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Research Article

Generation of spontaneous *Triticum durum* × *Aegilops tauschii* wheat synthetic amphiploids and their characterization

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Abstract

Aegilops tauschii **(2n=2x=14), the D-genome donor is the closest progenitor to modern wheat representing a large proportion of unexplored genetic variation. Flourishing in the adverse climatic conditions itcan be utilized in the wheat improvement programmes. Wetargeted to develop and characterize the primary synthetic amphiploids from nine different accessions of** *Ae. tauschii* **and one** *Triticumdurum* **cv. PBW114 through spontaneous chromosomal doubling.Being fertile with ~50% survival rate, the amphiploidswere selfed for five generations without any selection and 38 F_6 amphiploids were obtained. They were evaluated for chromosome number and pairing behaviour, different agro-morphological traits and diseases of leaf rust, yellow rust and powdery mildew. Nineteenamphiploids had 2n = 42 chromosomes, four had2n=28, two had 41 chromosomes.While in the remaining 12 amphiploids, the chromosome number varied from 24 to 42, some with abnormal pairing. Large variations were observed in agromorphological traits with 32 amphiploids showing better thousand grain weight than the hexaploid check varieties.. Response to the three targeted diseases varied widely across the panel. Twenty-fourD-genome specific SSRs used to assess the genetic diversity showed thatthe amphiploids from the same cross combinations weregrouped differently. We observed that the development of a new species is a complex event,and combining divergent genomes into one nucleus with chromosome doubling inflicts considerable stress on a newly emerged species with rapid genomic instability in nascent allopolyploid individuals to enable their immediate survival.However, this is an effective and novel means of creating diversityfor wheat improvement.**

Keywords: *Triticum durum, Aegilopstauschii,* synthetic amphiploid, leaf rust, yellow rust, powdery mildew, thousand grain weight

1. Introduction

Present day bread wheat (*Triticum aestivum* L., 2n=6x=42) originated through a natural hybridization between durum wheat (*T. turgidum* L. subsp. *durum,* 2n=4x=21) and *Aegilops tauschii* Coss. (2n=2x=14). Genetic diversity

of bread wheat progenitors is not fully represented in the hexaploid wheat as only a few progenitors were involved in the original hybridization event (Dreisigacker et al. 2008; Li et al. 2014). Repeated human interventions through domestication andselections for better cultivars further reduce this diversity (Feuillet et al. 2007; Singh et al. 2018). Due to involvement of few selective parents in the breeding programmes, the chances of identifying new variants/genes in the cultivated germplasm is further narrowed down,stagnating the wheat improvement(Tilman et al. 2011)as are the environmental impacts of agricultural expansion. Here, we project global demand for crop production in 2050 and evaluate the environmental impacts of alternative ways that this demand might be met. We find that per capita demand for crops, when measured as caloric or protein content of all crops combined, has been a similarly increasing function of per capita real income since 1960. This relationship forecasts a 100–110% increase in global crop demand from 2005 to 2050. Quantitative assessments show that the environmental impacts of meeting this demand depend on how global agriculture expands. If current trends of greater agricultural intensification in richer nations and greater land clearing (extensification. Thus along with strong breeding programmes, there is a need for well planned pre-breeding programmes to increase the wheat genetic diversity.

As a small fraction of the genetic diversity is represented in the elite cultivated wheat gene pool, the reversal of this breeding bottleneck might be attempted*via* reintroducing wild progenitors in the genetic background of wheat (Feuillet et al. 2007; Redden 2013). The wild relatives of wheat have high survived the unfavourable conditions since a long time without any human intervention and thus have acquired the adaptation potential and survival capability even under extreme conditions (McCouch 2004). Furthermore, as these wild species have been dispersed to different parts of the world from their centre of origin, a lot of variation for different traits is available in single wild species, and these gene pools can serve as a genetic stock for fastening the wheat improvement process(Tanksley and McCouch 1997). Using wild relatives can as such help in accelerating the process of wheat improvement by identifying desired genes/variants and identifying novel variants in the associated regulatory regions (Yumurtaci 2015). Since wheat does not have any hexaploid wild relative, in search of an efficient strategy to utilize the variation present in the wild progenitors, the recreation of cultivated bread wheat to produce 'synthetic hexaploidwheats' has gained much attention during the past few decades (Warburton et al. 2006; Cox et al. 2017).

Synthetic hexaploid wheat is the recreation of one of the most remarkable polyploidy crop in nature, wheat that arose by the cross of three different species via two rounds of genome hybridizations. A number of synthetic hexaploid wheats (SHWs) has been reported using different combinations of tetraploid and diploid wheats.Different reports of using *T. durum, T. dicoccum,T. dicoccoides,T. timopheevii*, *T. carthlicum*,*T. persicurn*var. *darginicurn*for the development of synthetic wheatsare available in the literature(Goncharov et al. 2007; Niwa et al. 2010; Megyeri et al. 2011; Mikó et al. 2014; Daskalova et al. 2016)AABB. Similarly, a number of different diploid progenitors like *T. boeoticum*, *T. monococcum*, *Ae. speltoides* and *Ae. tauschii, Ae. longissimi, Ae. kotschyi,*have been usedin different combinations with tetraploid wheats to develop synthetic hexaploid wheat (Cao et al. 2000; Valkoun 2001; Noori 2005; Rawat et al. 2009; Megyeri et al. 2011). Among these different attempts, *T. durum-Ae. tauschii* derived SHW has been defined as a successful,better yielding and quick outcome due to a close evolutionary relationship between the two (Yang et al. 2009; Zhang et al. 2010)research advances on the utilization of synthetic hexaploid wheat for wheat genetic improvement in China are reviewed. Over 200 synthetic hexaploid wheat (SHW. F_1s of the *T. durum-Ae. tauschii* based crosses are easily converted into hexaploid by one cycle of selfingthrough the formation of unreduced gametes. The hexaploids thus formed are stable and are identical to the natural bread wheat in genomic constitution(Zhang et al. 2010). An unlimited amount of diversity in available *Ae. tauschii*germplasmfor wheat improvement makes it an excellent choice to enrich genetic availability and broaden genetic diversity in wheat gene pool (Sharma et al. 2014)

Reported and utilized first time by the International Maize and Wheat Improvement Center (CIMMYT, Mexico) for major traits, *T. durum-Ae. tauschii* based SHW have proved thepractical value of this new diversity from the resistance to a range of biotic and abiotic stresses to different quality traits (Mujeeb-Kazi et al. 1996; van Ginkel and Ogbonnaya 2007; Lopes and Reynolds 2012;

Ogbonnaya et al. 2013; Jafarzadeh et al. 2016). SHWs are a proven source of genetic diversity to improve yield (van Ginkel and Ogbonnaya 2007; Reynolds et al. 2007; Ogbonnaya et al. 2013), soil-borne pathogen (Mulki et al. 2013)we employed a genome-wide association approach in which 332 synthetic hexaploid wheat lines previously screened for resistance to CCN and PN were genotyped with 660 Diversity Arrays Technology (DArT, insect (El Bouhssini et al. 2013; Joukhadar et al. 2013), and fungal disease resistance (Zegeye et al. 2014; Jighly et al. 2016), as well as heat (Jafarzadeh et al. 2016), boron (Emebiri and Ogbonnaya 2015) and salinity tolerance (Dreccer et al. 2004; Ogbonnaya et al. 2008). The present studyreports the generation and characterization *T. durum-Ae. tauschii*based synthetic amphiploids developed at Punjab Agricultural University, Ludhiana.

2. Materials and Methods

2.1 Plant material

The present study includes thirty-eight amphiploids derived by crossing *T. durum* cv.PBW114 (AABB) as female, with nine different accessions of *Ae. tauschii* (D^tD^t). In addition, three hexaploid wheat varieties *viz*., PBW550, HD2967, and PBW621 were used as checks(Table S1). *Ae. tauschii* accessions are designated as '*AT*' followed by their accession number given by Punjab Agricultural University (PAU), Ludhiana. Each of 38 amphiploid was designated as word 'Syn', followed by *Ae. tauschii* accession number and a serial number.Amphiploid generation and evaluation was done in the experimental fields of School of Agricultural Biotechnology, PAU, Ludhiana.

2.2 Meiotic observations

To study chromosome number and their pairing behaviour, immature spikes (prior to emergence from flag leaf) of each 38 amphiploidswas fixed in Carnoy's solution I (ethanol–chloroform–acetic acid; 6:3:1) for 24 h. Then the spikes were transferred to 70% ethanol for storage. Anthers were squashed in 2% acetocarmine and visualised under light microscope. Slides were prepared from pollen mother cells (PMCs) and different meiotic stages of prophase, metaphase and anaphase.

2.3 Phenotypic evaluation and data analysis

Field assessment was carried out in the experimental fields of School of Agricultural Biotechnology, PAU, Ludhiana for three years. Each amphiploid was planted in 1.5m paired rows with a row to row distance of 15cm and plant to plant distance of 5 cm in three replications in Randomised Complete Block Design (RCBD). The whole material was planted along with parental line *T. durum*cv. PBW114 and cultivated hexaploid wheat varieties, *viz.,* PBW550, HD2967, PBW621 as checks (Table S1). The tenrandom fully emerged spikes were tagged in each line and data were recorded on peduncle length (PL), spikelets per spike (SpS), spike length without awns (SLwoA),awn length (AL), days to flowering (DoF)and thousand grain weight (TGW) and meanswere calculated. PL was measured as the length of fully emerged peduncle from leaf sheath. The data was analysed using "Agricolae" version 1.3.1 package of Rstudio(Mendiburu 2019). The trait variation in different amphiploid groups was plotted using "GGplot2" version 3.3.2 and "GGpubr" version 0.4.0 packages of Rstudio(Wickham 2016; Kassambara and Kassambara 2020). The estimates of different genetic parameters *e.g*., mean, range, genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), broad sense heritability (h^2), genetic advance (GA) for different *AT* derived synthetic amphiploids were also estimated. GCV and PCV for were estimated using the formula:

$$GCV(\%) = \frac{\sqrt{\delta^2 g}}{x} \times 100$$

$$PCV(\%) = \frac{\sqrt{\delta^2 p}}{x} \times 100$$

where: $\delta^2 g$ = genotypic variance, $\delta^2 p$ = phenotypic variance and x =sample mean (Singh and Chaudhary 1977). Broad sense heritability (h^2) estimate of each trait was computed according to the procedure outlined by Falconer (1996) as:

$$h^2 = \frac{\delta^2 g}{\delta^2 p}$$

GCV and PCV are broadly classified in three categories, high (>20%), moderate (10-20%) and low (<10%) while $h^2$is categorized in four groups, low (<40%), medium (40-59%), moderately high (60-79%) and very high (>80%) (Johnson et al. 1955) that is, the fraction of variance in phenotypic expression that arises from genetic effects. However, the different methods employed do not necessarily estimate the same thing. For example, variance and regression methods of estimating heritability of F 2 plant differences estimate the same thing only if all gene effects are additive.

The nature of the selection units (plant, plot, mean of several plots, etc.. GAM is categorized as low (0- 10%), moderate (10-20%) and high (>20%) (Sivasubramanian and Madhavamenon 1973).Pearson correlation coefficients (r) among different traits were calculated using the cor() function in "stats" version 3.6.1 package of RStudio (R Core Team 2019) and the "corrplot" version 0.84 package was used to plot the results (Wei and Simko 2017).

Screening for leaf rust, stripe rust and powdery mildew

The amphiploids were screened against leaf rust (LR), yellow/stripe rust (YR) and powdery mildew (PM) by creating the epidemic of naturally occurring disease by planting infector rows after every 20 rows and all across the experimental field where amphiploids were grown.

2.4 Leaf rust and stripe rust screening

Two different set of amphiploids were grown in Punjab Agricultural University, Ludhiana (30.9°N 75.85°E) one for screening against LR and other against YR, both sown as a single row of 1.5m. Each rust was scored at least thrice to confirm the respective scores. Artificial rust epidemic was created by spraying the experimental material with the mixture of uredinospores of known leaf rust (77-5, 77-2, 104-2) and stripe rust (78S84, 100S119) races, mixed with local inoculum collected from farmer's field (1g of inoculum per 10L water, using one drop of Tween-20 as dispersant). The experimental area was regularly irrigated to create congenial conditions for rust development. The disease severity was scored as percentage of leaf area covered by rust following the modified Cobb's scale, as developed by Peterson et al. (1948) as no infection (0), resistant (R), moderately resistant (MR), moderately susceptible (MS) and susceptible (S). Leaves which showed no visible signs of chlorosis, necrosis or uredia were designated as Resistant (R) type, while moderately resistant (MR) type reaction had small uredia surrounded by chlorotic/necrotic area. Moderately susceptible (MS) type of reaction represented medium sized uredia surrounded by chlorotic area. While in Susceptible (S) type reaction, leaf was covered with large sized uredia with little or no necrosis. Further, trace severity of plant with a resistant type field response was designated as TR response, 5% severity with a MR field response was designated as 5MR and so on (www.wheatdoctor.org).

2.5 Powdery mildew screening

PM reactions were scored on a single row of the field-grown plants at Palampur, Himachal Pradesh, India (32.1°N, 76.5°E) under natural infection. The disease incidence was observed at three different intervals after flowering stage. The maximum score of disease incidence at the final stage was counted as the final disease score. The disease severity was evaluated at the adult plant stageafter 14 days of inoculation on a 0–9 infection type (IT) scale viz., 0 = free from infection; 1-3 = resistant; 4-6 = moderately resistant; 7-8 = moderately susceptible and 9 = highly susceptible (Saari and Prescott 1975).

2.6 Molecular marker analysis

The total genomic DNA from each genotype was extracted following the protocol of Saghai Maroof et al. (1994)71 variants were observed in a sample of 207 accessions of wild and cultivated barley. Analyses of wheat- barley addition lines and barley doubled haploids identified these variants (alleles. The quality and quantity of DNA was assessed using Nano-Drop spectrophotometer (Thermo Scientific NanoDrop™ 1000 Spectrophotometer). A set of 24 SSR primer pairs selected from seven D genome chromosomesof wheat (Table S2) were amplified to assess the molecular diversity among 38 amphiploids and one *T. durum* variety PBW114 (Table S1). The PCR was carried out in a final volume of 20µl containing 50-100ng template DNA, 0.2mM of each dNTPs, 1X PCR reaction buffer (10mM Tris HCl + 50mM KCl + 0.01% w/v gelatin, pH 8.3), 1.5mM $MgCl_2$, 0.25mM each forward and reverse primers and one unit of Taq Polymerase. Amplification was performed using polymerase chain reaction in a 96-well microtiter plate using Eppendorf Master Cycler. The initial denaturation step was performed at 94°C for 5 minutes followed by 35 cycles each consisting of 1-minute denaturation at 94°C, 1-minute annealing at 52-58°C and 1-minute polymerization at 72°C with a final extension for 10 minutes. Amplified products were resolved using 6% polyacrylamide gel prepared in 0.5X TBE buffer. The gels were visualized under UV light and photographed using photo gel documentation system (Alphaimager HP, Alpha Innotech). Scoring of the SSR alleles was performed manually for all the genotypes.

2.7 Genetic diversity evaluation

To evaluate genetic diversity of syntheticamphiploids, scoring of alleles amplified by SSRs weredone in a binomial format with '1' for the presence of an allele and '0' for the absence of an allele. Diffused bands or bands showing ambiguity in scoring were taken as missing data and designated '99'. DARwin5 software was used for clustering analysis and to compute pair wise similarity coefficients, a dice similarity matrix was generated using SSR data. Dendrogram was constructed using Unweighted Pair Group Method with Arithmetic Average (UPGMA) algorithm on neighbor-joining method with 1000 bootstraps (Perrier and Jacquemoud-Collet 2006).

2.8 Results

Nine different D^tD^t genome *AT*accessions were crossed as the male parent with *T. durum*cultivar PBW114 (AABB). The triploid (ABD) F_1s thus obtained (2n=x=21) on selfing had spontaneous chromosome doubling.The number of seeds obtained from selfing of different F_1s ranged from 5-20 of which 2-10 seeds germinated in each cross giving a 50% survival rate. Thereafter the selfingwas continued for five generations without any selection and38F_6amphiploids thus obtained were evaluated for different traits (Table S1, Fig. S1). The number of plants/amphiploids surviving fromcrosses of different *AT* accession varied from 9 and 8 from accessions At14328 and At14128 respectively,to one for accessions At14102 and At14200.

2.9 Cytological analysis

The data recorded on total chromosome number, number of univalents, bivalents and multivalents are given in Table S3. The chromosome number in the 38 amphiploids varied from 28 to 42 (Table S3, Fig 1) with 19 having chromosome number 2n = 42 and four amphiploids have 2n=28 with normal chromosome pairing and bivalent formation. Two amphiploids had 41 chromosomes while in 12 of the amphiploids, chromosome number varied from 24 to 42, some with abnormal pairing and presence of univalent and/or trivalents along with bivalents (Table S3). Even sister amphiploids derived from the cross of same *AT* accession showed variation in chromosome number.

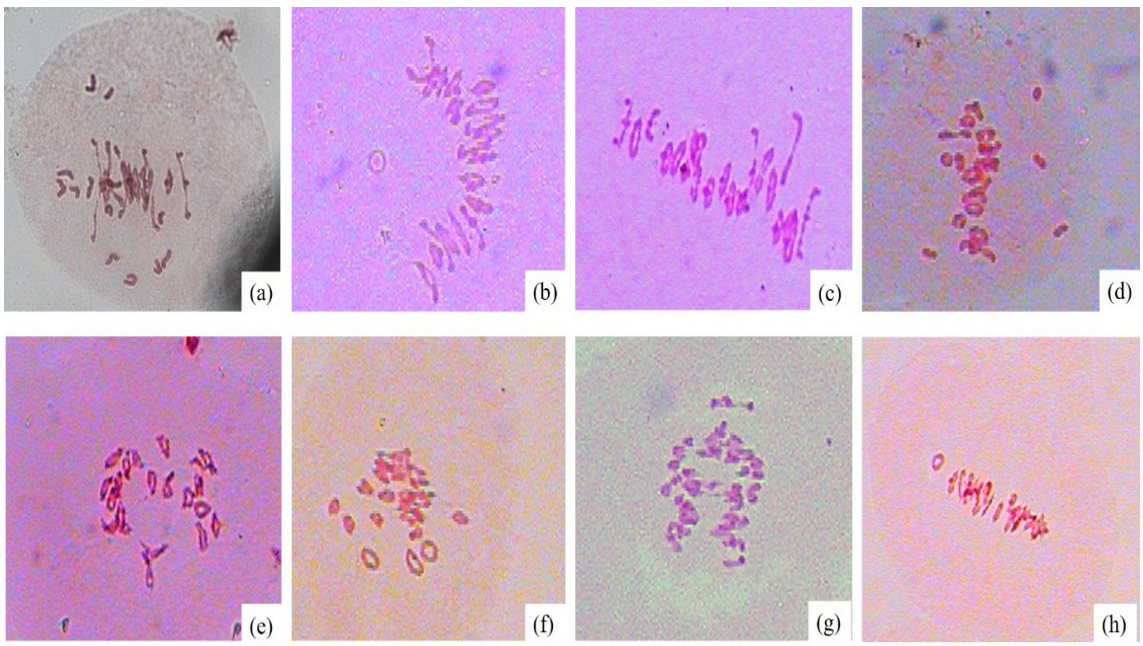

Fig 1 Meiotic observations for various amphiploids. a) TA13 (12 I + 11 II) b) TA69 (1 I + 20 II) c) TA104 (21 II) d) TA151 (7 I + 10 II +15 L) e) TA41 (21 II) f) TA78 (21 II) g) TA84 (21 II) h) TA99 (21 II)

2.10 Phenotypic evaluation of Spike traits

The synthetic amphiploids showed a substantial variation in the observed sixspike traits (Table S4, S5, Fig S2).PCV was ranged from moderate to high in all the traits except DoF. DoF had low PCV (3.14%)as well as GCV (2.26%), while TGW had moderate PCV (12.57%) coupled with low GCV (7.68%). PL, SLwoA andSpShad moderate PCV (16.07%, 17.33% and 14.92%respectively) and GCV (11.48%, 13.32% and 11.61%, respectively).A high PCV (27.92%) and GCV (24.56%) was estimated only for

AL.TGW had low h^2 (37.31) whilePL,SLwoAand DoFhad medium h^2 (51.06%, 59.05%, 51.89%) and SpS and AL had moderately high h^2(60.57%, 77.38%). Further, SLwoA (21.08%) and AL (44.51%) had high, PL (16.90%)and SpS (18.62%) had moderate, and DoF (3.35%) and TGW (9.66%) had low GAM.

Peduncle length(PL) in amphiploids ranged from 11cm in syn9810_TA97 to 24.47cm in syn14328_TA156. As compared to PBW114(19.33cm), 20 amphiploids had higher, 18 had lower, while threeamphiploids had PL similar to this durum parent.In hexaploid checks, HD2967 (19cm) have PL comparable to PBW114 while PBW621 (17.6cm) and PBW550 (14cm) had comparatively shorter PL (Table S5). Variation in PL could not be correlated to donor *AT*accession and the variation in chromosome number of amphiploids.Length of the spike (without awns) in amphiploids varied from 7.67cm (syn14170_TA51) to 13.73cm (syn14328_TA112) with SLwoA of PBW114 being shortest (7cm).Among amphiploids, *AT*acc 14328 derived amphiploids had maximum variation in SLwoAranging from 7.93cm (syn14328_TA113) to 13.73cm (syn14328_TA112) (Table S5). Four amphiploids with 2n=28 *viz.*, syn14170_TA51 (7.67cm), syn14128_ TA42(8.07cm), syn9809_TA74 (9.27cm) and syn14328_ TA113 (7.93cm) had SLwoA comparable to PBW114, but some amphiploids with 2n=42 also had SLwoA in similar range (Table S3, S5). However, two amphiploid syn14128_TA17 (12.00cm)and syn14128_TA43 (10.67cm) had a long spike similar to that of elite varieties despite having a chromosome number of 2n=28. Variation in SLwoAwas independent of donor *AT*accession and chromosome number of amphiploids.

PBW114 has longest awns of 12cmwith awn length of check varietiesranging from 3.67cmin HD2967 to 5.0cm in PBW621 and 5.67cm in PBW550. The AL of amphiploids had a variation of 4.6cm to 12.33cm. Of the five amphiploids with 2n=28 chromosomes, four amphiploids syn14170_TA51 (12.33cm), syn14128_TA42(10.93cm), syn9809_TA74 (10.27cm), andsyn14328_TA113 (11.67) had awn length comparable to PBW114, while syn14128_ TA17 had smaller (7.67cm) awns (Table S5).Amphiploids with 28 chromosomes had comparatively longer awns. The average values for SpS varied from 14.47 (syn14576_ TA139) through 24.20 (syn14128_TA45) in amphiploids. Eight amphiploids had fewer SpS less than PBW114 (17) while compared with hexaploid checks,all amphiploids had lesser SpSthan checks. (Table S5). No correlation of donor *AT* with SpS was concluded.

PBW114 took maximum days to 50% flowering (103.67 days), with syn14328_TA159 was the only amphiploid with DoF more than PBW114 (104.33 days). The check varieties, PBW550, PBW621 and HD2967 took 91.02, 94.06, and 98 days respectively for 50% flowering. Amphiploids took 96-104.33 days for 50% flowering (Table S5). TGW of PBW114 was 40.06gm while that of elite cultivars were 39.19gm (PBW621), 41.32 (HD2967) and 42.55gm (PBW550).In amphiploids,TGW varied from 37.94 to 58.16gm. Allthe amphiploidshad better TGW than PBW114 except Syn14170_TA51 (37.94gm) (Table S5).All amphiploids with 42 chromosomes had higher TGW than those with 28 chromosomes.

2.11 Disease reactionscore to rust and powdery mildew

YR and LR reaction of PBW114 was 20S and 10MR respectively while nine *AT*accessions also did not exceed 20S and 10MS (Table S5, Fig. S3a). However, amphiploids derived from these *AT*accessions showed highly variable reaction both for YR (TR-60S) and LR (0-60S). Five amphiploids were resistant with severityreaction of no to traces (TR) of YR, 17 were moderately resistant with YR severity of 5 to 20 percent (5-20MR), and 16 wereshowed susceptible reaction type with YR severity of 10 -80 percent (10-80S)(Table S5).For LR,27 of amphiploids were completely free from LR, four wshowedmoderate resistance with severity ranging from 5-20percent (5-20MR) and seven were susceptible with LR severityof 10 to 60 percent (10-60S). In amphiploids derived from all the *AT* accessions the disease reaction score varied with both higher and lower reaction scores than the parental lines.

T. durum cultivar, PBW114 was highly susceptible to PM with a score of 9. Among the nine *AT* accessions, one accession pau14128 was "R" with PM score of 1, two were "MR" (PM score 5-6), three were "MS" (PM score 7), and three were "S" with PM score of 9. Of 41 amphiploids only one of the amphiploid Syn14128_TA45 was resistant with infection score of 1 (Table S5, Fig. S3b), six were moderately resistant, 22 moderately susceptible and nine were susceptible with PM score of 9. The PM score of amphiploids was variable irrespective of PM score of *AT*. PM score of 8 amphiploids derived from "R" *AT*acc pau14128, ranged between 1-7. The amphiploids derived

from MR accessions of *AT*acc pau14102 (PM = 5) and pau14200 (PM = 6) showed disease severity score of 9 and 7 respectively. Similarly, PM score of amphiploids derived from "MS" *ATacc* pau9809 and pau9810 were ranged between 5-9. Same was the fate of PM in amphiploids derived from "S" accessions of *AT.*

2.12 Molecular characterization and genetic diversity

Summarized data for the number of alleles detected per primer pair and the PIC values for each of the 24 SSR primers are presented in Table S2. The 24 primers detected a total of 99 alleles in the given genotypes with an average of 4.13 alleles per primer. Polymorphic Information Content (PIC), a measure of allelic diversity at a locus, ranged from 0.14 (WMC 457) to 0.76 (GWM 269) with an average value of 0.54. The higher PIC values of SSR markers revealed their discriminating power in the amphiploids.The dendrogram generated from the dissimilarity matrix grouped all the 48 genotypes (including 41amphiploid, one *T. durum*, PBW114 and nine accessions of *AT*into three distinct clusters designated A, B and C (Table 1, Fig2) further divided into sub-clusters.

Cluster Ahad 20 genotypes (9*AT* accessions and 11 amphiploids) divided into threesub-clusters: A1, A2 and A3 (Fig 2). A1 sub-cluster has 2accessionsof *AT*(At_14576, At_14328) and three amphiploids derived from these accessions, TA159 (2n=42), TA137 (2n=39-42), TA139 (2n=37-39). A2 sub-cluster has five amphiploids from *AT*accession 14128 *viz.*,TA13 (2n=28-41), TA43 (2n=24-28), TA44 (2n=36-42), TA45 (2n=42) and TA48 (2n=41). A3 sub-cluster had seven *AT* accessions and three amphiploids syn14102_TA11, syn14128_TA41 and syn_9810TA99 all with 2n=42.

Cluster B had 21 genotypes and was further subdivided into three sub-clusters B1, B2 and B3 (Fig 2). B1 sub-cluster had 11 amphiploids derived from three different *AT* accessions of At_9810, At_14328, and At_3761. Four of these amphiploids have 2n=42 chromosomes while rest of the amphiploids have chromosome number varying from 37-42. Sub-cluster B2 has seven amphiploids derived from three different *AT* accessions of At_14170, At_9810 and At_9809. Chromosome number of five of these amphiploids were 42 while two have variable chromosome number. B3 sub-cluster has three amphiploids from At_14328, all with 2n=42 chromosomes.Group C includes the only *T. durum* cv PBW114, four supposedly amphiploids with 2n=28 (syn14128_TA42, syn14128_TA17, syn9809_TA74 and syn14170_TA51) and two amphiploid with 2n=42 (syn14328_TA113, syn14200_TA64) (Table 1).

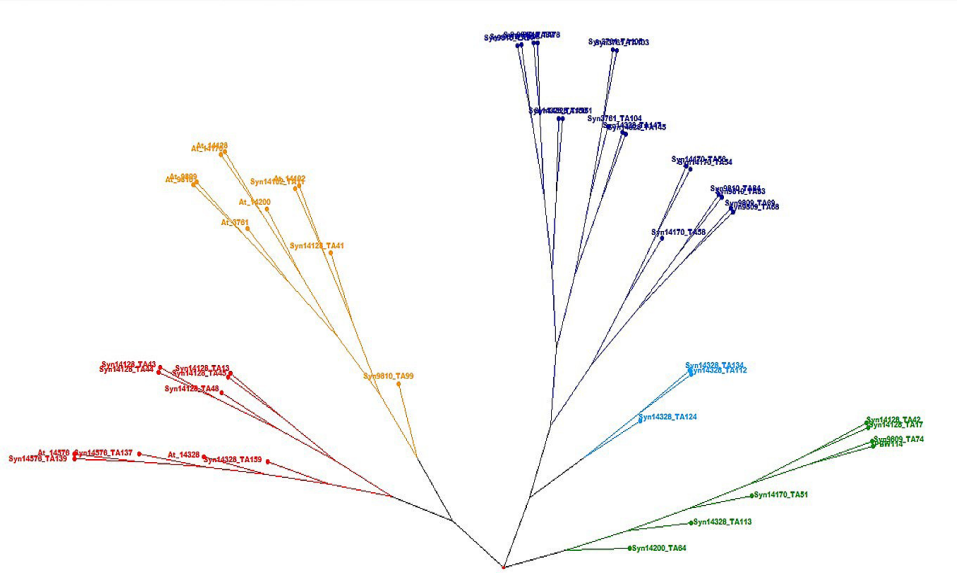

Fig 2 Dendrogram showing grouping of amphiploids, *Ae. tauschii* accessions and PBW114 into different clusters

Discussion 2 Meiosis is an event of high evolutionary importance and, insight into chromosome characteristics and their meiotic behaviour is essential for the utilization of any newly synthesized cultivar in a breeding programme. In the present study, we observed hexaploid (2n=42), tetraploid (2n=28) and aneuploid (2n=28-42) plants in the attempt to develop synthetic amphiploids from the crosses of nine accessions of *AT* with

Table 1 Clustering of amphiploids based on DARwin 5.0 software program

Cluster	Sub-cluster	Number of genotypes	Genotype
A	A1	5	At_14576, At_14328, Syn14576_TA137, Syn14576_TA139, Syn14328_TA159
	A2	5	Syn14128_TA13, Syn14128_TA43, Syn14128_TA44, Syn14128_TA45, Syn14128_TA48
	A3	10	At_9810, At_9809, At_3761, At_14170, At_14128, At_14200, At_14102, Syn14102_TA11, Syn14128_TA41, Syn9810_TA99
B	B1	11	Syn9810_TA78, Syn9810_TA89, Syn9810_TA95, Syn9810_TA97, Syn3761_TA103, Syn3761_TA104, Syn3761_TA105, Syn14328_TA145, Syn14328_TA147, Syn14328_TA151, Syn14328_TA156
	B2	7	Syn14170_TA54, Syn14170_TA56, Syn14170_TA58, Syn9809_TA68, Syn9809_TA69, Syn9810_TA83, Syn9810_TA84
	B3	3	Syn14328_TA112, Syn14328_TA124, Syn14328_TA134
C	C1	7	PBW 114, Syn14128_TA17, Syn14128_TA42, Syn14170_TA51, Syn14200_TA64, Syn9809_TA74, Syn14328_TA113

common *T. durum* PBW114. Since the triploid F_1s were allowed to self, we expected gametes with variable chromosome number due to presence of a single copy of chromosomes and lack of pairing counterparts during meiosis. Union of two unreduced (2n) gametes led to amphiploids with normal chromosome complement (2n=42) (Zhang et al. 2010; Fakhri et al. 2016) and may be affected by parental genotypes and genomic similarity. In the present study, five cultivars of Triticum aestivum and two tetraploid Aegilops species (i.e. Ae.triuncialis and Ae. cylindrica. Formation of unreduced gametes remained a major driving force in polyploidisation and plant speciation(Harlan and deWet 1975; Jauhar 2003) but are negligible. 2. The high frequency of unreduced gamete (upto 50%) in F_1 interspecific hybrids is mainly due to the absence of a common sub-genome among the parents (Fakhri et al. 2016)and may be affected by parental genotypes and genomic similarity. In the present study, five cultivars of Triticum aestivum and two tetraploid Aegilops species (i.e. Ae.triuncialis and Ae. cylindrica. Moreover,2*n* gametes are more adaptive, confer direct fitness advantage and positively selected over gametes with n chromosomes(Kreiner et al. 2017)our knowledge of the prevalence of and evolutionary mechanisms maintaining 2n gametes in natural populations is limited. We hypothesize that 2n gametes are deleterious consequences of meiotic errors maintained by mutation–selection balance and should increase in species with relaxed opportunities for selection on sexual processes (asexuality. Suppressing effect of *Ph1* locus on homeologous pairing also played key role in normal meiotic behaviour of these hexaploid amphiploids(Sears 1976; Matsuoka and Nasuda 2004). On the other hand, chance germination of aneuploid gametes on the stigmatic surface led to amphiploids with variable chromosome number. The occurrence of aneuploids in variable frequencies were observed in newly synthesized hexaploid wheats even in presence of *Ph1* locus (Matsuoka and Nasuda 2004; Zhang et al. 2008). Suppressing genetic factors have been reported in *AT* accessions influencing the expression of *Ph1* locus, leading to chromosome pairing abnormalities. (Fukuda and Sakamoto 1992a, b; Matsuoka et al. 2007)and 18 combinations of normal F1 hybrids were obtained. Their meiosis and the frequency of unreduced gamete formation were observed. From cytological observations, unreduced gametes were formed as follows; restitution of the first meiotic division, and normal second division followed by formation of dyads which developed into two fertile 2n pollen grains. Unreduced gametes were formed with high frequency in the following cases; (1. The sensitivity of one or more genomes to get eliminated during interspecific crosses also induce aneuploidy and different frequencies of aneuploidy were detected in amphiploids(Mestiri et al. 2010). Abnormal chromosome pairing with the presence of univalents and trivalents in many amphiploids could be due to chromosome elimination, but also there may be apparent euploidy due to loss of one and gain of another chromosome (Feldman and Levy 2012; Hao et al. 2013; Li et al. 2015; Gou et al. 2018).

The variation in the observed spike traits of amphiploids using single cultivar of *T. durum*, were attributed to different *AT* accessions. Variations were present not only across amphiploids derived from different accessions of *AT* but also in sister amphiploids derived from the same accession. Most of the amphiploids recorded higher values for SL, SpS and TGW, as compared to PBW114 suggesting the new gene combinations are responsible for longer rachis, internodes, TGW and spikelet number. For SL and SpS amphiploids values are close to hexaploid checks, but for TGW some of the amphiploids outpared the hexaploid checks. Synthetic wheats, wherever synthesized,have been reported to have better agronomic parameters related to plant vigour and yield (Trethowan and Mujeeb-Kazi 2008; Kazi et al. 2013; Dunckel et al. 2017). AL on the other hand was smaller in amphiploids than durum and close to hexaploid checks. Though mechanism underlying for awn length was not worked in amphiploids but a locus (*Antr*) for awnless present on chromosome arm 5D of *AT*is known to contribute towards shorter awn length (Nishijima et al. 2014; Ntakirutimana and Xie 2019). Three amphiploids with 2n=28, had longer awns with durum genome behaviour but one amphiploid Syn14170_TA51 with 2n=42 chromosomes also had longer awns, indicating some complex underlying mechanism. Awn length in the amphiploids, have also contributed to the enhanced TGW over the check varieties and can be used as a selection criterion for utilizing these amphiploids for wheat improvement (Blum 1985). PL showed a range with higher and lower values than PBW114. Effect of PL is expected to be more pronounced on the higher TGW in amphiploids. The upper part of peduncle is known to develop leaf-like autotrophic carbohydrate metabolism when exposed to high irradiance (Wardlaw 1965; Kong et al. 2010), accounting for about 9-12% of the photosynthesis of the stem (Wang et al. 2001). Gebbing (2003) also highlighted that the elongation of the peduncle is similar to leaf elongation in grasses, and its photosynthetic activity can indirectly contribute to grain weight.

Highly variable response to PM, YR and LR pathogens by crossing different *AT* accessions with the same *T. durum* represented the potential diversity of resistance genes and also a diversity in interactions between different genes responsible for modifying this resistance (Cox 1997; Bhatta et al. 2019). Though amphiploids showed variation in chromosome number, we could not correlate variations for disease resistance with the absence of some particular chromosome. The change in disease expression level than parental lines could be attributed to changes in gene expression by shifting from lower to higher ploidy (Assefa and Fehrmann 2000; McIntosh et al. 2011; Zegeye et al. 2014). A lower expression of resistance in amphiploids than parental lines could be due to action of suppressors of resistance to diseases of LR, YR, PM and stem rust (SR) in D genome (Kerber and Green 1980; Ma et al. 1995; Rafique et al. 2012). Hiebert et al. (2020) identified *SuSr-D1*, on D-genome, encoding Med15b. D subunit of the conserved mediator complex, act as suppressor of SR resistance. The presence of alternative alleles of suppressors in some accessions of *AT* may also have allowed the expression of rust resistance genes present in the A or B genome, leading to parallel level of resistance to that of parental lines (Lagudah et al. 1993). Further, mutual/additive effects of *AT* and *T. durum* resistance genes might have resulted in the increased resistance reaction (Szabo-Hever et al. 2018). Since most of the current cultivars established around a few resistance genes against yellow rust, this germplasm has the potential to reduce gene vulnerability issues (Bux et al. 2012).

The present studyshowedgrouping of synthetic amphiploids and their corresponding *AT* accessions into different groups, even the amphiploids from same cross combination fell into different groups. Since *T. durum* parent, PBW114 was common among all amphiploids, and any variation arose due to variation in different accessions of *AT*. Two *AT* accessions were in sub-cluster A1 and seven in A3 sub-cluster of group A, while amphiploids derived from it distributed into all the three groups formed. Amphiploids generated in present study had variable number of chromosomes from ranging from 28-42 representing no to seven D genome chromosomes. This led to variable chromosomal recombinations and rearrangements, affecting grouping based on the presence or absence of some D genome specific alleles. This was further added by different number of amphiploids derived from cross of different *AT* accession, ranging from one amphiploid from AT accession 14200 and 14102 to nine derived from AT accession 14328. A total of 99 alleles were detected by 24 D genome specific primers in 48 genotypes with an average of 4.13 alleles per primer. Higher PIC values represent the discriminating power of SSRs in the amphiploids under investigation. The PIC

values of some common SSR viz., CFD7 (0.87), CFD49 (0.60), CFD42 (0.73), WMC93 (0.51), GWM383 (0.82), CFD282 (0.76) and WMC112 (0.81) have also been represented by earlier study of our group (Chhuneja et al. 2010) on *AT* accessions. Chen & Li (2007) also reported the delineation of 95 synthetic hexaploid wheats into five major clusters in accordance with their pedigree.

3. Concluding remarks

Combining divergent genomes into one nucleus with chromosome doubling inflicts considerable stress on a newly emerged species with rapid genomic instability in nascent allopolyploid individuals to enable their immediate survival (Chen et al. 2009; Feldman and Levy 2009)but this software lacks genetic and epidemiological related functions. A general tool to do basic genetic and epidemiological analysis and data conversion for MS-Excel is needed. Findings. The SNP-tools package is prepared as an add-in for MS-Excel. The code is written in Visual Basic for Application, embedded in the Microsoft Office package. This add-in is an easy to use tool for users with basic computer knowledge (and requirements for basic statistical analysis.Multiple factors were governing in defining the final morphology of amphiploids, including cumulative action of *Ae. tauschii* and *T. durum* genomes, chromosomal rearrangements, chromosomal elimination, homeologous recombination, gene silencing, duplication, epigenetic changes, pseudogenization,action of suppressors and elimination of low and high copy sequences leading to variation (Levy and Feldman 2004; Feldman and Levy 2009, 2012; Ma et al. 2014). Combining multiple genomes in one nucleus causes interaction between different regulatory networks, the homeologous genomes and genes may follow non-functionalization, neo-functionalization or sub-functionalization with deletion, acquiring new functions or partitioning of ancestral functions respectively (Madlung et al. 2005; Chaudhary et al. 2009; Jackson and Chen 2010). Newly synthesized allopolyploids (amphiploids) can induce a variety of rapid and reproducible genomic changes. Inspite of so much variations in chromosome number and pairing behaviour, their morphology and reaction to different diseases, these are still useful pre-breeding material for improvement of various traits providing a backbone study for initiating future genetic analyses that would unravel interesting information around the chromosomal/gene contribution to resistance. Aneuploidy is not a limiting force here as due to allohexaploid nature, wheat can easily tolerate it and thus these aneuploids can even be utilised in variable studies, including wheat improvement.

Declarations: The authors declare that there is no conflict of interest

Ethics approval: NA

Authors' contributions: Generation and maintenance of synthetics amphiploids done by PC and SK. Disease data collection done by GSD, RS and SK. Agro-morphological data was collected by AK, SK and LKD. Molecular marker analysis was performed by Kunal. Cytogenetic analysis was performed by CK and DS. Final compilation of data, analysis, and preparing draft of manuscript was done by AK, LKD, GSD and SK.

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