Lefebvre · P. Devaux

Doubled haploids of wheat from wheat × maize crosses: genotypic influence, fertility and inheritance of the 1BL-1RS chromosome

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Abstract The wheat × maize cross as a technique for haploid induction in wheat was evaluated in a replicated block design comprising 18 wheat F_1 hybrids and five *Zea mays* L. parents. Haploid plants were regenerated at an average of 9.1 (4.4–14.7) plants per 100 florets processed. Genotypic differences for haploid production efficiency were recorded for both wheat and *Zea mays* L. Interaction between parents was significant for number of plants/100 florets. All 610 of the 1,703 regenerated plantlets that were analyzed by flow cytometry were haploid. At maturity, 70% (60–81%) of the colchicine-treated haploid plants were fertile, but the frequency of fertile and sterile plants was not consistent over the wheat hybrids from which they were derived. Flow cytometry performed using the first tiller which arose following colchicine treatment enabled prediction of fertility. The 1BL-1RS chromosome was found at the expected ratios in the F_2 and in the haploid progenies produced through the wheat × maize cross but deviated from the 1:1 ratio in the haploid progenies produced by anther culture.

Key words Wheat · Wheat × maize cross · Haploid · Doubled haploid · Distortion of segregation

Introduction

Unlike barley, very few wheat cultivars have been released as doubled haploids (Devaux 1992). Cultivar release is suitable to assess the efficiency of haploid production in self-pollinated crop species such as barley and wheat, for which techniques have been available for many years. Despite intensive efforts to increase anther culture response (reviewed by Henry and de Buyser 1990), its use has remained marginal in wheat breeding programs. The major limitation to a broad exploitation of anther culture has been its genotypic dependency (Lazar et al. 1984; Marsolais et al. 1984; Foroughi-Wehr and Zeller 1990). Crosses involving an anther culture-responsive parent such as a 1BL-1RS translocated line (Henry and de Buyser 1985) enabled the indirect recovery of haploid plants from recalcitrant genotypes. However, regenerated plants were skewed in the direction of the translocated parental type by the selective development of microspores or the derived embryoids (Agache et al. 1989; Devaux et al. 1990).

Wide crosses followed by elimination of the genome of one parent have been an alternate method for inducing haploid zygotic embryos and subsequent plants. The production of haploid plants from crosses between wheat and maize was first reported by Laurie and Bennett (1988). Refinements of the technique (Suenaga and Nakajima 1989; Laurie et al. 1990; Comeau et al. 1992) enabled haploid plants to be produced from many commercial wheat cultivars (Laurie and Reymondie 1991; Riera-Lizarazu et al. 1992) and hybrids with the aim of obtaining homozygous recombinant lines resistant to Russian wheat aphid (Kisana et al. 1993).

The objectives of the study presented here were (1) to assess doubled haploid (DH) production efficiency through wheat × maize or teosinte crosses over a range of wheat F_1 hybrids; this implies the regeneration of haploid plantlets and successful chromosome doubling to restore fertile DH plants; (2) to select superior maize genotypes that could increase haploid production efficiency; and (3) to compare the inheritance 1BL-1RS chromosome in haploids obtained through the maize method (MM) and by anther culture (AC) and hence to assess whether random gamete sampling takes place.

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D. Lefebvre · P. Devaux(✉)
Florimond Desprez, Laboratoire de Biotechnologie, B.P. 41, F-59242 Cappelle en Pévèle, France

Material and methods

Plant material

The 18 F_1 hybrids of winter wheat (*Triticum aestivum* L.) used in this study were numbered for later reference as follows: (1) 'Rossini'/FD91147, (2) 'Texel'/FD91091, (3) 'Trémie'/'Avital', (4) 'Sidéral'/'Eridane', (5) FD91072/'Soissons', (6) 'Fertil'/FD91072, (7) FD91072/FD91147, (8) FD0830/'Corsaire', (9) 'Vivant'/'Rialto', (10) 'Ritmo'/FD627, (11) Rialto/Trémie, (12) Texel/Sidéral, (13) FD5566/FD502, (14) FD90050/FD627, (15) 'Contra'/Soissons, (16) 'Cordial'/Soissons, (17) Ritmo/Sidéral, (18) Soissons/FD91143.

The male parents consisted of the two F_1 *Zea mays* L. hybrids cvs 'Saviero' (M1) and 'Earlibelle' (M2); FL, an early-type population of maize (M3); and one genotype of teosinte (*Zea mays* ssp. *mexicana*) (T4). A pollen mixture of the above four male parents (M5) were also used in this study.

Doubled haploid production

After germination, seedlings of wheat were transferred to a vernalization room held at a constant temperature of 4°C for 8 weeks, then planted out in a glasshouse with a temperature regime of approximately 20°/16°C (day/night). A 16-h photoperiod was supplied by Philips SON-T400 high pressure sodium lights when necessary. Emasculations and pollinations were as described in Laurie and Bennett (1986). The 18 wheat F_1 hybrids and the five *Zea mays* parents were compared in a seven-replicate randomized complete-block design. Wheat spikes were considered to be replicates in cross-combinations with *Zea mays*. The 2,4-D tiller and floret treatment was the same as that described in Laurie and Reymondie (1991). Fifteen days after pollination, spikes were collected and embryos were excised and cultured in vials containing B5 medium (Gamborg et al. 1968). Embryos were incubated in the dark at 22°C for 5–10 days and then transferred to a 16-h light regime at the same temperature. Rooted seedlings were transplanted into the glasshouse. At the three to five tiller stage, plants were treated with a 0.1% aqueous solution of colchicine according to Pickering (1980), and at maturity seeds were collected from each fertile plant.

Statistical analyses were performed using Statistix® 4.0 (1992) and Biom© (1989) analytical software packages.

Ploidy level determination

Of the regenerated plants 610 were checked for ploidy level by flow cytometry. Briefly, 40 mg of leaf tissue was chopped with a razor blade in 2 ml of buffer (Bergounioux et al. 1986) and 16 µl of a filter-sterilized solution of 4', 6-Diamidino-2-Phenylindole (DAPI, Sigma D-9542) at a concentration of 250 µg/ml. Samples were analyzed using a CA II flow cytometer (Partec GmbH, 4400 Münster, Germany).

Since the CA II flow cytometer was not precise enough to detect chromosome abnormalities such as aneuploids, root-tip cell chromosome counts were carried out for 4 plants which had a peculiar growth habit, i.e. many and small tillers, narrow leaves and reduced growth speed.

To assess chromosome doubling efficiency, flow cytometry was performed on 28 colchicine-treated haploid plants. For this purpose, leaf samples were cut off from the first tiller which had arisen a few weeks following colchicine treatment. Flow cytometry profiles were analyzed using the Dpac© 2.1 software provided by Partec.

Inheritance of the 1BL-1RS translocated chromosome

Among the wheat cultivars or advanced lines which had been used as parents, only 'Rialto' possessed a homozygous 1BL-1RS translocated chromosome. 'Rialto' was the parent of the 2 F_1 hybrids 9 and 11. As demonstrated by Ainsworth and Gale (1987), the glucose phosphate isomerase (GPI) system can be used as an indicator of the presence of the 1BL-1RS translocation in wheat lines, and therefore was used in the present study. Enzyme extraction from leaves of young plantlets, gel preparation, electrophoresis and enzyme visualization were the same as described in Wendel and Weeden (1989). Inheritance of the 1BL-1RS chromosome was investigated in the haploid progenies produced by the MM and by AC and in the F_2 progeny of the 2 hybrids 9 and 11. The AC method was the same as described in Devaux (1992). As a control, GPI analysis was carried out using the three parents, 'Rialto', 'Vivant' and 'Trémie', as well as the IR 'Chinese Spring' addition line (CS + 1R), Ditelo 1BL 'Chinese Spring' (D1BL) and cv 'Gabo', which has a 1BL-1RS chromosome. (CS + 1R, D1BL and 'Gabo' were provided by Dr. R. M. D. Koebner (IPSR Cambridge Laboratory, Norwich, UK).

Results

Haploid plant production

From the 18,716 wheat florets which were processed during this experiment, 15,342 (82%) of the ovaries enlarged after fertilization and 2,4-D treatment to reach a caryopses size similar to that of parental selfs of the same age. However, only 3,843 (25%) of the expanded ovaries contained an embryo. Attempts to identify those caryopses having an embryo by X-ray radiography were unsuccessful. A total of 1,703 plants (44.3% of the embryos) were regenerated. The number of embryos (%EMB/FL) and plants (%PL/FL) per 100 florets ranged from 26.1 (wheat hybrid no 1: whl) to 14.4 (whl8) and from 14.7 (whl) to 4.4 (whl8), respectively (Fig. 1). The effect of wheat genotype on the two characters was highly significant (Table 1). The *Zea mays* pollinator had a significant effect for %EMB/FL (highest value: 24.1 for M2, lowest: 15.2 for M3) and %PL/FL (highest value: 10.5 for M2, lowest: 6.8 for M3) (Fig. 1). The interaction between wheat and *Zea mays* genotypes was significant at the 0.01 level for %PL/FL (Table 1). The

Fig. 1 Effect of wheat and maize genotypes on haploid production of wheat. *Horizontal lines* represent homogeneous groups for number of plants per 100 florets ($P < 0.05$)

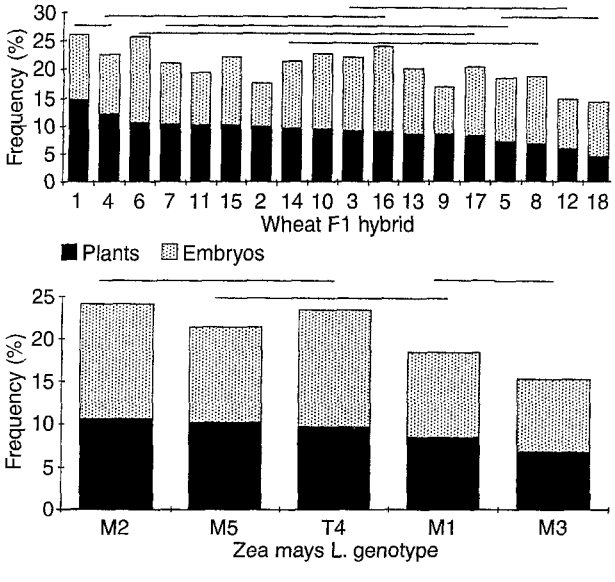

Table 1 Degrees of freedom and mean squares from analysis of variance for haploid embryo and plant production in wheat

Source	*df*	Embryos/100 florets MS	Plants/100 florets MS
Maize (M)	4	1708.5***	297.3***
Wheat (W)	17	380.9***	190.4***
M*W	68	145.4	84.0**
Error	540	115.1	51.9

,* Significant effect at $\alpha = 0.01$ and 0.001, respectively

highest %PL/FL (24.4) was obtained for wh1 crossed with M2, and the poorest (2.2) for whl8 with M3.

Heterogeneity *G*-tests (Sokal and Rolf 1981) for plant regeneration, i.e. the number of plants obtained from 100 embryos (%PL/EMB), were significant for both wheat ($G = 67.71$, $df = 17$) and *Zea mays* ($G = 9.74$, $df = 4$) genotypes at the 0.001 and 0.05 levels, respectively. The Pearson correlation coefficient between %EMB/FL and %PL/FL was $r = +0.630$ and reflects the difference between genotypes for regeneration ability. Of the cultured embryos 55.7% failed to establish themselves as plantlets; they just enlarged slightly or produced a coleoptile and/or roots.

Ploidy level and fertility of regenerants

The 610 plants which were checked for ploidy level by flow cytometry appeared to be haploid. This indicates that maize chromosome elimination was a consistent phenomenon in wheat × maize crosses and that no chromosome doubling had occurred during the process. Root-tip squashes revealed that the 4 plants which had a peculiar growth habit (see above) had 21 chromosomes. Noticeable differences existed in the proportion of diploid nuclei when tillers that developed after colchicine treatment were analyzed by flow cytometry (Fig. 2). The relative percentage of diploid over total nuclei, %DN/TN, of colchicine-treated haploid plants varied from 36 to 68 and revealed differences in chromosome doubling efficiency. % DN/TN was partially correlated to the number of grains collected from the whole plant at maturity ($r = +0.616$). All of the plants which had a % DN/TN of less than 42 were sterile. This suggests that a threshold exists under which sterility would be complete.

At maturity, 1,192 (70%) of the colchicine-treated haploid plants were fertile or partially fertile. The range was from 60% to 81% depending on the wheat hybrid from which the plants were derived. The contingency G

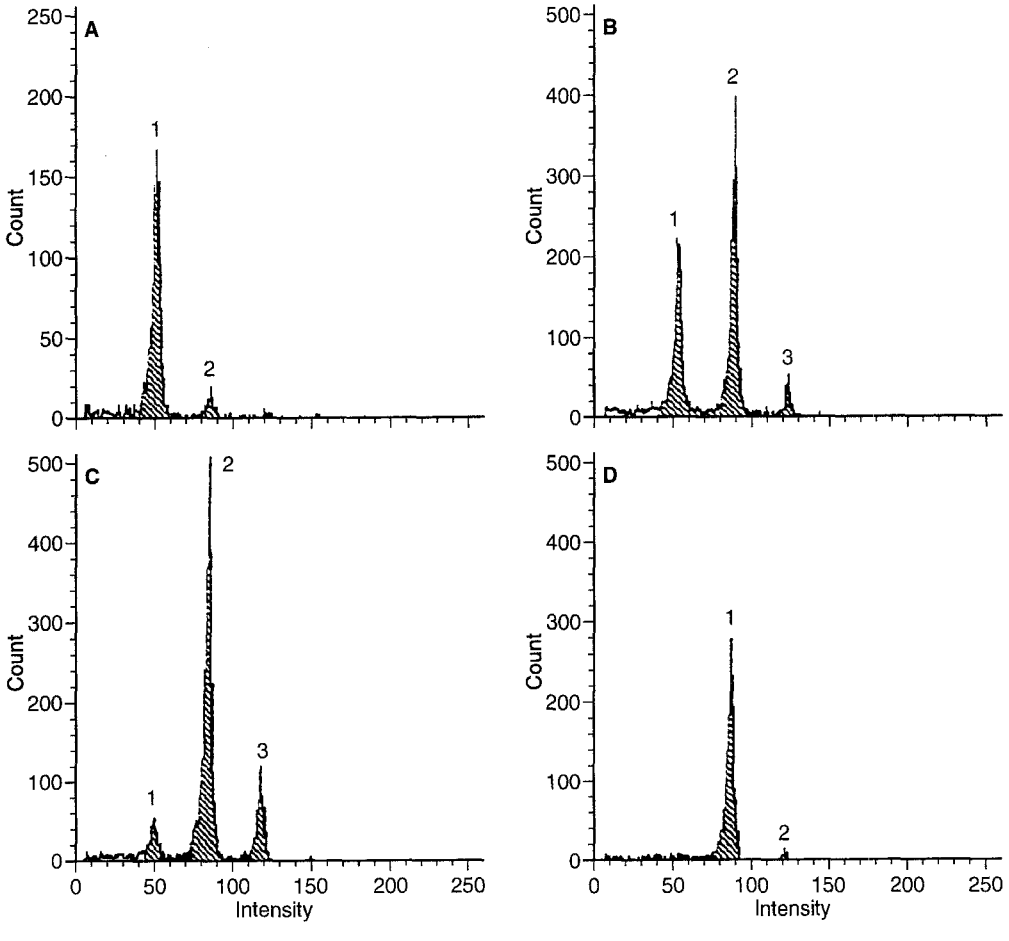

Fig. 2A–D Flow cytometric profiles of wheat plantlets derived from wheat × maize crosses. **A** haploid, **B** and **C** colchicine-treated haploids; **D** doubled haploid after one cycle of multiplication

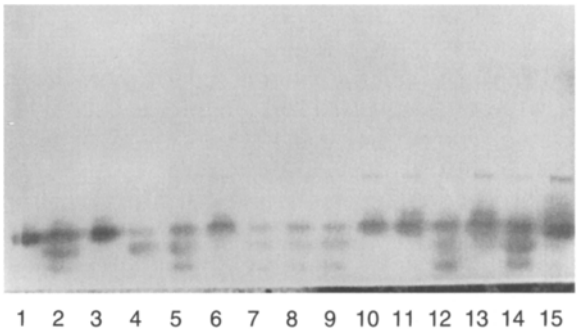

Fig. 3 Glucose phosphate isomerase (GPI) polymorphism of wheat. *Lanes 1–6* seedlings of various accessions and the parents of crosses 9 ('Vivant'/'Rialto') and 11 ('Rialto'/Trémie); *lanes 7–15* haploid plantlets derived from the cross 9 (*lanes 7–11*) and 11 (*lanes 12–15*) through wheat × maize hybridization. *1* Ditelo 1BL 'Chinese Spring', *2* 1R 'Chinese Spring' addition line, *3* cv 'Gabo' possessing a 1BL-1RS chromosome, *4* cv 'Vivant', *5* 'Trémie'. *6* 'Rialto'

value ($G_{(17)} = 30.86$) was significant at the, 0.05 level, indicating that the frequency of fertile and sterile plants was not consistent over genotypes.

Inheritance of the 1BL-1RS chromosome

Polymorphism for GPI between lines having the 1RS translocated chromosome and those with a normal 1B chromosome can be seen in Fig. 3. In 'Rialto', D1BL and 'Gabo', the two highest pI isozymes are missing. Segregation of alleles at the GPI locus did not deviate significantly from the 1:1 ratio in the haploid progenies derived from hybrids 9 and 11 by MM, while it did deviate in those produced by AC (Table 2). In the F_2 progenies derived from the same F_1 hybrids by self-fertilization, homozygous lines for the 1BL-1BS chromosome could not be distinguished without ambiguity from the structural heterozygotes 1BL-1RS/1BL-1BS through the GPI system. However, the segregation of parental phenotypes did not deviate from the 3:1 ratio (Table 2). This indicates that no selection pressure for the 1BL-1RS chromosome had occurred through self-fertilization or through fertilization with maize as it did for the regeneration of plants from microspores.

Discussion

In our study haploid plants were regenerated from all of the F_1 hybrids of wheat that were investigated at an average of 9.1 plants per 100 florets. This rate is quite acceptable with respect to the numerous advantages that haploids and derived DHs can bring into genetic studies and plant breeding (Kasha and Reinbergs 1982). Moreover, no recalcitrant genotype to green haploid plant production was encountered since the poorest responder yielded 4.4 haploid plants from 100 florets processed. In these respects, the MM showed an extraordinary advantage over AC for which recalcitrant and poor responder genotypes have been frequent (Ziegler et al. 1990; Devaux 1992; Orlov et al. 1993). However, genotypic differences for haploid production efficiency were found for both the wheat and *Zea mays* parents. In crop species for which the interspecific hybridization system has been exploited extensively for haploid production or for gene transfer (reviewed by Jiang et al. 1994), the influence of the cultivated target species used as the female parent has been reported in crosses of barley with *Hordeum bulbosum* (Pickering 1983; Bjørnstad 1986; Hayes and Chen 1989; Devaux et al. 1992), of potato with *Solanum phureja* (Hougas et al. 1964) and of bread and durum wheats with maize (Suenaga et al. 1991; Oury et al. 1993; Inagaki and Tahir 1990; Sarrafi et al. 1994). The genotype of the pollinator has also been reported to have a significant effect and, therefore, superior pollinators have been selected in *H. bulbosum* (Simpson et al. 1980; Pickering and Rennie 1990) and in *S. phureja* (Kotch and Peloquin 1987). Very little research has been devoted to the influence of the male parent component in the MM. Ushiyama et al. (1991) investigated over 39 maize genotypes and reported the superiority of one teosinte accession for haploid production. Inagaki and Tahir (1990) reported that differences between the pollen source were reflected in the frequencies of plant regeneration. It is clear from

Table 2 Inheritance of the 1BL-1RS chromosome in haploids produced by wheat × maize crosses (MM), anther culture (AC) and in F_2 progeny derived from two structural heterozygotes 1BL-1RS/1BL-1BS

Wheat cross	Group	1BL-1RS	1BL-1BS	1BL-1RS/1BL-1BS and 1BL-1BS/1BL-1BS[a]	χ^{2b}
9	MM	61	53	–	0.56
	AC	12	4	–	4.00*
	F_2	27	–	88	0.14
11	MM	65	74	–	0.58
	AC	59	30	–	9.45**
	F_2	20	–	77	0.99

[a] Homozygous lines for the 1BL-1BS chromosome could not be distinguished without ambiguity from the structural heterozygotes 1BL-1RS/1BL-1BS

[b] χ^2 test for deviation 1:1 ratio in haploids by MM and by AC, and from 3:1 in F_2

*,** Significant effect at $\alpha = 0.05$ and 0.01, respectively

the present study that there are superior genotypes of *Zea mays* and that these can be identified and used to improve the haploid production efficiency of wheat. However, the teosinte accession that was used was not different from the two best maize pollinators. Interactions between the two parent components were significant for plant production indicating that more than one *Zea mays* source should be used for the highest efficiency. As a pollen mixture of several *Zea mays* genotypes did not increase success rates, however, differences in fertilization efficiencies of *Zea mays* may exist, as in *H. bulbosum* (Pickering 1984).

At the time the spikes were collected for embryo excision, a high proportion (82%) of the wheat ovaries had enlarged. Suenaga and Nakajima (1989) reported that 2,4-D injected into Japanese wheat tillers after pollination with maize promoted ovary growth and embryo development. Similarly, 2,4-D was a prerequisite to the initiation of embryogenic callus or derivate suspension cultures (De Vries et al. 1988; Vasil et al. 1990). An average of 74.2% expanded ovaries was reported by Suenaga et al. (1991), this is similar to the rate found in our investigation. However, only a very small proportion of the expanded ovaries contained an embryo. In crosses between hexaploid wheat cv 'Chinese Spring' with Seneca 60 maize, Laurie et al. (1990) reported that 28% of the florets were fertilized. Since an average of 20.5 embryos per 100 florets were obtained, it is likely that most of the fertilized egg cells gave rise to an embryo. This is in agreement with the results reported in Laurie and Reymondie (1991). Laurie et al. (1990) identified different events as the causes of the fertilization failure. Further cytological observations of tetraploid wheat ovaries showed that maize pollen tubes exhibited various aberrations beyond the top of the ovule (O'Donoughue and Bennett 1994).

The reason why some of the cultured embryos failed to develop into plantlets is still unknown; however their frequency may be influenced by in vitro culture parameters. Comeau et al. (1992) showed differences between media can result in differences in the recovery efficiency of wheat haploid embryos. In carrot, the synthesis of extracellular glycoproteins promoting somatic embryogenesis is inducible by culture conditions (De Vries et al. 1988; Van Engelen and De Vries 1992). Plant embryogenesis is a very complex phenomenon which requires the expression of numerous genes (Lindsey and Topping 1993), and embryogenic-lethal mutants have been generated for these for example, in *Arabidopsis* (Meinke 1985) and maize (Sheridan and Clark 1993). It is well-established that growth regulators such as 2,4-D may induce chromosome alterations and mutations (Turkula and Jalal 1985; Lee and Phillips 1988; D' Amato 1990). Besides its beneficial effects, 2,4-D may induce mutations in the modifier or regulatory genes that control embryogenesis and seedling development; such mutants have been identified in *Arabidopsis* (Mayer et al. 1991). An alternative is that the dedifferentiating effects of 2,4-D that are so useful for inducing callus may be inhibiting proper embryo development. The failure of embryos to develop beyond the production of a coleoptile or roots may be due to the fact that the meristems are not properly formed.

The fertility of colchicine-treated haploid plants, a trait which can be assessed only after heading, is a critical and obviously an economical criterion for successful DH production. From this study, it can be predicted by an average approximation by flow cytometric analysis of the first tiller that arises after colchicine treatment. Further investigations would be necessary to increase the reliability of this predictor, especially by analyzing subsequent tillers. However, a threshold of %DN/TN seemed to exist under which there is no chance for a plant to be fertile. Consequently, those plants could be re-treated with colchicine for maximum efficiency.

Distortions in the segregation of marker alleles in anther culture-derived progenies have been reported. In barley, the most frequent allele found at some loci resulted in a significantly higher anther culture response (Devaux and Zivy 1994). Wheat cultivars which possess the 1BL/1RS translocated chromosome have been shown to have a higher anther culture regeneration ability than those with a normal 1B chromosome (Agache et al. 1989), and plants having this translocated chromosome are more frequent in populations derived from structural heterozygotes by AC. In contrast, this distortion of segregation did not occur in the MM-derived and selfed progenies of the same crosses, although Henry et al. (1993) reported that the IBL-IRS chromosome was transmitted through 45% of the egg cells. Further investigations are necessary to check for other markers spread over the genome. In this respect, Kleinhofs et al. (1993) reported distorted segregation for restriction fragment length polymorphism markers at several locations in the genome among barley DH lines derived by interspecific hybridization with *H. bulbosum*.

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