Spontaneous wheat-*Aegilops biuncialis, Ae. geniculata* and *Ae. triuncialis* amphiploid production, a potential way of gene transference

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Abstract

Some F_1 hybrid plants between three species of the *Aegilops* genus and different hexaploid wheat *Triticum aestivum* cultivars show certain self-fertility, with averages of F_1 hybrids bearing F_2 seeds of 8.17%, 5.12% and 48.14% for *Aegilops biuncialis*, *Aegilops geniculata* and *Aegilops triuncialis* respectively. In the *Ae. triuncialis*-wheat combination with 'Astral' wheat cultivar, the fertility was higher than that found in the other combinations. All the F_2 seeds studied were spontaneous amphiploids (2n=10x=70). The present study evidences the possibility of spontaneous formation of amphiploids between these three *Aegilops* species and hexaploid wheat and discusses their relevance for gene transference. Future risk assessment of transgenic wheat cultivars needs to evaluate the importance of amphiploids as a bridge for transgene introgression and for gene escape to the wild.

Additional key words: *Aegilops,* hybrid self-fertility, risk assessment, *Triticum aestivum,* wild relatives.

Resumen

Producción de anfiploides espontáneos entre *Aegilops biuncialis*, *Ae. geniculata* y *Ae. triuncialis* y el trigo, una vía potencial de transferencia de genes

Algunos híbridos F_1 entre tres especies del género *Aegilops* y diferentes cultivares de trigo hexaploide *Triticum aestivum* poseen cierta fertilidad por autofecundación, con un porcentaje medio de híbridos F_1 con semillas F_2 del 8,17%, 5,12% y 48,14% respectivamente para *Aegilops biuncialis*, *Aegilops geniculata* y *Aegilops triuncialis*. En las combinaciones *Ae. triuncialis*-trigo con el cultivar de trigo 'Astral' se obtuvo una fertilidad mayor que en las otras combinaciones. Todas las semillas F_2 que se estudiaron fueron anfiploides formados espontáneamente (2n=10x=70). Este estudio evidencia la formación espontánea de estos anfiploides entre estas tres especies de *Aegilops* y el trigo y discute su importancia en la transferencia de genes. Una futura evaluación del riesgo de los cultivares de trigo transgénico necesitará tener en cuenta la importancia de los anfiploides como puente para la introgresión de los transgenes y su escape a la naturaleza.

Palabras clave adicionales: *Aegilops*, especies silvestres relacionadas, evaluación de riesgo, fertilidad por autofecundación de los híbridos, *Triticum aestivum*.

Introduction

One of the concerns raised over the introduction of genetically modified crops is the stable transference of the transgenes to the wild relatives causing ecologically significant changes in fitness that could lead to increased weediness or invasiveness (Darmency, 1994), which constitutes an important basis for risk assessment studies. Therefore, over the last decade much attention has been paid to crop to weed and wild hybridization as potential avenues for the escape of transgenes into natural populations.

Prior to the commercialization of genetically modified crops the research on the natural hybridization between crops and related wild species was very limited. Most of the research was done with the purpose of breeding and with the aim of transferring desirable traits between species and crops were always used as female parent in

intergeneric and interspecific crosses. But the picture is quite different and numerous crops are known to have wild relatives that can hybridize with them somewhere in the world. Gene flow between cultivated species and their weedy and wild relatives has been documented in species such as oilseed rape (*Brassica napus* L.) (Jørgensen and Andersen, 1994), maize (*Zea mays* L.) (Doebley, 1990), sorghum (*Sorghum halepense* (L.) Pers) (Arriola and Ellstrand, 1996), sunflower (*Helianthus annuus* L.) (Arias and Rieseberg, 1994) and sugarbeet (*Beta vulgaris* L.) (Bartsch and Pohl-Orf, 1996).

Transgenic wheat varieties are being developed and field-tested and probably in the next few years certified cultivars will be commercially available. Potential risks should be examined before their widespread adoption. Gene transfer between cultivated wheat *Triticum aestivum* L. and the weedy *Aegilops cylindrica* Host. is known to occur and scientific literature confirms that herbicide resistance genes can move from herbicide tolerant wheat to *Ae. cylindrica* through hybrids (Seefeldt *et al.*, 1998; Zemetra *et al.*, 1998; Guadagnuolo *et al.*, 2001; Wang *et al.*, 2001). Other wild *Aegilops* species like *Ae. geniculata* Roth., *Ae. biuncialis* Vis. and *Ae. triuncialis* L. also form natural intergeneric hybrids with bread wheat where they grow in sympatry (van Slageren, 1994; Loureiro *et al.*, 2006; Zaharieva and Monneveux, 2006), a phenomenon underlining the close genetic links of the two genera. Hybrids between *Ae. geniculata* and *Ae. triuncialis* and wheat have been found in several countries of Europe, mainly in Spain and France, while *Ae. biuncialis*-wheat natural hybrids have been described in Lebanon (van Slageren, 1994). These natural hybrids are highly sterile, although seeds may occasionally be found in *Ae. geniculata* hybrids (van Slageren, 1994; Loureiro *et al.*, 2008).

Crop-to-wild transgene transference may occur through recombination in homoeologous chromosome pairs, translocation, or chromosome retention (Schoenenberger *et al.*, 2006). Another possible transference mechanism is the formation of amphiploids. Spontaneous chromosome doubling usually results from unreduced female and male gametogenesis and the union of those unreduced gametes leads to the formation of a spontaneous amphiploid from an interspecific hybrid (Ramsey and Schemske, 2002). It is known that amphiploidy has played a fundamental role in the evolution in the Triticeae tribe: durum wheat *Triticum turgidum* L. probably evolved in one step as an amphiploid between the wild grasses *Ae. speltoides*

Tausch and *T. urartu* Tumanian ex Gandilyan; that durum wheat crossed naturally with *Aegilops tauschii* Coos, which resulted in the first hexaploid wheat, *T. spelta* L. (Stebbins, 1946; Maan and Sasakuma, 1977; Jauhar, 2003). In the amphiploids every chromosome of the original hybrids is represented twice and each chromosome has a homologous partner to pair at meiosis. Thus, meiosis is regular and fertile derivatives can be obtained from hybrids whose sterility was caused by chromosomal unbalance and irregularity (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998; David *et al.*, 2004). In their review on hybridization between wheat and its relatives, Zaharieva and Monneveux (2006) mentioned that Tschermak and Bleier (1926) were the first to obtain an amphiploid species as a result of the spontaneous doubling of the chromosomes of a wheat hybrid produced by the cross of *T. turgidum dicoccoides* with *Ae. geniculata*. Amphiploidy is a mechanism that can also provide a valuable genetic resource for the introgression of desirable genes from alien species to cultivated wheats. Induced amphiploidy was indeed used to transfer *Ae. geniculata* chromosomes carrying disease resistance into bread wheat (Landjeva and Ganeva, 1998, 1999).

In nature, amphiploids could serve as an effective bridge for gene flow over the interspecific and intergeneric barriers. David *et al.* (2004) reported an estimated frequency of 10^{-6} of spontaneous amphiploidy between the tetraploid wheat *T. turgidum* and *Ae. geniculata* in field sympatric populations, that was higher (10^{-3}) in nursery conditions and with different genotypes. Genomic *in situ* hybridization proved that fertile amphiploids had arisen through unreduced gametes and that some of them carried wheat−*Ae. geniculata* recombinant chromosomes.

The objective of this paper is to report the production and frequency of spontaneous amphiploids in the self-progenies of hybrids between hexaploid wheat *T. aestivum* and the *Aegilops* species *Ae. biuncialis*, *Ae. geniculata* and *Ae. triuncialis*.

Material and methods

Experiments were carried out at the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) experimental station in Madrid, Spain (40º 27´ North; 3º 44´ West).

Ae. biuncialis, *Ae. geniculata* and *Ae. triuncialis* are relatively easy to hybridize with wheat. *Aegilops × T. aes-*

tivum hand crosses made during several years (from 2004 to 2007) yielded abundant F_1 hybrid seeds, at rates from 30 to 70% of the pollinated flowers (Loureiro, 2005). The plant material employed in the experiments was the F_2 seed progeny produced by selfing of the F_1 hybrids between the wheat wild relatives *Ae. geniculata* (2n=4x=28 chromosomes, genomic constitution *MM*UU), *Ae. biuncialis* (2n=4x=28 chromosomes, UU*MM*) and *Ae triuncialis* (UUCC), and the *T. aestivum* (2n=6x=42, AABBDD) cultivars 'Chinese Spring' (CS), 'Castan' and 'Astral'. Nomenclature of the wild *Aegilops* spp. is according to van Slageren (1994) and the genomic constitution is according to Kimber and Tsunewaki (1988). The F_1 hybrids were sown each year in autumn in a greenhouse in which lateral walls were open and temperature and relative humidity were similar to outdoor conditions. There was no temperature or humidity control. Two to three days prior to anthesis, hybrid spikes from each plant were covered with cellophane bags to avoid any cross-pollination. The fertility of the hybrids was estimated as the number of seeds obtained per 100 spikelets after selfing. A Kruskal-Wallis test was carried out to determine significant differences between the F_2 seed set in the different *Aegilops*-wheat combinations, followed by a Mann-Whitney U-test for multiple comparisons. A confidence level of 95% ($p < 0.05$) was considered significant.

Seeds collected on hybrids were placed on filter paper moistened with distilled water in 9-cm diameter Petri dishes and once germinated were subjected to cytological analysis to confirm amphiploidy. Root meristems for mitotic chromosome number counts were collected from each germinated seed and were pre-treated in α-bromonaphthalene at 4ºC during 16 hours, fixed in a 90% acetic acid solution during 30 min,

washed twice with 95% ethanol and stored in 70% ethanol. After a minimum of 10-14 days, root meristems were ready to be stained in Schiff reactive for 60 min after a 10-12 min hydrolysis at 60ºC HCl 1N and squashed in a 1% Belling's aceto-carmin solution prior to light microscopy observation.

The F_2 plantlets were further grown to maturity in the greenhouse.

Results

A total of 182, 150 and 26 hybrids between hexaploid wheat and *Ae. biuncialis*, *Ae. geniculata* and *Ae. triuncialis* respectively, were studied. These F_1 first-generation hybrids were pentaploids (2n=5x=35) (Figure 1A). F_1 plants were grown to maturity in order to study the self-fertility. Some of the plants gave F_2 seeds, with a percentage of F_1 plants bearing at least one F_2 seed that varied significantly in the distinct *Aegilops*-wheat combinations (Kruskal-Wallis test: H=24.06, $p<0.001$) (Table 1). The highest self-fertility was obtained for the *Ae. triuncialis*-wheat combination with frequencies of 1.82 seeds in 100 spikelet and percent averages of 48.14% F_1 plants with F_2 seeds. All the hybrids obtained between *Ae. triuncialis* and the wheat cultivar 'Astral' were fertile (Table 1), their fertility varied from 1.06 to 8.88 seeds in 100 spikelets among the five plants studied. The fertility of the hybrids with 'Astral' was significantly higher than the obtained with the other wheat cultivars (Mann-Whitney U tests, $p < 0.05$) that did not show differences among them (Mann-Whitney U tests, $p > 0.05$). Fertility was lower for *Ae. biuncialis* and *Ae. geniculata*-
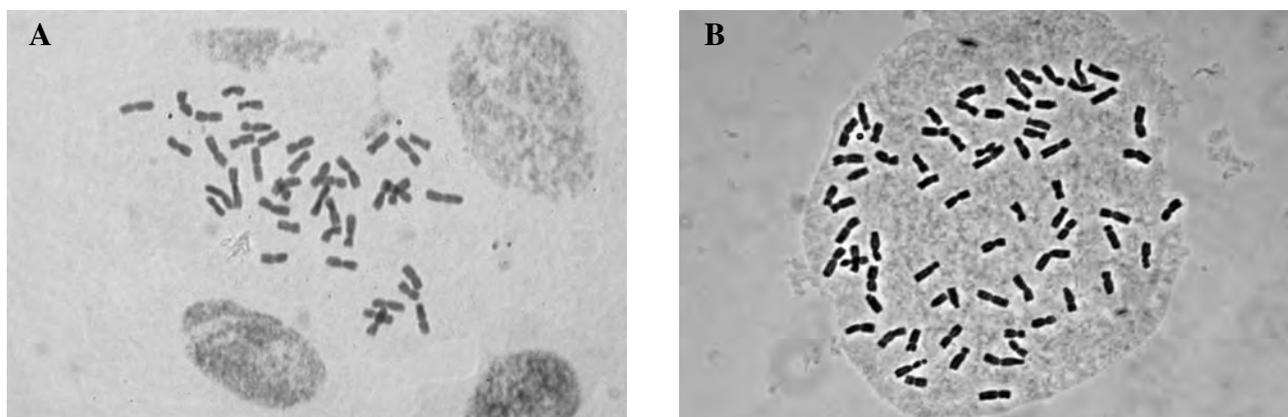

Figure 1. A) F_1 hybrid between *Aegilops biuncialis* and *Triticum aestivum* 'CS' with 35 chromosomes. B) Amphiploid of *Aegilops triuncialis* × *T. aestivum* 'Astral' hybrid with 70 chromosomes formed by the union of two unreduced gametes.

Table 1. Fertility of the three *Aegilops* × wheat F_1 hybrids by selfing and evidence of formation of amphiploids. The fertility of the hybrids was estimated as the number of seeds obtained per 100 spikelets after selfing

	Number of					Number of			
	F_1 hybrids	Spikes	Spikelets	F_2 seed set	Fertility	F_1 plants with F_2 seeds (%)	F_2 seed studied	Germinated F_2 seeds	Amphiploids
Ae. biuncialis									
× CS	161	1488	8987	6	0.07	5 (3.10)	3	1	1
× Castan	14	118	765	1	0.13	1 (7.14)	1	1	1
× Deganit	7	53	234	2	0.85	1 (14.28)	1	0	0
Total	182	1659	9986	9	0.09		5	2	2
Mean						8.17 ± 5.66			
Ae. geniculata									
× CS	70	605	3959	2	0.05	2 (2.86)	1	1	1
× Castan	40	342	2529	4	0.16	4 (10.00)	2	2	2
× Deganit	40	332	1988	1	0.05	1 (2.50)	1	1	1
Total	150	1279	8476	7	0.08		4	4	4
Mean						5.12 ± 4.23			
Ae. triuncialis									
× CS	3	22	181	2	1.10	1 (33.33)	2	1	1
× Castan	18	129	1174	2	0.17	2 (11.11)	2	2	2
× Astral	5	72	570	31	5.44	5 (100.00)	15	14	14
Total	26	223	1925	35	1.82		19	17	17
Mean						48.14 ± 46.26			

wheat combinations with values of 0.09 (0.07-0.85) and 0.08 (0.05-0.16) seeds in 100 spikelets respectively, and without significant differences among these two combinations according to the Mann-Whitney U test ($p = 0.097$).

Not all the F_2 hybrid seeds obtained were germinated and used for amphiploidy study; some of them were conserved for subsequent studies (Table 1). F_2 seeds were mostly well formed with a germination rate of 89%, and all the germinated *Aegilops* × wheat F_2 hybrid seeds showed 70 chromosomes (Figure 1B). On the assumption that all the obtained F_2 seeds are amphiploids, the mean frequency of amphiploid formation (new decaploid F_2 plants per single pentaploid F_1 hybrid) would be of 0.049 for *Ae. biuncialis*, 0.047 for *Ae. geniculata* and 1.34 for *Ae. triuncialis*-wheat hybrid combinations.

The decaploid F_2 plants were grown in the greenhouse. The two F_2 *Ae. biuncialis*-wheat plants were not viable and died at the seedling stage. In the case of the *Ae. geniculata* and *Ae. triuncialis*-wheat hybrid plants, the phenotype of the F_2 amphiploids resembled that of the normal *Aegilops* × *T. aestivum* F_1 hybrids with regard to their leaf size, height and spike morphology (Figure 2). The plants showed a vigorous vegetative growth.

Discussion

The hybrids between tetraploid *Aegilops*-species and hexaploid wheat are often considered self-sterile; however, seeds can occasionally be found (Maan and Sasakuma, 1977; van Slageren, 1994). This high sterility may be due to the expected highly disrupted meiosis in these F_1 hybrids, whose parental plants differ in chromosome number and in chromosome pairing homology due to the relative divergences between *Aegilops* genomes (U, C and M in the case of the species studied) and wheat genomes (A, B and D) (Sears, 1941). Structural differences in chromosomes between parents and cytoplasmic differences may also cause sterility in certain hybrids (Stebbins, 1950; Maan and Sasakuma, 1977).

Our data reveals certain degree of self-fertility in some *Aegilops*-wheat F_1 hybrid combinations. The most plausible explanation would be the fecundation of an unreduced female gamete (2n=5x=35) of an F_1 hybrid with another unreduced male gamete (2n=5x=35), resulting in a decaploid plant (2n=10x=70). As happens with the self-fertility of intergeneric hybrids, the formation of unreduced gametes was also considered to be infrequent in general on the assumption that the occurrence of 2n gametes
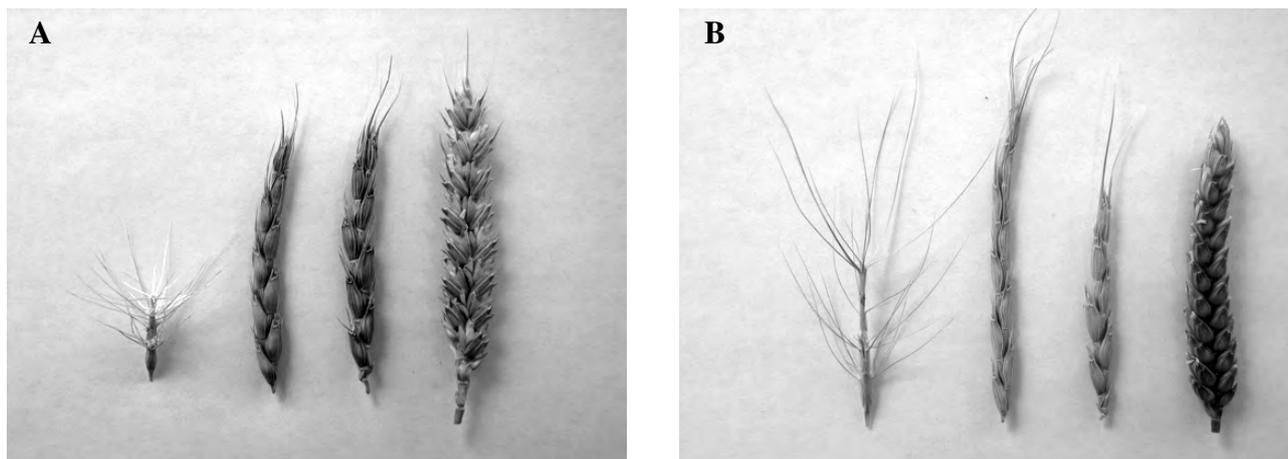
Figure 2. Spikes of A) *Ae. geniculata* (left), F_1 hybrid between *Ae. geniculata* and *T. aestivum* 'Castan', F_2 amphiploid obtained by selfing the F_1 hybrid and 'Castan' (right) and B) *Ae. triuncialis* (left), F_1 hybrid between *Ae. triuncialis* and *T. aestivum* 'Astral', F_2 amphiploid obtained by selfing the F_1 hybrid and 'Astral' (right).

in plants is rare and sporadic. But contrary to this assumption, Harlan and de Wet (1975) showed that almost all plant species can produce 2n gametes in some frequencies and that all polyploids have originated through functional 2n gametes. Many of the intergeneric hybrids have disturbed chromosome pairing and produce high frequencies of 2n male and female gametes. Both phenomena have been reported to occur in the F_1 hybrids between *T. turgidum* and *Ae. tauschii* (Xu and Joppa, 1995). There is clear evidence that viable seeds are produced by functional 2n gametes in durum wheat haploids (Jauhar *et al.*, 2000; Jauhar, 2003). Similarly, David *et al.* (2004) observed that fertile *Ae. geniculata* × *T. turgidum durum* hybrids always produced total or partial amphiploids in their offspring.

In the case of our *Ae. triuncialis*-wheat F_1 hybrids from 'Astral' wheat, the F_2 seed set was quite elevated and all plants obtained were fertile. The number of spikes and spikelets per plant was greater in these hybrids, which could contribute to their higher fertility. This wheat genotype effect is also a factor that has an influence on the frequency of 2n gamete formation (Maan and Sasakuma, 1977; Bretagnolle and Thompson, 1995; Ramsey and Schemske, 2002). However, the *Ae. triuncialis*-wheat F_1 hybrid plants flowered later due to the *Aegilops* parent's life cycle and suffered higher temperatures during their meiosis, so it cannot be excluded that this increase in their fertility may be the result of the effects of those temperatures which can also induce meiotic abnormalities promoting unreduced gamete production (Sax, 1937; Ramsey and Schemske, 1998).

Hybridization rates between wheat and *Ae. biuncialis* and *Ae. geniculata* under semi-natural conditions are around 0.3% (Loureiro *et al.*, 2007) while the frequencies of amphiploid formation obtained in this study vary from 3.7 to 28.5 per 100 F_1 hybrids studied between *Ae. biuncialis* and wheat and from 2.5 to 10 amphiploids for *Ae. geniculata*-wheat hybrids, depending on the *Aegilops*-wheat parental combination. Thus, finding spontaneous amphiploids in nature would not be completely exceptional. In the case of *Ae. triuncialis*, although the frequency of hybridization with wheat under natural conditions is unknown, it is a matter of concern that between 11 and 620 amphiploids may be formed per 100 F_1 hybrids. As stated above, fertility rates were significantly higher with 'Astral' and these data on amphiploid formation cannot be generalized for all *Ae. triuncialis*-wheat hybrid combinations.

However, the production of unreduced gametes and the formation of a new polyploid is only one step towards the establishment of this polyploid, which must be competitive in order to persist. Even if the fertility and/or viability of F_1s were low, these traits often increase in each successive hybrid generation (Rieseberg, 1997). Therefore if the F_1 sets results in viable seed at all, this will provide a second generation of hybrids that might be more fertile. These will breed, among themselves and with their parents, and might provide a third hybrid generation comprising more and fitter individuals than those of the second one. Once gene dispersal has occurred, it is important to understand whether these genes will persist and establish in the natural populations of free-living relatives; since

there is a lack of information it follows that more studies are necessary.

Herbicide-resistant wheat, primarily glyphosate tolerant wheat (Blackshaw and Harker, 2002; Zhou *et al.*, 2003), has the potential to improve the efficiency of weed management. The risk of gene transfer to wild and weedy relatives that grow in sympatry and with overlapping flowering times should be studied, in order to evaluate the likelihood that such an event occurs. *Ae. geniculata*, *Ae. biuncialis* and *Ae. triuncialis* are colonizing species with the capacity to develop large stands, up to many hectares, that could increase their invasiveness under the selection pressure of the herbicide if they acquire the herbicide resistance gene. The risk of introgression of the wheat transgenes into the genome of these *Aegilops* species is related to the occurrence of meiotic recombination during chromosome pairing in the meiosis of the F_1 hybrids. The introgression may be achieved either by hybridization with transgenic wheat and recurrent backcrossing with the *Aegilops* parent or by the "bridge" of the amphiploids. This second route of spontaneous amphiploid production, via doubling the chromosomes can permit a good bivalent pairing and overcome the sterility of the F_1 hybrids. In the case of *Ae. geniculata*-wheat, the F_2 fertility varied between 0 and 36 seeds in 100 spikelets among combinations, with one *Ae. geniculata* × 'Castan' F_2 plant that reached up to 66.7 seeds in 100 spikelets (Loureiro *et al.*, 2008), while the F_2 amphiploids between *Ae. triuncialis* and wheat are also fertile and, indeed, plants producing up to 11 F_3 grains can be found (Loureiro, unpublished). In both cases F_3 seeds were well-developed.

Further studies of the recombination in the meiosis of the amphiploid hybrids are essential in order to determine whether a transgene may be transferred to the wild genome. In addition, the possible fertility of these amphiploids increases the likelihood of them becoming a new species in which a transgene would be maintained easily. Data on amphiploid frequency and fertility will without doubt be useful in assessing the potential risks of future transgenic wheat cultivars.

Acknowledgements

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