Phenotypic effects of A^m genomes in nascent synthetic hexaploids derived from interspecific crosses between durum and wild einkorn wheat

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Abstract

Background Allopolyploid speciation is a major evolutionary process in wheat (*Triticum* spp.) and the related *Aegilops* species. The generation of synthetic polyploids by interspecific crosses artificially reproduces the allopolyploidization of wheat and its relatives. These synthetic polyploids allow breeders to introduce agriculturally important traits into durum and common wheat cultivars. This study aimed to evaluate genetic and phenotypic diversity in wild einkorn (*Triticum monococcum* ssp. *aegilopoides*) accessions, to generate a set of synthetic hexaploid lines containing the various A^m genomes from wild einkorn, and to reveal their agronomic characteristics.

Results We examined the genetic diversity of 43 wild einkorn accessions using simple sequence repeat markers covering all the chromosomes and revealed two genetically divergent lineages, L1 and L2. The genetic divergence between these lineages was linked to their phenotypic divergence and the climate of their habitats. L1 accessions were characterized by early flowering, fewer spikes, large spikelets, and low-density spikelets compared to L2 accessions. These trait differences could have resulted from adaptation to their different habitats. We then developed 42 synthetic lines containing the AABBA^mA^m genome through interspecific crosses between *T. turgidum* cv. Langdon (AABB genome) as the female parent and the wild einkorn accessions (A^mA^m genome) as the male parents. Two of the 42 AABBA^mA^m synthetic lines exhibited hybrid dwarfism. Phenotypic variation, especially for days to flowering and spikelet-related traits, in the synthetic lines was significantly correlated with that in their wild einkorn parents. The phenotypic divergence between L1 and L2 accessions also reflected phenotypic differences among the synthetic lines. Furthermore, the AABBA^mA^m synthetic lines had longer spikelets and grains, long awns, high plant heights, soft grains, and late flowering, which are distinct from other synthetic hexaploid wheat lines such as AABBA and AABBDD.

Conclusions Wild einkorn genetically and phenotypically diverged into two lineages. The phenotypic variations in the synthetic lines reflected the divergence among wild einkorn. Utilization of various A^m genomes of wild einkorn resulted in wide phenotypic diversity in the AABBA^mA^m synthetic hexaploids and provides promising new breeding materials for wheat.

Background

Allopolyploid speciation is one of the major evolutionary processes in wheat and its relatives. For the emergence of common wheat (*Triticum aestivum* L., AABBDD genome), triploid hybrids with the ABD genome were produced from interspecific crosses between tetraploid wheat (*Triticum turgidum* L., AABB) and diploid wild wheat (*Aegilops tauschii* Coss., DD), and unreduced gametes were formed in pollen and egg cells of the ABD hybrids. This evolutionary process can be reproduced through artificial crosses, and the resulting allohexaploid plants are called synthetic wheat hexaploid lines [1]. Synthetic wheat hexaploids have been useful in the study of genetic and epigenetic modifications in chromosomes during allohexaploidization and common wheat speciation [2, 3, 4, 5]. The synthetic wheat lines with the AABBDD genome have been used as bridges to introduce agriculturally important traits from *Ae. tauschii*

into common wheat [6, 7, 8]. Besides the AABBDD synthetic hexaploids, various synthetic allopolyploid lines have been exploited among wheat and its wild relatives, and alien chromosome addition lines and introgression lines have been produced to study the effects of the added and introduced chromosomal regions of the wild relatives.

Einkorn wheat (*Triticum monococcum* L., A^mA^m) includes two subspecies: ssp. *monococcum* as the cultivated form and ssp. *aegilopoides* (Link) Thell. (syn. *T. boeoticum* Boiss) as the wild form. *Triticum urartu* Tumanian ex Gandilyan is the A-genome donor of tetraploid and common wheat [9] (Fig. 1a). Interspecific hybrids between *T. monococcum* and *T. urartu* are almost sterile, and many chromosomal rearrangements occur between the A and A^m genomes [10, 11], indicating genetic differentiation of the two genomes. Wild einkorn wheat, ssp. *aegilopoides*, displays wide genetic diversity [12, 13, 14] and is a valuable resource for disease resistance and grain quality-related characteristics in wheat breeding [15, 16, 17, 18, 19, 20, 21], as well as drought stress tolerance [22].

Synthetic hexaploid wheat lines containing the A^m genome have been produced and used for breeding. For example, AABBA^mA^m synthetic lines were produced from crosses between *T. turgidum* and *T. monococcum* ssp. *monococcum* [15, 23, 24] and the AAGGA^mA^m synthetic lines from crosses between *Triticum timopheevi* Zhuk. and *T. monococcum* ssp. *monococcum* [25]. Triploid F₁ plants between tetraploid wheat and *T. monococcum* are sterile, whereas the AABBA^mA^m amphiploids are meiotically stable and fully fertile [23]. Recently, an A^m genome-specific single nucleotide polymorphism (SNP) marker set was developed to characterize the introduced chromosomal segments of *T. monococcum* ssp. *monococcum* in common wheat [26]. Expression of the high-molecular-weight glutenin subunit gene *Glu-1* and the stripe rust resistance from *T. monococcum* ssp. *monococcum* was successfully confirmed in the AABBA^mA^m synthetic lines [7, 15]. Two powdery mildew resistance genes were transmitted to common wheat through a cross between the F₁ progeny (ABA^m) of tetraploid wheat and *T. monococcum* ssp. *aegilopoides* with common wheat [27]. A stripe rust resistant quantitative trait locus (QTL) was also transferred from *T. monococcum* ssp. *aegilopoides* to common wheat using tetraploid wheat as a bridging species [19]. Therefore, AABBA^mA^m synthetic lines are expected to be useful as bridges to introduce desirable genes into durum and common wheat cultivars.

The durum wheat cultivar Langdon (Ldn) is an efficient female parent used to produce synthetic hexaploid wheat lines with the AABBDD genome [1], and many of the synthetic hexaploids have been developed through crosses between Ldn and diverse *Ae. tauschii* accessions [28, 29]. The AABBDD synthetic lines are useful for evaluating the effect of D-genome diversity in the hexaploid background. Synthetic hexaploid lines were recently developed through crosses of Ldn and various accessions of *Aegilops umbellulata* Zhuk. [30, 31]. The AABBUU synthetic lines are expected to enlarge the diversity in grain hardness, heading/flowering date, and spike/spikelet morphology in common wheat. In this study, we generated a set of synthetic hexaploid lines with the various A^m genomes from accessions of *T. monococcum* ssp. *aegilopoides*. The nascent AABBA^mA^m synthetic hexaploids should be differentially affected by the different A^m genome of each ssp. *aegilopoides* accession. First, we evaluated genetic

diversity and phenotypic variations in 43 wild einkorn accessions. Then, we generated the AABBA^mA^m synthetic hexaploids using the 43 wild einkorn accessions as the pollen parents and Ldn as the female parent and examined phenotypic variations in the synthetic lines to evaluate the transmission of traits. Finally, we compared phenotypes between the AABBA^mA^m synthetic lines and previously developed synthetic lines that shared the common tetraploid wheat female parent Ldn and revealed unique characteristics of the AABBA^mA^m synthetic lines.

Results

Population structure of the wild einkorn wheat accessions

First, we characterized the genetic diversity and traits of 43 wild einkorn wheat (*T. monococcum* ssp. *aegilopoides*) accessions. To clarify the intraspecific genetic diversity and population structure of wild einkorn, simple sequence repeat (SSR) marker-based PCR analysis was performed using the 43 wild einkorn accessions that were used for creating the synthetic hexaploids (Additional file 1: Table S1). One *T. urartu* accession was used as an outgroup species. Forty-two SSR markers covering all the chromosomes were selected based on the linkage maps of the A genome [32, 33] (Additional file 1: Table S2). The genotype accumulation curve plateaued at 15 SSR markers, indicating that these markers were enough to discriminate between the genotypes of the tested accessions (Additional file 2: Fig. S1). All the tested markers were polymorphic and had two to seven alleles (Additional file 1: Table S3). Mean Simpson's index, expected heterozygosity, and evenness for the SSR markers were 0.4639, 0.4747, and 0.6993, respectively.

To estimate the population structure of the 43 wild einkorn accessions, we conducted Bayesian clustering analysis. The delta K for differing number of subpopulations presumed two clusters ($k = 2$) to be optimal (Fig. 1b, c). These two clusters corresponded to the two distinct lineages in the previous RNA-seq-based polymorphism analysis [34]. The lineage mainly distributed in southern Turkey, Iraq, and Iran was called Lineage 1 (L1), and the other lineage distributed in Turkey, Greece, and Armenia was called Lineage 2 (L2) (Fig. 1d). These two lineages were also detected in the UPGMA phylogenetic tree (Additional file 2: Fig. S2). *T. monococcum* ssp. *aegilopoides* KU-8143 was classified into L1 in Bayesian clustering analysis (Fig. 1c), but it belonged to L2 in the tree (Additional file 2: Fig. S2). KU-8143 could be a recombinant between L1 and L2. We designated KU-8143 as L1 for subsequent analyses.

To evaluate the genetic diversity of L1 (29 accessions) and L2 (14 accessions), we estimated the Simpson's index and expected heterozygosity for these lineages. The Simpson's index for L1 and L2 were 0.9655 and 0.9286, respectively. Expected heterozygosity for L1 and L2 was 0.3771 and 0.4571, respectively. These results indicate that the genetic diversity for L2 was higher than that for L1.

Phenotypic differences between the two lineages of wild einkorn wheat

Plant architecture was distinct between the two wild diploid wheat species, *T. monococcum* ssp. *aegilopoides* and *T. urartu* (Fig. 1a, Table 1, Additional file 1: Table S4, Table S5). Heading date (HD) and

flowering date (FD) were earlier, plant height (PH) was higher, and spike length (SL) was longer in wild einkorn than in *T. urartu*. The number of spikelets per spike (NSp) was higher in wild einkorn than in *T. urartu*. To estimate phenotypic variations in the 43 wild einkorn accessions, we evaluated 26 traits in two seasons. Sixteen traits, including flowering date and heading date; awn-related traits including top awn length (TAL), middle awn length (MAL), and bottom awn length (BAL); 5th internode length (5InL), leaf width (LW), and flag leaf width (FLW); and spikelet-related traits including spikelet length (SpL), spikelet width (SpW), number of spikelets (NSp), spikelet density (SpD), and length-width ratio (SpLWr), showed significant correlations between the two seasons, implying that the variations of these traits among the accessions were stable. By contrast, leaf length, flag leaf length, spike width (SW), and spike length and stem-related traits including plant height, 2nd internode length (2InL), 3rd internode length (3InL), 4th internode length (4InL), and stem length (StL) showed no significant correlations between the seasons. Variations of these traits were not stable and could be influenced by environmental factors (Additional file 2: Fig. S3). To compare the extent of variation among the traits, we calculated the coefficient of variation (CV). Top awn length, 5th internode length, and self-seed fertility (SSF) were highly variable among the accessions (CV > 0.46), whereas heading date, flowering date, spikelet length, and spikelet width were less variable (CV < 0.10) (Table 1, Additional file 1: Table S4).

Table 1. Variation in traits of *T. monococcum* ssp. *aegilopoides* and the AABBA^mA^m synthetic hexaploids in the season 2017–2018.

		<i>T. monococcum</i> ssp. <i>aegilopoides</i>		AABBA ^m A ^m synthetic hexaploids	
		Mean ± SD	CV	Mean ± SD	CV
HD	Heading time (days)	170 ± 9.99	0.059	156 ± 2.99	0.019
FD	Flowering time (days)	176 ± 9	0.051	160 ± 2.12	0.013
FLL	Flag leaf length (cm)	6.75 ± 2.025	0.300	25 ± 4.62	0.185
FLW	Flag leaf width (m)	0.513 ± 0.1069	0.208	1.366 ± 0.16	0.120
LL	Leaf length (cm)	12.9 ± 2.85	0.221	36.4 ± 5.41	0.149
LW	Leaf width (cm)	0.724 ± 0.144	0.199	1.434 ± 0.13	0.091
TAL	Top awn length (cm)	6.42 ± 2.985	0.465	10.02 ± 2.06	0.206
MAL	Middle awn length (cm)	10.37 ± 2.7	0.260	14.59 ± 1.61	0.111
BAL	Bottom awn length (cm)	9.06 ± 2.237	0.247	11.74 ± 1.46	0.124
1InL	1st Internode length (cm)	31 ± 7.48	0.241	57.5 ± 11.65	0.203
2InL	2nd Internode length (cm)	16.4 ± 2.96	0.180	25.8 ± 3.05	0.118
3InL	3rd Internode length (cm)	11.18 ± 1.8	0.161	20.8 ± 2.81	0.135
4InL	4th Internode length (cm)	8.18 ± 1.58	0.193	18.28 ± 2.55	0.140
5InL	5th Internode length (cm)	5.02 ± 2.88	0.574	13.08 ± 7.14	0.546
StL	Stem length (cm)	71.6 ± 11.27	0.157	135.5 ± 15.94	0.118
PH	Plant height (cm)	81.3 ± 11.7	0.144	147.1 ± 16.00	0.109
SW	Stem width (cm)	0.119 ± 0.021	0.176	0.246 ± 0.03	0.133
SL	Spike length (cm)	9.69 ± 1.411	0.146	11.58 ± 1.29	0.111
SLwA	Spike length with awn (cm)	19 ± 2.868	0.151	23.8 ± 1.76	0.074
SN	Number of spikes	6.76 ± 2.34	0.346	4.99 ± 1.34	0.269
NSp	Number of spikelets	25.1 ± 4.44	0.177	24.5 ± 2.73	0.111
SpD	Spikelet density	2.6 ± 0.314	0.121	2.13 ± 0.24	0.112
SpL	Spikelet length (cm)	1.18 ± 0.09511	0.081	1.67 ± 0.09	0.054
SpW	Spikelet width (cm)	0.263 ± 0.02587	0.098	0.544 ± 0.04	0.070
SpLWR	Length-width-ratio of spikelet	4.52 ± 0.342	0.076	3.08 ± 0.21	0.069
GAS	Grain area size (mm ²)	6.09 ± 1.65	0.271	13.9 ± 2.87	0.206
GPL	Perimeter length of grain (mm)	15.2 ± 1.75	0.115	20.3 ± 1.84	0.091
GL	Grain length (mm)	6.65 ± 0.75	0.113	8.84 ± 0.78	0.088
GW	Grain width (mm)	1.22 ± 0.26	0.215	2.13 ± 0.36	0.169
GC	Grain circularity	0.327 ± 0.05	0.155	0.42 ± 0.05	0.110
SSF	Selfed seed fertility (%)	45.1 ± 22.3	0.494	92.3 ± 8.20	0.089
HGW	Hundred-grain weight (g)	0.798 ± 0.27	0.340	3.17 ± 0.65	0.206

To evaluate phenotypic differences between L1 and L2 of wild einkorn, we compared the 16 stable traits in addition to five grain-related traits, including grain area size (GAS), grain length (GL), grain width (GW), grain circularity (GC), and perimeter length of grain (GPL), between these lineages. Of the 21 traits, 19 showed significant differences between the two lineages (Fig. 2a). Flowering date of L1 was earlier than that of L2. L1 had longer awns than L2. Number of spikes in L1 was smaller than that in L2, implying that L1 had a smaller number of tillers. Spikes of L1 were characterized by a small number of spikelets, low spikelet densities, and large spikelets and grains compared to the spikes of L2.

Given that most L1 and L2 accessions were geographically separated, these genetically and morphologically divergent lineages were assumed to have adapted to the local temperature and precipitation of their habitats. Average temperature (°C) per month and average precipitation (mm) per month were estimated in the habitats of each lineage based on WorldClim global climate datasets (Fig. 2b). The average temperature per month in the L1 habitats from March to November was significantly higher than that in the L2 habitats. The average precipitation per month in the L1 habitats from January to April was greater than that in the L2 habitats, while the average precipitation per month in the L1 habitats from June to September was more than that in the L2 habitats. Overall, during the growing season of wild einkorn, the habitats of L1 accessions were characterized by relatively high temperatures, more precipitation in winter, and rapid drying after May. The habitats of L2 accessions were characterized by relatively low temperature, little precipitation in winter, and gradual drying after May.

Generation of synthetic wheat hexaploids with the A^m genome

In total, 42 synthetic lines were generated through interspecific crosses between Ldn and 42 accessions of *T. monococcum* ssp. *aegilopoides* (Fig 3a). These synthetic hexaploid lines produced self-pollinated seeds. We conducted genomic *in situ* hybridization (GISH) analysis to evaluate the somatic chromosomes of the ABA^m synthetic lines (Fig 3b, c, d). Forty-two somatic chromosomes were observed in the root cells of the F₃ plants as expected. GISH analysis using the wild einkorn DNA as probes detected 28 chromosomes of wild einkorn descent in the ABA^m synthetic line (Fig 3c, d). Fourteen chromosomes were stained with relatively stronger probe signals among the 42 chromosomes of the ABA^m polyploids, and the weaker-stained chromosomes were presumed to be the B-genome chromosomes (Fig 3c, d). This observation indicated that the A^m-genome chromosomes could not be clearly distinguished from the A-genome chromosomes by the GISH analysis using the wild einkorn DNA.

Table 2. The A^m-chromosome-specific CAPS markers developed in this study.

Marker name	Chr.	Position (bp)	Forward and reverse primer sequences (5' to 3')	Restriction enzyme	Annealing temp. (°C)
1A-9	1A	301,133,756	CGCAATGCAGCTCCCGAAAA TGTGTGAGGGGATGCTCAGC	<i>BstPI</i>	58
2A-32	2A	111,410,167	ACTGTAGCAGGATCGTCCGC CGCAGGCCAGCAGTGTATC	<i>HhaI</i>	60
3A-9	3A	714,155,627	TCTCCAATGCAACTTTCACG CATTGGGGCTAAACCAGTTG	<i>MboI</i>	54
4A-55	4A	238,382,739	CTGGCAGTCGGAGTGGTACA GAAAGCTTTCAGGCCCACT	<i>DdeI</i>	62
5A-169	5A	118,666,482	TTGAACTCGGGACCTCTCGC AGCCCCAGCGAAATGGATCA	<i>SfaNI</i>	58
6A-51	6A	294,652,546	AGGAAGCGAAGTGGATGGGG CACGTCGACACCCAACTTGC	<i>KpnI</i>	62
7A-20	7A	314,410,689	CGTGTATCTCGACGGCCCAT AGAAGGCAAGCGCGAGAAGA	<i>SfaNI</i>	60

The position of each marker was deduced according to information for the chromosomal position of the SNP site distinguished between the A and A^m genomes (Michikawa et al. 2019).

In nascent allohexaploid wheat, whole-chromosome aneuploidy has been reported [5]. To confirm the A^m-genome chromosomes in the ABA^m synthetics, we conducted PCR analysis with A^m-chromosome-specific SSR markers (Fig 4, Table 2). The SSR markers used for this confirmation showed clear polymorphisms between Ldn and the wild einkorn accessions. For each of the A^m-genome chromosomes, the ABA^m synthetics contained both Ldn- and wild einkorn-derived PCR bands. Thus, all tested ABA^m synthetic lines contained a set of the A^m-genome chromosomes. Two of the 42 ABA^m synthetic lines exhibited a hybrid dwarf phenotype (Fig 5a, b). The two lines showing hybrid dwarfism contained the expected 42 chromosomes including a set of the A^m-genome chromosomes in the root tip cells (Fig 5c).

Phenotypic variation in the AABBA^mA^m synthetic lines

To examine phenotypic variation in the AABBA^mA^m synthetic lines, 26 traits were evaluated over two growing seasons. Significantly positive correlations between years were detected in 14 traits (Additional file 2: Fig. S4). Plant height, stem length, and 1st internode length showed high correlation coefficients. The correlation coefficients for traits related to flowering, awn, and spikelet morphology were significantly positive. To compare the extent of phenotypic variation between the tested traits, we calculated the

coefficient of variation (CV) for each trait (Table 1). The CV was lower in the AABBA^mA^m synthetic hexaploids than in their wild einkorn parents, indicating that the degree of phenotypic trait variation within the synthetic hexaploids was less than that within their wild einkorn parents. The correlation coefficient of CV between the synthetic hexaploids and wild einkorn was 0.77 ($p = 1.54e-07$). When excluding SSF, which was an outlier trait, the correlation coefficient of CV was 0.89 ($p = 4.92e-12$). The significantly positive correlation coefficient of CV indicates that the diversity of each trait in wild einkorn was transmitted into the synthetic hexaploids.

Two of the 42 AABBA^mA^m synthetic lines (Ldn/ssp. *aegilopoides* KU-8267 and Ldn/ssp. *aegilopoides* KU-8276) showed hybrid dwarfism. The stem length and plant height of these two lines were significantly shorter than those of the other synthetic lines (Additional file 2: Fig. S5). These two lines are hereafter called HDW for hybrid dwarf. The heading and flowering dates of the HDW lines were delayed. The number of spikelets for the HDW lines was also smaller than those of the other AABBA^mA^m synthetic lines, but awn length, leaf-related traits, and selfed seed fertility were not different between them.

Transmission of the A^m-genome variations to the AABBA^mA^m synthetic lines

To evaluate transmission of trait variation within the wild einkorn *T. monococcum* ssp. *aegilopoides* accessions to the AABBA^mA^m synthetic lines, the correlation coefficient for the 26 traits between the pollen parents and the synthetic lines was calculated. A significantly positive correlation was detected for nine traits in both seasons (Additional file 2: Fig. S6, Fig. S7). The traits with significant correlations were related to flowering, awn, and spikelets, but the traits involved in plant height and leaf morphology were not significant.

We conducted clustering analysis of correlation coefficients of the traits between the two seasons in each of the synthetic lines and the pollen parents in addition to correlation coefficients of the traits between the synthetic lines and the pollen parents. The traits were grouped into three major clusters: A, B, and C. In some traits of clusters A and B, the reproducibility of the traits between the seasons in the AABBA^mA^m synthetic lines was different from that in the pollen parents (Fig. 6). Plant heights, stem length, and 1st internode length showed high correlation coefficients in the AABBA^mA^m synthetic lines, but not in the wild einkorn accessions. In contrast, 5th internode length showed a significant correlation in the wild einkorn accessions, but not in the AABBA^mA^m synthetic lines.

In the traits of cluster C, both of the comparisons between the seasons in the AABBA^mA^m synthetic lines and the wild einkorn accessions showed high correlation coefficients. High correlation coefficients were also observed in the comparisons between the AABBA^mA^m synthetic lines and the wild einkorn accessions. Cluster C included flowering, awn, and spikelet-related traits. These results indicated that phenotypic variations in these traits within the wild einkorn accessions were transmitted into the AABBA^mA^m synthetic lines.

We examined whether phenotypic divergence between the wild einkorn accessions in L1 and L2 reflected phenotypic variations in the AABBA^mA^m synthetic lines (Fig. 7). Of the 16 traits showing significant divergence in the wild einkorn accessions between L1 and L2, 14 traits were also significantly different in the synthetic lines. In addition, significant differences in flag leaf width, 5th internode length, and stem length with awn between the L1 and L2 accessions were uniquely detected in the synthetic lines. The synthetic lines derived from L1 accessions had earlier flowering, longer awns, bigger spikelets and grains, and lower density spikelets than those from L2 accessions. These trait characteristics in the synthetic lines were clearly inherited from their wild einkorn pollen parents.

Characterization of grain morphology of the AABBA^mA^m synthetic lines

Five grain-related traits, grain area size, grain length, grain width, grain circularity, grain perimeter length, and hundred-grain weight, were evaluated in the AABBA^mA^m synthetic lines. Grains of the AABBA^mA^m synthetic lines were longer than those of their Ldn and wild einkorn parents (Fig. 8a). Compared with the AABBDD synthetic wheat, the shape of grains of the AABBA^mA^m synthetic lines was slender, suggesting that addition of A^mA^m into AABB genomes changed grain shape from round to slender. Grain area size, perimeter length of grain, and grain length of the AABBA^mA^m synthetic lines were highly correlated with those of their einkorn pollen parents (Additional file 2: Fig. S8). All the grain-related traits showed significant differences between the synthetic lines derived from L1 and L2 einkorn accessions (Fig. 7). The variations of grain shapes within the synthetic lines were transmitted from the wild einkorn parents.

Grain hardness of wheat is an essential parameter for characterizing flour. Hard grain is used for Italian-style pasta, and soft grain is used for other types of noodles, such as ramen and udon. Grain hardness of the 40 AABBA^mA^m synthetic lines, excluding the HDW lines, was measured using a single-kernel characterization system (SKCS), which is algorithmically forced to have a value of 75 for hard wheat and 25 for soft wheat [35]. Grain hardness of the AABBA^mA^m synthetic lines ranged from 18.55 to 42.31 (Fig. 8b). Average grain hardness was 29.65 ± 6.00 , indicating that grains of the AABBA^mA^m synthetic lines were soft grains.

A scanning electron microscope was used to observe cross sections of seeds of three AABBA^mA^m lines (Ldn/ssp. *aegilopoides* KU-101-3, Ldn/ssp. *aegilopoides* KU-8001, and Ldn/ssp. *aegilopoides* KU-8201) (Fig. 8c). The surface of starch granules of all the lines was smooth. We also detected holes formed by starch granules that had fallen out of the endosperm during sample preparation. These observations were consistent with the characteristics of soft grains [30].

Phenotypic comparisons among AABBA^mA^m, AABBA, and AABBDD synthetic lines.

Phenotypic comparisons between AABBDD synthetic wheat and AABBUU synthetic hexaploids indicated that differences in phenotypic traits between these nascent synthetic hexaploids reflect the genomes of their pollen parents [31]. To test whether this observation could extend to the other nascent synthetic hexaploids, we compared phenotypic traits of the AABBDD synthetic wheat with those of the AABBA^mA^m

and AABBA^mA^m synthetic hexaploids (Fig. 9, Additional file 2: Fig. S5). The two HDW lines and the other 40 AABBA^mA^m synthetic lines were separately analyzed in the comparisons among the synthetic hexaploids. Heading and flowering dates of the AABBA^mA^m synthetic lines were later than those of the AABBDD synthetic wheat and almost the same as those of the AABBA^mA^m synthetic hexaploids. Heading and flowering dates of the HDW lines were significantly delayed. The plant height and stem length of the AABBA^mA^m synthetic lines were longer than those of the AABBDD and AABBA^mA^m synthetic hexaploids. The awn length of the AABBA^mA^m synthetic lines was also longer than those of the AABBDD and AABBA^mA^m synthetic lines. Compared with the AABBDD synthetic wheat, spike length in the AABBA^mA^m synthetic lines was shorter and the number of spikelets was larger, resulting in the high density of spikelets in the AABBA^mA^m synthetic lines. The grain length of the AABBA^mA^m synthetic lines was longer and grain width was shorter, forming the more slender grain of the AABBA^mA^m synthetic lines compared with the other synthetic hexaploids.

Discussion

Two divergent lineages in *T. monococcum* ssp. *aegilopoides* are linked to the climate of their habitats

The tested accessions of *T. monococcum* ssp. *aegilopoides* were separated into two lineages, L1 and L2, based on polymorphisms for SSR markers that covered all the chromosomes (Fig. 1, Additional file 2: Fig. S2). These separate lineages were also observed in the phylogenetic analysis based on RNA-seq-based polymorphisms [34] and whole-genome polymorphisms in wild einkorn [36]. The habitats of L1 accessions were southern Turkey, northern Iraq, and Iran, which correspond to the Fertile Crescent. L2 accessions were mainly distributed from Greece to Turkey. The genetic differences in wild einkorn reflected the phenotypic traits (Fig. 2a). Heading and flowering dates in L2 accessions were later than those in L1 accessions. Leaf length and spike length of L2 accessions was longer than those of L1 accessions. The number of spikes and spikelets in L2 accessions was larger than that in L1 accessions. These observations suggest that L2 accessions have longer vegetative phases than L1 accessions, allowing for large leaves and a large number of spikes and spikelets. On the other hand, the spikelet size of L1 accessions was larger than that of L2 accessions. Grain length, grain width, and perimeter length of grain of L1 accessions were also larger than those of L2 accessions. By suppressing the number of spikes and spikelets, L1 accessions could invest more nutrients in each of the spikelets and grains, resulting in their larger size.

The amplified fragment length polymorphism (AFLP) analysis for 321 wild einkorn wheat accessions revealed three distinct einkorn races, a, b, and g [13]. The a race is distributed over the Fertile Crescent. The b race is observed in a restricted area of southern Turkey. Given that the distribution of the a and b races overlapped with that of L1, our tested accessions in L1 could belong to the a or b race. In addition, the habitat of the g race is Greece and western Turkey, which overlapped with that of L2. Therefore, this lineage could correspond to the g race. Heading date of the g race is later than that of the a and b races. Stem length of the g race is longer than that of the a and b races. The differences in heading date

between these einkorn accessions are consistent with the differences between L1 and L2 (Fig. 2a), although differences in stem length were not validated in our study due to low reproducibility of plant height-related traits between the two seasons.

The temperature and precipitation were significantly different between L1 and L2 habitats (Fig. 2b). The habitat of L1 showed relatively high temperatures and abundant precipitation in winter and rapid drying after May. Due to the high temperatures and sufficient rainfall in winter, the shorter vegetative period of wild einkorn may be sufficient in L1 accessions. The earlier heading and flowering dates in L1 accessions could be essential for reproduction before the start of the dry season. On the other hand, L2 accessions are subjected to lower temperatures in winter, which may slow plant growth and necessitate more time for the vegetative period. The habitats of L2 are not as dry as those of L1 in May and June. The delayed flowering of L2 accessions could be a trait naturally selected to adapt to the habitat.

Transmission of traits from the pollen parents into the nascent synthetic hexaploids

Analysis of correlations in phenotypic variations between the AABBA^mA^m synthetic hexaploids and their *T. monococcum* ssp. *aegilopoides* pollen parents revealed the transmission of phenotypic traits from the pollen parents into their corresponding synthetic lines (Fig. 6, Additional file 2: Fig. S6, Fig. S7, Fig. S8). Variations of traits related to flowering, spikelets, and awn length in the wild einkorn accessions directly reflected those in the AABBA^mA^m synthetic hexaploids. Significantly positive correlations between the two seasons were detected in these traits of both the wild einkorn accessions and the AABBA^mA^m synthetic hexaploids, implying that these traits were genetically stable and were less influenced by environmental or other factors. Due to the stable characteristics of these traits, the transmission of phenotypic variation in the wild einkorn accessions to the synthetic lines was clearly detected. Phenotypic divergence between L1 and L2 wild einkorn accessions was also detected between their corresponding synthetic lines, but direct transmission of the leaf morphological traits and the plant height-related traits was not clear in the AABBA^mA^m synthetic lines. As observed in the wild einkorn parents, correlation coefficients between the seasons for these traits were low.

In the traits showing transmission from the pollen parents into the synthetic lines, the extent of variations in the AABBA^mA^m synthetic lines was reduced compared with those in the wild einkorn accessions (Table 1). This was also found in the relationship between the other synthetic hexaploids (AABBDD and AABBUU) and their diploid parents of *Ae. tauschii* (DD) and *Ae. umbellulata* (UU) [31, 37]. The AABBA^mA^m synthetic lines were derived from the same mother parent, Ldn. Additive effects of the Ldn and wild einkorn genomes could result in the reduction of phenotypic variations in the AABBA^mA^m synthetic lines. Additionally, additive effects from Ldn could provide a more robust response to environmental factors. Although the variations of plant height, stem length, and 1st internode length in the wild einkorn accessions were unstable (Fig. 6, Additional file 2: Fig. S3), the variations of these traits in their synthetic lines showed significantly positive correlations between the seasons (Fig. 6, Additional file 2: Fig. S4).

The variations of spike length in wild einkorn and in the AABBA^mA^m synthetic hexaploids were stable between the seasons (Fig. 6, Additional file 2: Fig. S3, Fig. S4). Variations of spike length, however, were not significantly correlated between the wild einkorn accessions and the AABBA^mA^m synthetic hexaploids (Fig. 6, Additional file 2: Fig. S6, Fig. S7). This inconsistency could be explained by interactions between the AB genome and the A^m genome, which may cause non-additive effects on spike length.

Characteristics of the AABBA^mA^m synthetic hexaploids.

By comparing the traits among the AABBDD, AABBUU, and AABBA^mA^m synthetic hexaploids sharing the common tetraploid wheat female parent, Ldn [31], the phenotypic traits of the AABBA^mA^m synthetic hexaploids are clearly distinct from those of the AABBDD, AABBAA, and AABBUU synthetic hexaploids. The AABBA^mA^m synthetic hexaploids were characterized by higher plant height, more spikelets, slender spikelets and grains, longer awns, and relatively late flowering. Given that the same mother parent *T. turgidum* cv. Langdon was used to generate the synthetic hexaploids, the phenotypic characteristics of these synthetic hexaploids are considered to reflect those of the diploid species used as their pollen parents. Takumi et al. 2009 [28, 37] and Okada et al. 2020 [31] investigated phenotypic traits of *Ae. tauchii* (DD) and *Ae. umbellulata* (UU) that were the pollen parents of the AABBDD and AABBUU synthetic hexaploids in the same fields. We compared the phenotypic traits of wild einkorn with *Ae. tauschii*, *Ae. umbellulata*, and *T. urartu* (Additional file 1: Table S6). Wild einkorn had the longest 1st internode, resulting in the tallest plant. Wild einkorn also had the longest awns and bloomed later than the other species. Wild einkorn also had slender spikelets and a large number of spikelets, distinct from the others. Although these phenotypic data for each species were collected in different years, the observations suggest that the phenotypic features of the pollen parents are characteristic of those of the synthetic hexaploids.

Grain hardness of the AABBDD synthetic wheat and the AABBUU synthetic hexaploids was characterized using SKCS and revealed that most of the AABBDD synthetic wheat have soft grains and the AABBUU synthetic hexaploids have hard grains [30, 38]. Distribution of grain hardness was compared between these synthetic hexaploids and the AABBA^mA^m hexaploids. Distribution of grain hardness of the AABBA^mA^m hexaploids mostly overlapped with that of the AABBDD synthetic wheat [38]. The distribution of grain hardness of the AABBUU hexaploids was completely separate from that of the AABBA^mA^m hexaploids [31]. The soft grain characteristics of the AABBA^mA^m hexaploids suggest a potential for use in making Asian-style noodles such as udon.

Conclusions

The analysis of SSR-based polymorphisms in the 43 wild einkorn accessions revealed that wild einkorn genetically and phenotypically diverged into two lineages, L1 and L2. These divergences were linked to their habitats' climate, suggesting that the traits of each lineage might have evolved to adapt to the local environment. Through interspecific crosses between *T. turgidum* cv. Langdon and the wild einkorn

accessions, 42 nascent synthetic lines of AABBA^mA^m were generated. The phenotypic variations of the AABBA^mA^m synthetic hexaploids were transmitted from their wild einkorn pollen parents and reflected the L1 and L2 divergence among wild einkorn. Introduction of various A^m genomes of wild einkorn into the synthetic hexaploids provides increased phenotypic diversity and unique characteristics that can be used for breeding of new and improved wheat cultivars.

Methods

Plant materials

In this study, 43 accessions of *Triticum monococcum* ssp. *aegilopoides* (Link) Thell., an accession (KU-199-16) of *T. urartu* Tumanian ex Gandilyan, and a tetraploid wheat (*T. turgidum* L. ssp. *durum*) cultivar Langdon (Ldn) were used. The wild einkorn and *T. urartu* accessions are listed in Additional file 1: Table S1. These seeds, except for PI427634, were supplied by the National BioResource Project (NBRP)-Wheat, Japan (<https://shigen.nig.ac.jp/wheat/komugi/>).

After removing anthers from the immature florets of Ldn, pollen of each of the diploid wheat accessions were crossed to Ldn as the female parent. Seedlings of the F₁ hybrids were treated with 0.1% colchicine (Wako, Osaka, Japan) for 5 hours and grown to maturity in a glasshouse at Kobe University (34°43'N, 135°13'E) to obtain the selfed seeds (F₂ generation). Thus, the synthetic lines with the AABBA^mA^m genome (ABA^m hexaploids) share the A and B genomes from Ldn and contain the A^m genome derived from diverse *T. monococcum* ssp. *aegilopoides* accessions. The somatic chromosome numbers were determined from root-tip mitotic preparations of the F₃ plants using the standard acetocarmine squash method. Four lines of synthetic hexaploid wheat with the AABBDD genome (ABD hexaploids), Ldn/KU-2097 (Syn6214), Ldn/IG126387 (Syn6240), Ldn/PI476874 (Syn6256), and Ldn/KU-2069 (Syn6262), were also used and grown under the same conditions as the ABA^m hexaploids.

SSR-based PCR analysis of the wild einkorn parental accessions

The simple sequence repeat (SSR) markers used in this study are listed in Additional file 1: Table S2. Information on the SSR markers and the respective annealing temperatures was obtained from the NBRP KOMUGI website (<http://www.shigen.nig.ac.jp/wheat/komugi/strains/aboutNbrpMarker.jsp>) and the GrainGenes website (<http://wheat.pw.usda.gov/GG2/maps.shtml>). For SSR genotyping, 40 cycles of PCR were performed using 2x Quick Taq HS DyeMix (TOYOBO, Osaka, Japan) under the following conditions: 20 s at 94°C, 30 s at the annealing temperature, and 30 s at 68°C. PCR products were resolved in 2% agarose or 15% non-denaturing polyacrylamide gels, stained with ethidium bromide, and visualized under UV light according to our previous report [39]. To quantify genetic diversity in wild einkorn, Simpson's index, expected heterozygosity, evenness, and Nei's genetic distance for the SSR markers were calculated using the R packages Poppr version 2.8.3 [40] and ape version 5.3 [41].

The population structure and proportion of membership (Q) for the wild einkorn accessions were inferred using STRUCTURE v2.2.3 software [42]. The STRUCTURE analysis was conducted using the admixture model without prior population information of the wild einkorn accessions. A number of populations (K) ranging from 1 to 5 were examined. Ten runs were performed for each K with a burn-in period of 0.5×10^6 iterations followed by 1×10^6 iterations of Markov chain Monte Carlo methods. The ΔK method [43] was applied to infer the optimal K value. The Q-matrix from 10 runs under the optimal K value was summarized using CLUMPP version 1.1.2 [44]. The wild einkorn accessions were sorted into the lineages based on the most probable Q-values. Phylogenetic trees were constructed based on the SSR polymorphisms. MEGA, version 10 [45], was used for construction of the unweighted pair group method using an arithmetic average (UPGMA) tree. Bootstrap probability was calculated for 1000 replications.

Genomic *in situ* hybridization

GISH analysis of mitotic metaphase chromosomes was performed according to a previously described fluorescence *in situ* hybridization protocol with minor modifications [31, 46]. Genomic DNA was extracted from young leaves of a *T. monococcum* ssp. *aegilopoides* accession, KU-3620. Genomic DNA of KU-3620 was labeled using Biotin-Nick Translation Mix (Roche Diagnostics, Japan), incubated at 16°C for 24 hours, and then digested with the restriction enzyme *Hae*III at 37°C for 5 hours. The biotin-labeled DNA was used as a probe. After chromosomes were incubated in SSC including 70% formamide at 80°C for 2 minutes for denaturation, they were hybridized with the biotin-labeled probe. The biotin-labeled probe was visualized using rhodamine-conjugated Alexa Fluor 555 (Life Technologies Japan Ltd., Japan). Chromosomes were counterstained with 0.1 µg/ml 4,6-diamino-2-phenylindole (DAPI). GISH signals and DAPI-stained chromosomes were captured using a fluorescence microscope (Axioskop2, Carl Zeiss, Oberkochen, Germany), and images were pseudo-colored and processed using ZEN software blue edition (Carl Zeiss).

PCR analysis of the wild einkorn accessions and the AABBA^mA^m synthetic lines

For validation of the added A^m chromosomes in the synthetic hexaploids, PCR analysis with the A^m-genome-specific marker of each A^m chromosome was conducted according to our previous study [34]. The A^m-genome-specific markers were cleaved amplified polymorphic sequences (CAPS) converted from RNA sequencing-derived SNP information [34]. Total DNA was extracted from leaves of the parental accessions and synthetic hexaploid lines. PCR conditions for genotyping were described in our previous report [31]. Marker names, amplification conditions, and restriction enzymes of the A^m-genome-specific markers developed in the present study are shown in Table 2. PCR products and their digests were resolved in 2% agarose or 15% nondenaturing polyacrylamide gels.

Phenotype measurement and statistical analysis

To evaluate the phenotypic traits, four F₂ and F₃ seeds of each synthetic hexaploid line were sown in November 2017 and 2018, respectively. These F₂ and F₃ plants were grown in seasons 2017–2018 and

2018–2019 using pots arranged randomly in the glasshouse in which the temperature was not regulated. The parental wild einkorn wheat accessions were grown in seasons 2017–2018 and 2018–2019 using pots in a field at Kobe University. The examined traits were related to flowering days, spike and spikelet shapes, awn length, leaf shape, and plant height. Abbreviations of the examined traits are listed in Table 1. The traits of four individuals per synthetic hexaploid line and two individuals per wild parental accession were evaluated in each season. Stem length corresponded to the length from the crown to the neck of the spike. Plant height was the sum of stem length and spike length. Stem width was measured at the middle of the 1st internode. The number of spikelets was counted for three spikes per individual. Spikelet length and spikelet width were measured for three spikelets per spike. Self-seed fertility was estimated as the ratio of seeds that bear fruit per 10 florets in three replications per individual.

These data were statistically analyzed using R Studio ver. 1.2.1335 software (<http://www.rstudio.com>) with R software ver. 3.6.1 according to our previous study [31]. Student's *t*-test was used to compare traits between lineages L1 and L2 of the wild einkorn accessions and their synthetic lines. The statistical differences were assessed after Bonferroni correction. The correlations among the morphological traits, estimated based on Pearson's correlation coefficient values, were conducted using R software ver. 3.6.1. Statistical comparisons of the traits among Ldn and the AABBA^mA^m, AABBA^a, and AABBD^d synthetic hexaploids were evaluated using the Tukey-Kramer method. Average temperature per month and average precipitation per month for each habitat of the tested accessions from 1970 to 2000 were estimated based on WorldClim global climate datasets version 2.1 using the R package "raster" [47].

Evaluation of grain-related traits

Grain size and shape were measured in each synthetic hexaploid line using *SmartGrain* software ver. 1.2, which was developed for high-throughput phenotyping of rice (*Oryza sativa*) seeds [48]. Six parameters for grain size and shape, including grain area size, grain perimeter length, grain length, grain width, grain length-width ratio, and grain circularity, were recorded for at least 50 seeds of each accession and line according to the *SmartGrain* protocol.

Four grain-related traits, grain hardness, weight, diameter, and moisture were evaluated using SKCS 4100 (Perten, Stockholm, Sweden). The SKCS hardness index was obtained from crushing a sample of at least 50 kernels from Ldn and each synthetic line, similar to our previous report [30, 31].

A transverse section of grain was observed by a scanning electron microscope (S-3400N, Hitachi High-Technology, Tokyo, Japan) after the grain was snapped in the middle as previously described [49]. SEM observation was conducted without any pretreatment at an accelerating voltage of 8.00 kV under low vacuum conditions of 70 Pa at –25°C, and these conditions were similar to our previous reports [30, 49].

List Of Abbreviations

HD: Heading time

FD: Flowering time

FLL: Flag leaf length

FLW: Flag leaf width

LL: Leaf length

LW: Leaf width

TAL: Top awn length

MAL: Middle awn length

BAL: Bottom awn length

1InL: 1st Internode length

2InL: 2nd Internode length

3InL: 3rd Internode length

4InL: 4th Internode length

5InL: 5th Internode length

StL: Stem length

PH: Plant height

SW: Stem width

SL: Spike length

SLwA: Spike length with awn

SN: Number of spikes

NSp: Number of spikelets

SpD: Spikelet density

SpL: Spikelet length

SpW: Spikelet width

SpLWR: Length-width-ratio of spikelet

GAS: Grain area size

GPL: Perimeter length of grain

GL: Grain length

GW: Grain width

GLWR: Length-width-ratio of grain

GC: Grain circularity

SSF: Selfed seed fertility

HGW: Hundred grain weight

HDW: hybrid dwarf

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets and materials analysed and/or generated during the current study are available from the corresponding author on reasonable request.

Competing interests

All the authors declare that there exist no competing interests.

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Authors' contributions

AM and MO evaluated phenotypic traits. ST generated the synthetic hexaploids. AM conducted genotyping. AM and KY analyzed the data. AM and NK performed cytological experiments. AM and TMI characterized grains of the synthetic hexaploids. AM, ST and KY wrote the manuscript.

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Figures

Figure 1

Subpopulation structure of *T. monococcum* ssp. *aegilopoides* (a) Morphological differences of spikes between *T. monococcum* ssp. *aegilopoides* KU-3620 and *T. urartu* KU-199-16. White bars indicate 5 cm. (b) Estimation of the number of sub-populations (k) from STRUCTURE results for differing numbers of subpopulations. Delta K peaked at K = 2. (c) Proportion of membership of the 43 accessions for K = 2, as calculated by STRUCTURE software based on the polymorphisms detected by SSR markers. Multiple runs for each K were concatenated using CLUMPP. (d) Geographic distribution of the L1 and L2 accessions of wild einkorn and the one accession of *T. urartu*.

Figure 2

Phenotypic comparison of the L1 and L2 subpopulations of *T. monococcum* ssp. *aegilopoides* (a) Comparisons of the 16 stable traits and five grain-related traits, heading date (HD), flowering date (FD), top awn length (TAL), middle awn length (MAL), bottom awn length (BAL), flag leaf length (FLW), leaf width (LW), 5th internode length (5InL), spike length (SL), spike length with awn (SLwA), number of spikes (SN), spikelet length (SpL), spikelet width (SpW), spikelet density (SpD), length-width-ratio of spikelet

(SpLWr), number of spikelets (NSp), grain area size (GAS), grain length (GL), grain width (GW), grain circularity (GC), and perimeter length of grain (GPL) between L1 and L2 accessions of *T. monococcum* ssp. *aegilopoides*. Box plots with jitter points are shown in each trait. The violet and blue boxplots correspond to the L1 and L2 accessions. Student's t-test was used to statistically compare the traits. P-values after Bonferroni correction are shown by asterisks. (b) Average temperature and precipitation per month from 1970 to 2000 in the habitats of the L1 and L2 accessions. Significant differences with Student's t-test are marked by asterisks. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NS: non-significant.

Figure 3

Confirmation of the Am-genome chromosomes in the synthetic allohexaploid wheat The presence of the Am-genome chromosomes in the 40 AABBAmAm synthetic hexaploid lines was confirmed based on the amplification of Am-chromosome-specific CAPS markers. Their parents (Ldn and wild einkorn accessions) were used as controls. Restriction enzyme and marker names are shown in parentheses following the chromosome names on the left of each gel image. Details of the CAPS markers are described in Table 2. Size differences in amplicons between the AB and Am genomes were observed. Both amplicons from the AB and Am genomes were detected in the synthetic hexaploid lines. The gel images are cropped from different gels. The full-length gel images are shown in Additional file 3.

Figure 5 placeholder

Figure 5

Wild-type and hybrid dwarf plants of the synthetic allohexaploids Wild-type plants (a). Hybrid dwarf plants (b). Confirmation of the Am-genome chromosomes in the synthetic lines showing hybrid dwarf phenotypes (c). The presence of the Am-genome chromosomes in the synthetic hexaploid lines was confirmed based on amplification of Am-chromosome-specific CAPS markers. Their parents (Ldn and wild einkorn accessions) were used as controls. Restriction enzyme and marker names are shown in parentheses following the chromosome names on the left of each gel image. Details of the CAPS markers are described in Table 2. Size differences between the AB and Am genomes were observed. Both amplicons from the AB and Am genomes were detected in the synthetic hexaploid lines. The gel images are cropped from the same gel or different gels. The full-length gel images are shown in Additional file 4.

Figure 6 placeholder

Figure 6

Transmission of the Am-genome variations to the AABBAmAm synthetic lines Hierarchical clustering of correlation coefficients of the traits between two seasons in each of the synthetic lines and the pollen parents in addition to correlation coefficients of the traits between the synthetic lines (AABBAmAm) and the pollen parents (AmAm). The values of correlation coefficients were displayed as a heatmap. Darker red indicates more positive correlation, while darker blue indicates more negative correlation. The heatmap shows that the stable traits between the two seasons in both the synthetic lines and their parents (Cluster C) were more clearly transmitted from the pollen parents to the synthetic lines compared with the unstable traits (Clusters A and B). 2018 and 2019 represent the 2017–2018 season and the 2018–2019 season, respectively. The abbreviations of traits are described in Table 1.

Figure 7

Comparisons of the 21 traits that were tested in Fig. 2 between the synthetic lines derived from lineage 1 (L1) and lineage 2 (L2) *T. monococcum* ssp. *aegilopoides* accessions. Box plots with jitter points are shown for each trait. The violet and blue boxplots correspond to the synthetic lines derived from the L1 and L2 accessions. Student's t-test was used to statistically compare the traits. P values after Bonferroni correction are shown by asterisks. *p < 0.05, **p < 0.01, ***p < 0.001. NS: non-significant. The abbreviations for traits are provided in Fig. 2 and Table 1.

Figure 8

Seed shape among the AABBAmm synthetic lines, their parental wild einkorn accessions, the AABDD hexaploid lines, and Ldn (a). Scale bar corresponds to 1 cm. Frequency distribution of the SKCS hardness values in 40 AABBAmm hexaploid lines (b). Scanning electron microscopy of the transverse sections in Ldn/*ssp. aegilopoides* KU-101-3, Ldn/*ssp. aegilopoides* KU-8001, and Ldn/*ssp. aegilopoides* KU-8201

grains (c). The characteristics of all the AABBAmAm hexaploid lines were consistent with those of soft grains of common wheat.

Figure 9

Phenotypic comparisons of the AABB (Ldn), AABBAmAm, AABBDD, AABBAA, and HDW AABBAmAm hexaploids. Boxplots of each trait are shown. No significant difference was observed between groups with the same letters. The abbreviations for traits are provided in Table 1.

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