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Cytological, Phenological and Molecular Characterization of B (S)-Genome Synthetic Hexaploids (2n = 6x = 42; AABBSS)

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The B(S) genome diploids (2n = 2x = 14) are a unique reservoir of genetic diversity that can provide wheat breeders a rich source of allelic variation for stress traits that limit productivity. Restricted in practical use essentially due to their complex chromosomal behavior, these diploids have been in limited practical usage. The classic utilization example has been the suppression activity of the *Ph* locus and role in alien genetic transfer aspects that has been a standard in cytogenetic manipulation studies. For applied efforts focusing on *Aegilops speltoides* researchers in CIMMYT initiated an ambitious program to make AABBBB(SS) synthetics and made progress by generating over 50 such synthetics. Of these 20 were available for this study in which phenology and powdery mildew screening were evaluated. Four of these 20 synthetics appeared to be useful sources for further exploitation in breeding. These were entries 6, 9, 10 and 11 suited for exploitation in pre-breeding, with positive phenological characters particularly high thousand-kernel weight and are cytologically near euploid at 2n = 6x = 42. The subtle hyper (43) and hypoploid number would not negate their applied use potential. Preference however goes to genotypes 9 and 11.

Keywords: *Triticum aestivum,* phenology, cytology, fingerprinting

Introduction

Conventional wheat breeding programs utilize diverse germplasm cross-combinations with diversity residing in the same gene pool that easily undergoes genetic recombination followed by trait segregation, evaluation selection and ultimate varietal release. In order to amplify the genetic diversity of the crop, novel genetic resources have become a focus for which the close progenitors of wheat are preferred (Mujeeb-Kazi and Hettel 1995). These are numerous diploid accessions of the A, B (S) and D genomes (2n = 2x = 14). Within this

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spectrum, A and D genomes have greater advantage than B essentially because of the proximity order (homology) of the D and A genomes to related genomes present in bread wheat (2n = 6x = 42; AABBDD) based upon cytogenetic test analyses that indicate greater homology of the 7 chromosomes of the D genome than the B genome chromosomes with their respective D and A genome chromosomes. Accessions of these two diversity sources reside in the primary gene pool, can be hybridized with ease, allow for swift gene transfer via homologous recombination and have extensive diversity for global biotic/abiotic stress/constraints that limit wheat production (Mujeeb-Kazi 2006; Ogbonnaya et al. 2013).

Greater genetic proximity (up to 7 bivalents at meiosis) tilts the optimum choice towards the exploitation of the D genome diploid *Aegilops tauschii* (2n = 2x = 14). It is also preferred because only a few of its accessions were involved in the natural hybridization/amphiploidization event, thus giving rise to a crop with an extremely narrow genetic base (Metakovsky et al. 1984). Complementary to this are the observations of Kihara (1944) and McFadden and Sears (1946) associated with the *Ae. tauschii* role, which have enabled current investigators to focus their wheat improvement efforts around this wild diploid via direct (Alonso and Kimber 1984; Gill and Raupp 1987) and bridge crossing protocols (Mujeeb-Kazi and Asiedu 1995).

The polyploid *Aegilops* and *Triticum* species sharing one genome with wheat are included in the secondary gene pool, which also includes the five diploid species of the Sitopsis section. Genetic transfers are routine within homologous genomes but require manipulative protocols between non-homologous types. Embryo rescue is a complementary aid for obtaining hybrids. Very limited practical usage has emerged for wheat improvement but suggestions have been made for exploiting the Sitopsis diploid accessions with *Ae. speltoides* (2n = 2x = 14: BB or B^sB^s or SS) as the priority choice for both durum and bread wheat improvement. Breeding protocols are more complex since manipulation strategies associated with alien gene transfer often incorporate undesirable traits together with the interesting target gene as a consequence of disturbed meiotic normalcy due to the suppression of the *Ph* locus.

Special emphasis is currently given to the use of *Ae. speltoides* as the putative donor of the B-genome and its accessions (2n = 2x = 14; BB or SS) were considered as the initial choice for general wheat improvement via hexaploid amphiploid bridge-crossing route (2n = 6x = 42, AABBSS). These newly produced amphiploids have shown initial promise for resistances to *Cochliobolus sativus, Fusarium graminearum, Septoria tritici,* barley yellow dwarf virus (BYDV), leaf rust and stripe rust. More testing for the above stresses together with exploiting the potential of other Sitopsis species diploids hence appears logical.

Within the Sitopsis section, *Ae. longissima* and *Ae. searsii* have been studied to a considerable extent but more common have been attempts to exploit *Ae. speltoides* across basic research scenarios. Its various accessions have demonstrated their potential in addressing biotic stress resistances and impact has been seen through the contribution of rust genes (Faris et al. 2008; Mago et al. 2009; Zhixia et al. 2011) from the accessional resource and the development of the Ph^I stock that promotes homoeologous pairing (Chen

et al. 1994). Attempted here is an effort to see if the SS genome wide exploitation of this diploid is possible for wheat improvement.

Materials and Methods

Germplasm

The AABBSS stock germplasm was obtained from CIMMYT in 2004 and entry numbers kept similar to the data base maintained in CIMMYT Wide Crosses program in Mexico. Pedigrees details are given in Table 1. Since the initial production of the B (S) genome hexaploids, the number available for exploitation dropped to 34 as several of the 54 were poorly adapted to the Pakistani conditions at Islamabad from which a set of 20 B-genome synthetics with ample seed was fingerprinted and phenologically characterized (Table 2).

DNA extraction was done on young seedlings prior to inoculation using the protocol of Weining and Langridge 1991 with minor modifications. A total of 89 SSR primers (Röder et al. 1998) were applied on each set to detect genetic polymorphism at DNA level (Table S1*). The protocols for meiosis, C-banding, PCR conditions and software used are provided in online supplementary material.

Results

Cytological analysis

The B(S)-genome hexaploids had weaker plants and showed aneuploid meiotic associations (Figs 1a and b) with all expressing a co-dominant spike phenotype (Fig. S1). At meiosis, normalcy was altered and open rod bivalents increased in number with the increase of multiple chromosomal associations categorized as trivalents, quadrivalents and few

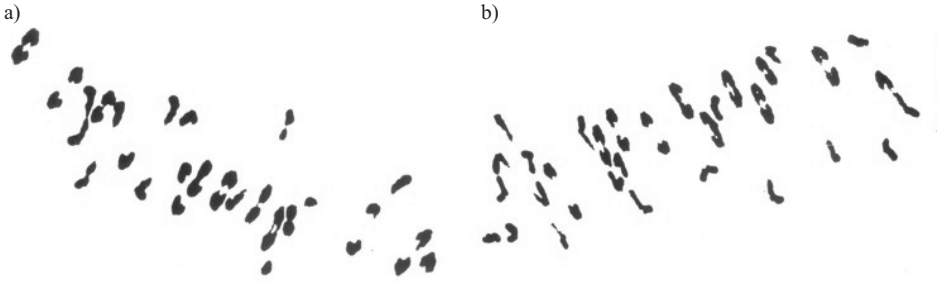

Figure 1. Meiotic associations at metaphase I of some B-genome Synthetic amphiploids (hexaploids) genomically AABB BB(SS) showing in a and b the following meiocyte details: (a) An aneuploid synthetic with 41 chromosomes with 1 trivalent (arrowed) and a mixture of ring and rod bivalents at the bivalent separation stage; (b) An aneuploid synthetic derivative with 41 chromosomes with univalents, ring + rod bivalents and 1 quadrivalent association (arrowed)

* Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

Table 1. Pedigrees of B-genome synthetic hexaploids

S. No.	Parentage/pedigree
1	CPI/GEDIZ/3/GOO//JO/CRA/4/AE.SPELTOIDES (124)*
2	CETA/AE.SPELTOIDES (124)
3	CPI/GEDIZ/3/GOO//JO/CRA/4/AE.SPELTOIDES (125)
4	CETA/AE.SPELTOIDES (125)
5	CPI/GEDIZ/3/GOO//JO/CRA/4/AE.SPELTOIDES (127)
6	CETA/AE.SPELTOIDES (127)
7	CPI/GEDIZ/3/GOO//JO/CRA/4/AE.SPELTOIDES (129)
8	CETA/AE.SPELTOIDES (129)
9	CPI/GEDIZ/3/GOO//JO/CRA/4/AE.SPELTOIDES (133)
10	ARLIN_1/AE.SPELTOIDES (134)
11	CPI/GEDIZ/3/GOO//JO/CRA/4/AE.SPELTOIDES (135)
12	CETA/AE.SPELTOIDES (135)
13	CETA/AE.SPELTOIDES (139)
14	ARLIN_1/AE.SPELTOIDES (152)
15	ALTAR 84/AE.SPELTOIDES (133)
16	CROC-1/AE.SPELTOIDES (134)
17	CROC_1/AE. SPELTOIDES (137)
18	ALTAR 84/AE.SPELTOIDES (141)
19	CROC_1/AE.SPELTOIDES (149)
20	CETA/AE. SPELTOIDES (140)
21	ARLIN_1/AE. SPELTOIDES (141)
22	ARLIN_1/AE.SPELTOIDES (126)
23	D67.2/P66.270//AE.SPELTOIDES (126)
24	ARLIN_1/AE.SPELTOIDES (128)
25	ARLIN_1/AE.SPELTOIDES (130)
26	ARLIN_1/AE.SPELTOIDES (131)
27	D67.2/P66.270//AE.SPELTOIDES (126)
28	ARLIN_1/AE.SPELTOIDES (132)
29	D67.2/P66.270//AE.SPELTOIDES (132)
30	ARLIN_1/AE.SPELTOIDES (138)
31	ARLIN_1/AE.SPELTOIDES (142)
32	ARLIN_1/AE.SPELTOIDES (143)
33	CPI/GEDIZ/3/GOO/JO/CRA/4/AE.SPELTOIDES (143)
34	ARLIN_1/AE.SPELTOIDES (144)
35	CETA/AE.SPELTOIDES (144)
36	ARLIN_1/AE.SPELTOIDES (145)
37	ARLIN_1/AE.SPELTOIDES (146)
38	CETA/AE.SPELTOIDES (146)
39	CPI/GEDIZ/3/GOO/JO/CRA/4/AE.SPELTOIDES (146)
40	ARLIN_1/AE.SPELTOIDES (147)
41	CPI/GEDIZ/3/GOO/JO/CRA/4/AE.SPELTOIDES (147)
42	ARLIN_1/AE.SPELTOIDES (148)
43	ARLIN_1/AE.SPELTOIDES (150)
44	CPI/GEDIZ/3/GOO/JO/CRA/4/AE.SPELTOIDES (150)

Table 1 (cont.)

S. No.	Parentage/pedigree
45	ARLIN_1/AE.SPELTOIDES (156)
46	CPI/GEDIZ/3/GOO/JO/CRA/4/AE.SPELTOIDES (156)
47	ARLIN_1/AE.SPELTOIDES (157)
48	CPI/GEDIZ/3/GOO//JO/CRA/4/AE.SPELTOIDES (157)
49	ARLIN_1/AE.SPELTOIDES (158)
50	CPI/GEDIZ/3/GOO/JO/CRA/4/AE.SPELTOIDES (158)
51	ARLIN_1/AE.SPELTOIDES (159)
52	D67.2/P66.270//AE.SPELTOIDES (160)
53	ARLIN_1/AE.SPELTOIDES (161)
54	ARLIN_1/AE.SPELTOIDES (162)
55	CPI/GEDIZ/3/GOO/JO/CRA/4/AE.SPELTOIDES (162)
56	D67.2/P66.270/AE.SPELTOIDES (162)

* Accession number in the Wheat Wide Crosses working collection in CIMMYT, Mexico

Table 2. Phenological and disease characterization of 20 B-genome synthetic hexaploids (2n = 6x = 42; AABBBB/SS)

S. No.	FLOW	HT	AWN	P.MA	GWT	G/S	SL
6	113	72	Y	161	52.0	22	10.0
7	140	80	Y	160	40.0	40	12.5
9	113	75	Y	160	60.3	56	14.0
10	133	90	AW	164	62.7	28	15.0
11	112	85	AW	163	44.8	60	12.0
12	129	98	Y	164	18.0	5	13.0
13	133	93	Y	162	44.6	24	14.5
18	133	66	LB	166	60.0	30	13.0
19	139	78	AW	163	40.0	36	11.3
22	131	85	AW	165	40.0	34	12.6
23	130	89	Y	162	40.0	36	12.8
24	130	92	AW	164	50.0	6	9.3
25	145	74	AW	166	13.2	14	11.0
26	113	65	AW	161	44.8	42	8.5
32	146	90	Y	165	18.0	12	16.0
34	134	87	AW	160	22.4	1	13.0
36	141	68	Y	166	15.0	9	16.0
47	118	63	AW	161	28.6	46	8.0
48	134	77	LB	162	11.0	8	14.0
49	133	79	AW	164	19.5	27	18.5

Abbreviations in the first row are as follows: FLOW: Days to Flowering; HT: Plant Height at Maturity (cm); AWN: Awn color (LB = light brown, AW = Emery white, Y = yellow, DB = dark brown); P. MA: Days to Physiological Maturity; GWT: 1000-grain weight (g); G/S: No. of grains/spike; SL: Spike length (cm)

pentavalents (Table S2). The aneuploid prevalence appears to be the cause of weak plant growth, limited per spike seed set and shrivelled seed. The B(S) genome showed a tall plant habit (100 to 130 cm) and late maturity (145 to 155 days). Seed fertility was satisfactory in those that were adapted but the seed was shrivelled. The crossability frequency across all combinations obtained by researchers in CIMMYT was high (Table S3) and regeneration of the plated embryos was generally over 90 percent with colchicine induced doubling to yield the AABBBB(SS) amphiploids also of a similar or higher level. C-banding validation of the presence of four B genomes in the AABBBB amphiploids was evident from the cytological validity a somatic cell (Fig. S2).

Phenological parameters

The attributes of each of the phenologically best genotypes were:

- Genotype 6: Days to flowering 113, 1000-kernel weight of 52 g.
- Genotype 9: Days to flowering 113, 1000-kernel weight of 60.3 g, grains/spike (56) and spike length (14 cm).
- Genotype 10: good for 1000-kernel weight (62.7 g) and spike length (15 cm).
- Genotype 11: Days to flowering 112 and grains/spike (60).

Genetic diversity evaluation using SSR primers

Genetic analysis was performed only on the scorable bands. Every single band was considered as a single locus/allele. The loci were scored as present/absent. Bivariate data 1–0 were used to estimate genetic distances (GD). Unweighted Pair Group of Arithmetic Means (UPGMA) function (Nei and Li 1979) estimated genetic distances between the genotypes as follows: $GD_{xy} = 1 - d_{xy}/d_x + d_y - d_{xy}$, where GD_{xy} = Genetic distance between two genotypes, d_{xy} = Total number of common loci (bands) in two genotypes, d_x = Total number of loci (bands) in genotype 1 and d_y = Total number of loci (bands) in genotype 2. Simple Sequence Repeats (SSR) primers were used for genetic diversity evaluation of B-genome synthetic hexaploids. Population genetic analysis showed that the B (S)-genome synthetic hexaploids scored total 327 alleles with 299 polymorphic reaching the percentage of 91.43%. Bivariate analysis was conducted to generate a similarity matrix and dendrogram using Nei and Li's coefficient (1979) to estimate genetic diversity. The similarity coefficient in B(S)-genome synthetic hexaploids ranged from 58.2% (13 and 49) to 88.1% present between 24 and 32 (Fig. 2).

Discussion

The yield of wheat is determined by number of spikes per plant, number of grains per spike and grain weight. The latter is one of the most important yield contributing traits and has been an important selection criteria of higher yielding plants (Röder et al. 2008). Grain weight is usually represented in plant breeding programs by thousand-grain weight (TGW) and is determined by grain length, grain width and grain thickness (Campbell et al. 1999). The physiological factors controlling grain weight in wheat were resolved to some

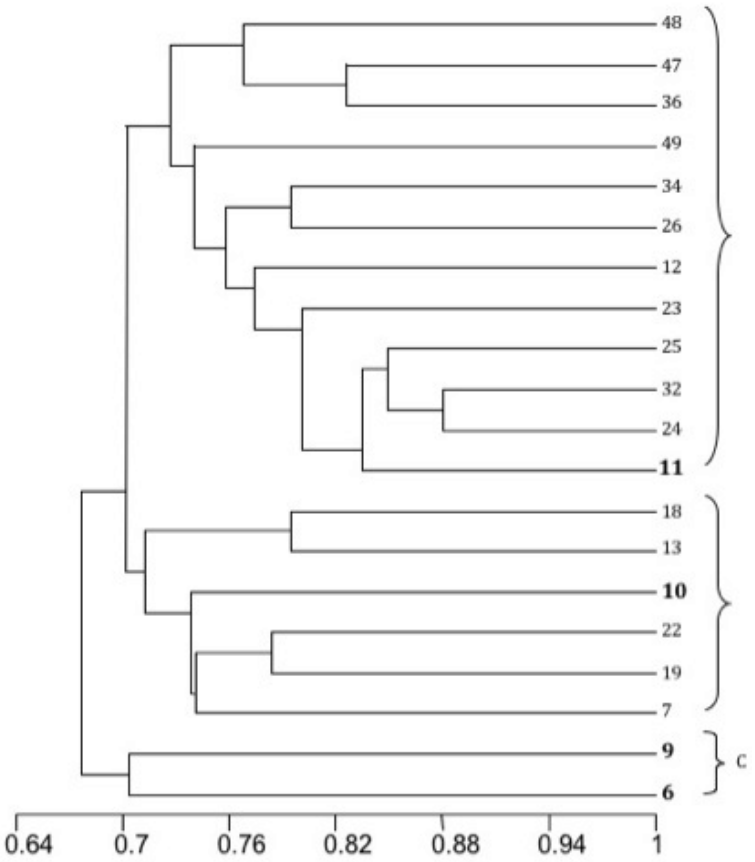

Figure 2. SSR based cluster formation of 20 genotypes of the B-genome synthetic hexaploids (2n = 6x = 42; AABBBB/SS)

extent by Brocklehurst (1977) who reported that grain weight was mainly dependent on the rate of accumulation of dry matter, which in turn was governed by the number of endosperm cells formed. These cell numbers in the endosperm seemed to be regulated by supply of assimilates available to the grain during the first two weeks after anthesis.

Synthetic hexaploid wheats are the products of artificial crossing between *Triticum turgidum* L. (2n = 4x = 28; AABB) and *Aegilops tauschii* Coss. (2n = 2x = 14; DD) accessions, the evolutionary progenitor of common bread wheat (Mujeeb-Kazi et al. 1996). Due to the same genomic constitution both synthetic hexaploids and bread wheats can be readily crossed making synthetic hexaploids a unique germplasm resource for bread wheat breeding. Synthetic hexaploids can act as a vehicle for the introduction of specific characters from these numerous D-genome progenitor accessions into bread wheat backgrounds. The natural hybridization events that formed bread wheat are thought to be lim-

ited, thus the genetic diversity within the recently produced synthetic hexaploid wheats possess novel alleles and genes for biotic and abiotic stress tolerances not currently represented within the bread wheat gene pool. Increased grain size in bread wheat has a favorable effect on milling yield. Large grains have higher endosperm to surface area ratios thus improving milling yields with reduced by-products. Increases in grain yield over the last 40 years have come partly from the use of gibberellic acid-sensitive dwarfing genes (*Rht-1* and *Rht-2*), which were globally distributed during the 'green revolution'. In addition to reducing plant height, these genes increased seed set and the number of kernels per m^2 compared to normal stature wheats. These changes have been accompanied by reductions in grain size. Synthetic hexaploids have been proposed as sources of genetic material for the improvement of thousand-grain weight in bread wheat breeding (Calderini and Orliz-Monasterio 2003). Some synthetic hexaploids have also achieved yields similar to those of check cultivars under drought stress (Trethowan and Mujeeb-Kazi 2008). Backcrossing and top-crossing strategies have been employed to exploit primary synthetic hexaploids for these characters in both elite CIMMYT and local bread wheat backgrounds (Dreccer et al. 2007).

From the limited number of 20 BB (SS) genome hexaploids studied, identifying 4 of superior potent value for usage in breeding is a fairly high frequency and encouraging. The meiotic behavior during the maintenance of these stocks is a notch below that of the A genome hexaploids as more rod bivalents/aneuploidy were observed but with adequate seed set to permit generation advance and promise to exploit for breeding targets. Trait value coupled with molecular diversity status and a unique genetic resource sparsely used in breeding make this germplasm important for further exploitation and additional stock production.

There has been a surge in use of primary gene pool Triticeae species for exploiting novel alleles for wheat improvement. Maximum usage has been of the D-genome accessions of *Ae. tauschii* followed by those of the A-genome. These synthetic hexaploids (AABBDD and AAAABB) are a novel conduit for transferring stress traits from the diploid progenitor to wheat via direct or bridge crossing. Gaining impetus from the above and in an effort to further widen the gene pool, focus shifted to the diploids of the Sitopsis section and narrowed here initially to *Ae. speltoides;* a resource under-exploited in applied agriculture. Its importance in cytogenetic manipulation has text-book coverage but utility in applied agriculture has been minimal other than what has been documented for the few rust genes the diploid has contributed (Chen et al. 1994; Faris et al. 2008; Mago et al. 2009; Zhixia et al. 2011).

Another classical contribution of *Ae. speltoides* has been the production of the Ph^I stock (Chen et al. 1994) that promotes homoeologous wheat/alien chromosome pairing crucial for wide crossing programs. Whether AABBSS synthetics could be produced and maintained was a challenge but if possible then having synthetic amphiploid stocks would be a valuable genetic reservoir of global importance as the full potential of the diploid progenitor accessions could be of usage. Their scientific novelty has been demonstrated and opened doors for harnessing the B-genome (S) diversity akin to what is currently being utilized through the D-genome source and the A. We believe that the stigma of not exploit-

ing the Sitopsis section in direct wheat breeding programs is somewhat minimized through the present observations.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1.* Mean meiotic chromosomal associations at metaphase I of AABBBB(SS) synthetic hexaploids (amphiploids) involving *Triticum turgidum* cultivars and B genome diploid accessions of *Aegilops speltoides*

Electronic Supplementary *Table S2.* The mean crossability data of some B genome synthetic hexaploids; *Triticum turgidum* crossed with *Aegilops speltoides*

Electronic Supplementary *Table S3.* Molecular fingerprinting pattern by SSRs in B-genome synthetic hexaploids (2n = 6x = 42; AABBBB(SS))

Electronic Supplementary *Figure S1.* Spike morphology of B(S)-genome hexaploids derived from *Triticum turgidum* (2n = 4x = 28; AABB)/*Aegilops speltoides* crosses (2n = 2x = 14; BB or SS) from left to right (a to c) as follows: (a) *Triticum turgidum*; (b) *Triticum turgidum* cv. Cerceta/*Aegilops speltoides* (B-13); (c) *Triticum turgidum* cv. Cerceta/*Aegilops speltoides* (B-02)

Electronic Supplementary *Figure S2.* A C-banded 2n = 6x = 42; AABBBB(SS) synthetic hexaploid somatic cell at metaphase showing 4 doses of the B-genome chromosome

Electronic Supplementary *Protocol for meiotic counts*

Electronic Supplementary *Protocol for constitutive heterochromatin banding (C-banding)*

Electronic Supplementary *Single sequence repeats (SSR) analysis*

Electronic Supplementary *Statistical analysis*