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Distinct genetic regulation of flowering time and grain-filling period based on empirical study of D-genome diversity in synthetic hexaploid wheat lines

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Aegilops tauschii Coss., the D-genome progenitor of hexaploid wheat, has a wide species range in central Eurasia. Large natural variation in *Ae. tauschii* offers potential for improving modern varieties of common wheat. Maturation time, an important agronomic trait for wheat breeding, is tied to flowering time and grain-filling period. Here, we established 82 wheat synthetic lines derived through endoreduplication, forming triploid gametes, in interspecific hybrids obtained by crossing a tetraploid wheat cultivar, Langdon, with 69 *Ae. tauschii* accessions. Empirical study of the hexaploid synthetics showed abundant variation in heading, flowering, and maturation time and the grain-filling period throughout the two growth seasons examined. The wide variation observed in heading time in *Ae. tauschii* was maintained in the hexaploid background of the synthetic wheat lines. Significantly positive correlations were observed among heading, flowering, and maturation time in the hexaploid synthetics. On the other hand, no significant correlations were found between grain-filling period and the other traits examined. Some comparisons between two selected synthetic wheat lines having similar flowering time stably exhibited differences in their grain-filling periods. These observations suggest that two major genetic pathways independently determine wheat maturation time; one controls heading and flowering time and the other regulates grain-filling period.

Key Words: *Aegilops tauschii* Coss., allopolyploidy, maturation time, natural variation, senescence-associated gene, *Triticum aestivum* L..

Introduction

Maturation time is one of the most important traits for improvement of modern wheat varieties, especially in Japan, where the harvesting season of common wheat overlaps with the rainy season. Maturation time is determined by the sum of flowering time and the grain-filling period. In wheat, flowering time is controlled by three major genetic factors, vernalization requirement, photoperiodic sensitivity and narrow-sense earliness (reviewed in Murai *et al.* 2005). There is little information about the genetics of the grain-filling period, but in contrast, a lot of genetic studies on wheat flowering time have been reported. Especially regarding the vernalization requirement and photoperiodic sensitivity, *Vrn-1* and *Ppd-1*, respectively, have been elucidated as the major controlling genes (Beales *et al.* 2007, Murai *et al.* 2003, Trevaskis *et al.* 2003, Turner *et al.* 2005, Yan *et al.* 2003a). Narrow-sense earliness (earliness *per se*) is defined as earliness in flowering of fully vernalized plants under long-day conditions, and is controlled by several quantitative trait loci (QTLs) (Cockram *et al.* 2007). In terms of genes controlling narrow-sense earliness, only the *Eps-1* locus has been physically mapped; it is on the long arm of chromosome $1A^m$ in einkorn wheat (Faricelli *et al.* 2010). Little is known on the molecular nature of narrow-sense earliness in common wheat.

Aegilops tauschii Coss., a diploid self-pollinating goatgrass, is the D genome donor of common wheat (Kihara 1944, McFadden and Sears 1944). The genome of *Ae. tauschii* was introduced to that of common wheat through a spontaneous species cross with tetraploid wheat (AABB genome) and subsequent amphidiploidization about 8,000 years ago (Nesbitt and Samuel 1996). Habitats of *Ae. tauschii* are widely distributed from northern Syria and Turkey to western China in Eurasia. The birthplace of common wheat is considered to lie within the area comprising Transcaucasia and the southern coastal region of the Caspian Sea (Dvorak *et al.* 1998, Tsunewaki *et al.* 1966). The *Ae. tauschii* populations that participated in the origin of common wheat are limited to a narrow distribution range relative to the entire species range, suggesting that this species has huge genetic diversity that is not represented in common wheat (Feldman 2001, Mizuno *et al.* 2010a, 2010b). Natural variation in *Ae. tauschii* populations offers potential for improving modern varieties of common wheat. In fact, some disease and pest resistance genes have been transferred from *Ae. tauschii* to common wheat (Kerber 1987, Ma *et al.* 1995, Mujeeb-Kazi *et al.* 1996, Zhu *et al.* 2005). In addition, some studies have

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reported higher levels of genetic diversity of glutenin subunits and gliadin in *Ae. tauschii* than in the D genome of common wheat (Gianibelli *et al.* 2001, 2002, Giles and Brown 2006, Yan *et al.* 2003b, 2003c).

Despite the potential for wheat breeding, naturally occurring variation in *Ae. tauschii* has not necessarily been evaluated systematically or comprehensively. In our recent studies using more than 200 accessions of *Ae. tauschii*, natural variation in flowering time and morphological traits showed significant longitudinal and latitudinal clines (Matsuoka *et al.* 2008, 2009, Takumi *et al.* 2009a). Extensive flowering time variation was present in *Ae. tauschii* accessions, and the early-flowering accessions were spread mainly in eastern habitats such as Afghanistan and Pakistan, implying that the early-flowering phenotype contributed to eastward dispersal and adaptation to these habitats (Matsuoka *et al.* 2008). Our previous study on the population structure of *Ae. tauschii* identified two major lineages, lineages 1 (L1) and 2 (L2), and an HG17 minor lineage (HGL17), and showed that L1 and L2 are respectively divided into six and three sublineages (Mizuno *et al.* 2010a). Only four of the six L1 sublineages had dispersed from western habitats in the Transcaucasus and northern Iran to eastern habitats such as Pakistan and Afghanistan.

Allohexaploid wheat plants can be artificially produced through hybridization of tetraploid wheat and *Ae. tauschii* and are called synthetic hexaploid wheat (Kihara 1944, Kihara and Lilienfeld 1949, McFadden and Sears 1944). Use of the tetraploid wheat cultivar Langdon (Ldn) as a female parent allows efficient production of hexaploid synthetic wheat lines through unreduced gametogenesis in F_1 triploid hybrids having A, B and D genomes (Matsuoka and Nasuda 2004, Takumi *et al.* 2009b). Allopolyploidization is often accompanied by genetic and epigenetic modification of the genomes, as observed in synthetic polyploids in wheat and *Arabidopsis* (Kashkush *et al.* 2002, Madlung *et al.* 2002, Ozkan *et al.* 2001, Shaked *et al.* 2001, Wang *et al.* 2004). In addition, allopolyploidy brings new epistatic interactions to genes belonging to different genomes and possibly allows the formation of defective heteromeric protein complexes (Comai 2000, Terashima and Takumi 2009, Wendel 2000). Therefore, transcript accumulation levels and patterns are not necessarily additive in interspecific hybrids and allopolyploids (Chagué *et al.* 2010, Kurahashi *et al.* 2009, Pumphrey *et al.* 2009, Wang *et al.* 2004, 2006). It is unknown whether individual phenotypic variations carried by the *Ae. tauschii* population will appear in the hexaploid genetic background of synthetic wheat derivatives. Our previous empirical study using only 27 wheat synthetics showed that the degree of variation in the parental *Ae. tauschii* accessions is reduced in the hexaploid derivatives for many of the traits examined (Takumi *et al.* 2009b). The wide variation observed in heading and flowering time in *Ae. tauschii* was well maintained in the hexaploid background of the 27 synthetic wheat lines, although no significant correlation was observed between the diploid and hexaploid backgrounds. Our comparative analysis of maturation time using three selected lines of wheat synthetics revealed that, after vernalization, a synthetic wheat containing a D-genome from an early-flowering *Ae. tauschii* accession showed an early-flowering phenotype (Fujiwara *et al.* 2010). This observation suggests that the flowering time genes of *Ae. tauschii* function in the hexaploid background and affect flowering time.

Here, we established 82 synthetic hexaploid wheat lines through hybridization between Ldn and 69 *Ae. tauschii* accessions and compared their maturation time-related traits to apply the genetic diversity in flowering time in the *Ae. tauschii* population to hexaploid wheat breeding. Through this empirical study, we discuss the genetic relationship between flowering time and the grain-filling period in wheat.

Materials and Methods

Plant materials and production of synthetic hexaploids

A total of 69 *Ae. tauschii* accessions representing the entire natural habitat range of the species was used in this study (Table 1). Their passport data, including the geographical coordinates of the original collection sites, have been provided in previous reports (Matsuoka *et al.* 2007, 2008, 2009). For each accession, we used seeds propagated from a single plant. Plants of all *Ae. tauschii* accessions were grown in a field of Kobe University (34°43′N, 135°13′E) for verification of the species classification and evaluation of heading time. The data on heading time were previously published (Matsuoka *et al.* 2008).

Tetraploid wheat accession *Triticum durum* cv. Langdon (Ldn) was used as the female parent and was crossed with each of the 76 *Ae. tauschii* accessions. Langdon contains a spring habit gene *Vrn-A1* and no *Ppd-1* dominant allele because of its high sensitivity to photoperiods (Fujiwara *et al.* 2010, data not shown). The F_1 progeny were grown and selfed to produce synthetics (herein designated the F_2 generation) as previously reported (Mizuno *et al.* 2010b, Takumi *et al.* 2009b). All 82 synthetics were independently generated through unreduced gamete formation in each of the triploid F_1 hybrids (Matsuoka and Nasuda 2004). Out of the 82 synthetic hexaploids, 47 lines have been previously reported (Kurahashi *et al.* 2009, Takumi *et al.* 2009b). The synthetics thus contained the A and B genomes from Ldn and the diverse D genomes originating from the *Ae. tauschii* pollen parents. Some of the triploid F_1 hybrids between Ldn and *Ae. tauschii* show abnormal growth, such as hybrid necrosis and severe growth abortion (Matsuoka *et al.* 2007, Mizuno *et al.* 2010b). Such hybrids showing necrosis and severely aborted growth were excluded from the 82 synthetics. The synthetic hexaploids except for three hybrid lines showing chlorosis grew normally (Table 1). Somatic chromosome numbers were determined from root-tip mitotic preparations of two F_3 seeds from one F_2 plant of each synthetic, using the standard acetocarmine squash method.

Table 1. Strain numbers and sources of the 69 parental *Ae. tauschii* accessions as the D-genome donors to synthetic hexaploids

Afghanistan (n = 3)
KU-2022[c,1], KU-2059[c,1,an], PI476874[c,1]
Armenia (n = 5)
KU-2810[a,1], KU-2811[a,2], KU-2814[a], KU-2816[a,1], KU-2824[a,1]
Azerbaijan (n = 1)
IG47202[a,2]
China (n = 6)
AT47[c,1], AT55[c,1], AT76[c,1], AT80[c,1], PI499262[a,1], PI508262[a,1]
Georgia (n = 3)
AE454[d,17], AE929[d,17], KU-2829A[d,17]
India (n = 1)
IG48042[a,1]
Iran (n = 39)
KU-2069[a,2], KU-2074[a,2,s], KU-2075[a,2,s], KU-2076[b,2,s], KU-2078[b,2,s], KU-2079[b,2,s], KU-2080[b,2,s], KU-20-8[a,2], KU-20-9[b,2,s], KU-20-10[b,2,m], KU-2083[a,2], KU-2088[b,2,s], KU-2090[b,2,s], KU-2091[b,2,s], KU-2092[b,2,s], KU-2093[a,2,s], KU2096[b,2], KU-2097[a,2], KU-2098[b,2], KU-2100[b,2,m], KU-2101[b,2], KU-2102[b,2], KU-2103[b,2], KU-2104[a,2], KU-2105[b,2], KU-2106[b,2], KU-2108[b,2,m], KU-2109[a], KU-2118[a,2], KU-2124[a,2], KU-2126[a,2], KU-2144[a,1], KU-2152[a,1], KU-2155[a,2], KU-2156[a,2], KU-2157[a,1], KU-2158[a,2,m], KU-2159[a,2], KU-2160[b,2,m]
Kazakhstan (n = 1)
AE1090[c,1]
Kyrgyzstan (n = 1)
IG131606[c,1]
Pakistan (n = 3)
IG46663[c,1], CGN10768[c,1], CGN10770[c,1]
Syria (n = 2)
IG46623[b,2], IG47259[a,1]
Tajikistan (n = 1)
IG48554[a,1]
Turkey (n = 2)
KU-2132[a,1], KU-2136[a,1]
Turkmenistan (n = 1)
IG126387[c,1]

Underlined accessions were used as pollen parents for two independently produced synthetics.
KU: Plant Germ-Plasm Institute, Faculty of Agriculture, Kyoto University, Japan.
PI: National Small Grains Research Facility, USDA-ARS, USA.
IG: International Center for Agricultural Research in the Dry Areas (ICARDA).
CGN: Centre for Genetic Resources, The Netherlands.
AE: Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK).
AT: Kenji Kato, Okayama University.
[a] HG7 lineage (n = 32), [b] HG9 lineage (n = 21), [c] HG16 lineage (n = 13), [d] HG17 lineage (n = 3) in Takumi *et al.* (2009a).
[1] L1 (n = 26), [2] L2 (n = 38), [17] HGL17 (n = 3) in Mizuno *et al.* (2010a).
[s] ssp. *strangulata* (n = 12), [an] var. *anathera* (n = 1), [m] var. *meyeri* (n = 5).

Phenotype measurement and statistical analysis

Selfed seeds (F_3 generation) derived from one F_2 plant of each synthetic were sown in November 2007 and 2008, and plants were grown individually in pots arranged randomly in the field and in a glasshouse of Kobe University. The temperature of the glasshouse was not regulated. A set of the 82 synthetic wheat lines was repeatedly grown and measured in seasons 2007–2008 and 2008–2009. Heading and flowering time were recorded as days after sowing. Maturation time was evaluated as the number of days that had passed when the ear neck turned yellow. The heading, flowering, and maturation time were measured for the five earliest tillers of each plant, and mean values were calculated using data from four plants in each synthetic hexaploid line. The grain-filling period was defined as the number of days from flowering to maturation. The data were statistically analyzed using JMP software ver. 5.1.2 (SAS Institute, Cary, NC, USA). The correlations among the examined traits were estimated based on Pearson's correlation coefficient values.

Water content in grains was measured using at least eight grains per ear with a Riceter m2 grain moisture tester (Kett Electric Laboratory, Tokyo, Japan) according to the manufacturer's protocol. The day when the water content became lower than 30% was recorded for each synthetic hexaploid grown in the glasshouse.

Total RNA extraction and gene expression analysis

Total RNA was extracted by guanidine thiocyanate from flag leaves of the wheat synthetics grown under the standard growth temperature (23°C). About 0.5 g of developing and maturing seeds of the individual wheat synthetics, grown in the glasshouse, was collected and ground to a fine powder in liquid nitrogen. Total RNA was extracted using an SDS-phenol method (Kawakami *et al.* 1992). First-strand cDNA was synthesized from DNase I-treated mRNA samples with oligo-dT primers using the high fidelity ReverTra Ace reverse transcriptase (Toyobo Co., Ltd., Osaka, Japan). Quantitative RT-PCR was performed using a LightCycler 480 System II (Roche, Basel, Switzerland) and primer sets specific to two wheat senescence-associated genes, *TaSAG5* and *TaSAG6* (Kajimura *et al.* 2010). *TaSAG5* and *TaSAG6* putatively encode methylcrotonoyl-CoA carboxylase and seed imbibition protein, respectively. Here, we used these genes as developmental markers to monitor the physiological state of developing seeds. The wheat actin gene (*Act*) was used as an internal control. Information on the primer sequences and PCR products is in our previous report (Kajimura *et al.* 2010). The rate of amplification was monitored using THUNDERBIRD SYBR qPCR mix (Toyobo) according to the manufacturer's protocol. Results were obtained as $2^{-\Delta Ct}$, where Ct is the difference in number of PCR cycles required to reach the log phase of amplification of the target gene relative to *Act*; the values were expressed relative to the transcript levels in the first samples of each time course experiment.

Results

Establishment of the synthetic wheat series

We previously produced 47 lines of synthetic hexaploids, each independently derived from different *Ae. tauschii* accessions (Kurahashi *et al.* 2009, Takumi *et al.* 2009b). In this

study, 35 additional synthetic wheat lines were developed from triploid hybrids between Ldn and *Ae. tauschii* accessions. In these 35 synthetic lines, 13 *Ae. tauschii* accessions were repeatedly used as pollen parents to produce triploid hybrids. Finally, 82 synthetic wheat lines were established from 69 *Ae. tauschii* accessions, in which 3 lines exhibited weak hybrid chlorosis and no synthetics showed any symptoms of hybrid necrosis or extensive growth abortion. All the synthetics were fertile and there was no segregation of plants with excessively reduced height (indicative of haploidy or aneuploidy) in the F_3 populations (data not shown), indicating that the synthetics were hexaploid and genetically stable. Somatic chromosome numbers were 42 in all F_3 seeds examined from single F_2 plants of each synthetic.

In the 69 parental *Ae. tauschii* accessions, 39 (56.5%) were originally collected from Iran (Table 1). The *Ae. tauschii* accessions contributing to the synthetic hexaploids were found in the eastern habitats (<60° longitude) such as Afghanistan and Pakistan. Also, a small number of the accessions from Armenia and Azerbaijan were successfully used to form synthetic hexaploids. Therefore, a geographical bias in the genetic variation in the parental *Ae. tauschii* accessions might be considered in relation to the entire variation in the species. In our previous study, the *Ae. tauschii* accessions were genealogically classified into six sublineages of L1, three sublineages of L2 and a haplogroup lineage 17 (HGL17) (Mizuno *et al.* 2010a). The 69 parental *Ae. tauschii* accessions used here covered the four sublineages of L1, three sublineages of L2 and HGL17. Only sublineages 1 and 3 of L1 were excluded from the parental accessions. Although the 82 synthetics did not necessarily extend across the entire D-genome diversity in *Ae. tauschii*, most of the genetic variation was included in the D-genomes of the established synthetic lines. Five and twelve accessions of variety *meyeri* and subspecies *strangulata*, respectively, contributed to the production of the synthetic wheat lines, and the synthetic hexaploids were established from all parental accessions belonging to variety *meyeri* and subspecies *strangulata*. All accessions of variety *meyeri* and subspecies *strangulata* belonged to L2 (Mizuno *et al.* 2010a).

Variations in maturation time-related traits

For evaluation of the D-genome diversity in the 82 synthetics, four maturation time-related traits, i.e., heading time, flowering time, maturation time, and grain-filling period, were measured under the field and glasshouse conditions in two seasons, 2007–2008 and 2008–2009 (Supplemental Tables 1, 2). Large variations were found for the four maturation time-related traits under the both conditions (Fig. 1). Some synthetic lines showed earlier-heading or -flowering phenotypes than those of Ldn in the field and glasshouse. Shorter grain-filling periods were also observed in the synthetic hexaploids than in Ldn. The minimum values of maturation time were at least two weeks shorter than the maximum values for the wheat synthetics. All wheat synthetics grown in the glasshouse showed earlier heading, flowering, and maturation times than those in the field, whereas the grain-filling duration of the wheat synthetics in the glasshouse was generally shorter than those in the field (Fig. 1 and Table 2). Significantly positive correlations ($P < 0.001$) were observed in heading, flowering, and maturation times between the 2007–2008 and 2008–2009 seasons (Table 2). The significance was independent of the growth location of the plants, i.e., glasshouse or field. However, no correlation was found for grain-filling period between the growth seasons.

In terms of heading time, the parental *Ae. tauschii* accessions showed larger variation than the synthetics in the glasshouse and field (Fig. 1A). Scatter plots of the heading time showed clearly significant correlations between the diploid and hexaploid wheat backgrounds in the 2008–2009 season (Fig. 2). Therefore, the wide variation observed in heading time in *Ae. tauschii* was well maintained in the hexaploid background of the synthetic wheat lines. The correlation coefficient values in the glasshouse were higher than those in the field for both seasons examined. A significant difference in heading time ($P < 0.001$) was observed among eight sublineages of the parental *Ae. tauschii* accessions (Fig. 3A). Sublineage 6 of L1 (L1S6) showed the earliest heading time in the eight sublineages, whereas heading times in sublineage 3 of L1 (L1S3) and sublineage 2 of L2 (L2S2) was late. On the other hand, no significant differences in heading time was found among the eight sublineage-derived synthetic wheat lines, either in the glasshouse or in the field (Fig. 3B, 3C). Differences between early- and late-heading sublineages were narrower in the hexaploid background than in the diploid background.

Thirteen pairs of the synthetic wheat hexaploids were independently derived from the same parental combinations, which indicates that the paired synthetic lines contained

Table 2. Correlation of each examined trait between the two growth seasons, 2007–2008 and 2008–2009

Traits	glasshouse			field		
	Mean ± standard deviation		Correlation coefficient	Mean ± standard deviation		Correlation coefficient
	2007–2008	2008–2009		2007–2008	2008–2009	
Heading time	134.90 ± 5.06	153.51 ± 3.04	0.46***	145.75 ± 3.05	167.15 ± 2.96	0.47***
Flowering time	142.20 ± 4.52	159.86 ± 2.97	0.46***	152.09 ± 3.39	173.86 ± 2.65	0.52***
Maturation time	179.27 ± 2.26	192.46 ± 2.88	0.38***	185.74 ± 3.02	203.14 ± 1.78	0.43***
Grain-filling period	36.68 ± 3.57	32.61 ± 1.09	0.04	33.65 ± 2.05	29.28 ± 1.78	–0.04

Significant correlation coefficients are indicated by asterisks (*** $P < 0.001$).

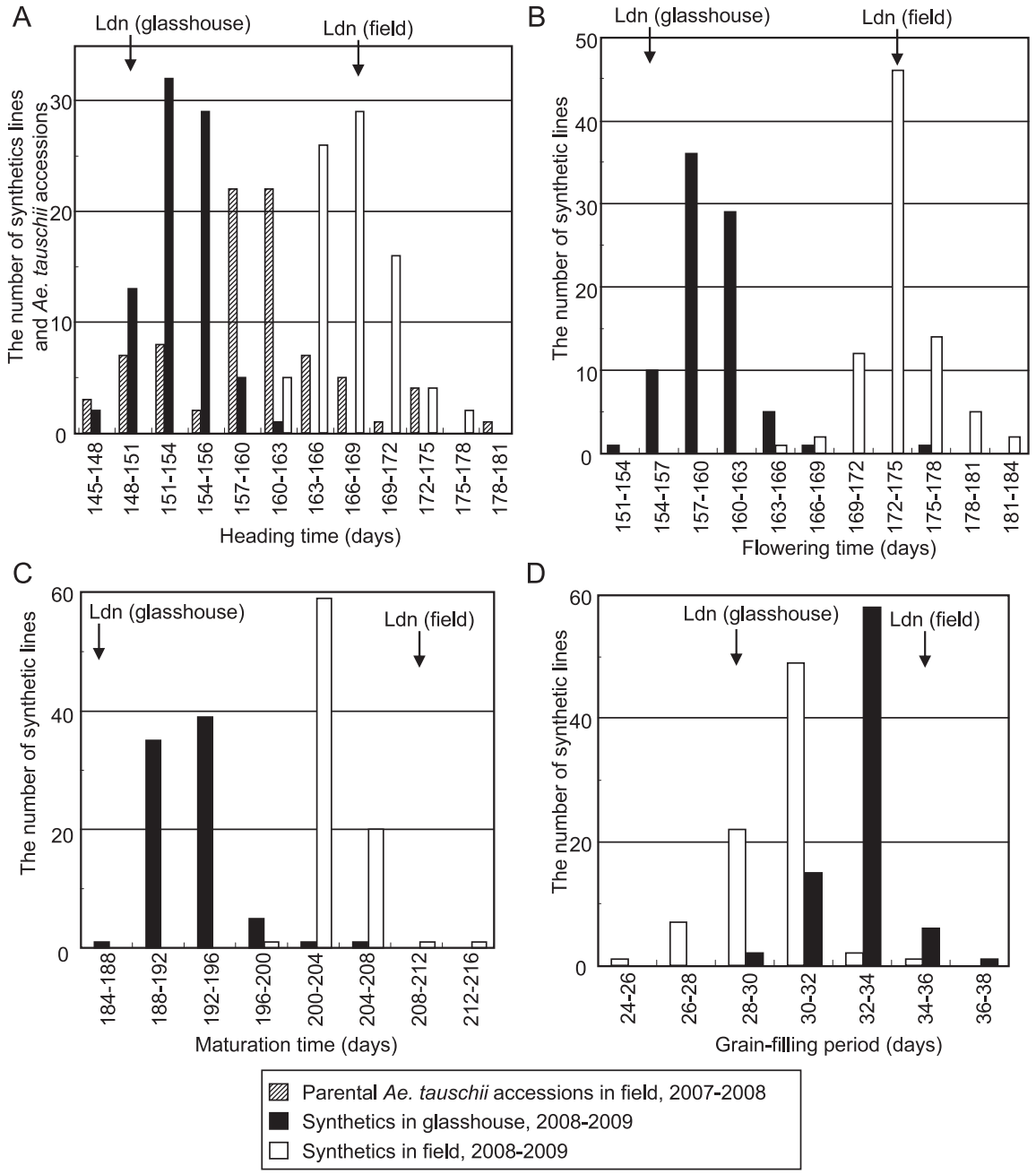

Fig. 1. Frequency distributions of the four traits examined in the 82 wheat synthetics. Arrows indicate the data for Ldn in the 2008–2009 season. (A) Heading time variation in the synthetic lines and their parental *Ae. tauschii* accessions. The *Ae. tauschii* accessions were grown under field conditions. (B) Flowering time variation in the synthetic lines. (C) Maturation time variation in the synthetic lines. (D) Grain-filling period variation in the synthetic lines.

identical genetic information if any mutation did not occur in the genomes through hexaploidization. In fact, three pairs of wheat synthetics showed no significant differences in the traits examined in the 2007–2008 and 2008–2009 seasons (Table 3). However, the other synthetic pairs exhibited significant differences in multiple traits. In particular, highly significant differences were observed on the most traits examined in two pairs of wheat synthetics in both growth seasons (Ldn × KU-2090 and Ldn × KU-2126).

Correlation coefficients among the traits examined were compared for both the 2007–2008 and 2008–2009 seasons (Table 4). Highly significant correlations were observed among heading, flowering, and maturation time under the same growing conditions. Positive correlations among heading, flowering, and maturation time were also found to be significant between the two conditions of glasshouse and field. On the other hand, the grain-filling period was negatively correlated with heading and flowering time in the glasshouse and in the field, although the correlation in the glasshouse in 2007–2008 season was not significant. The

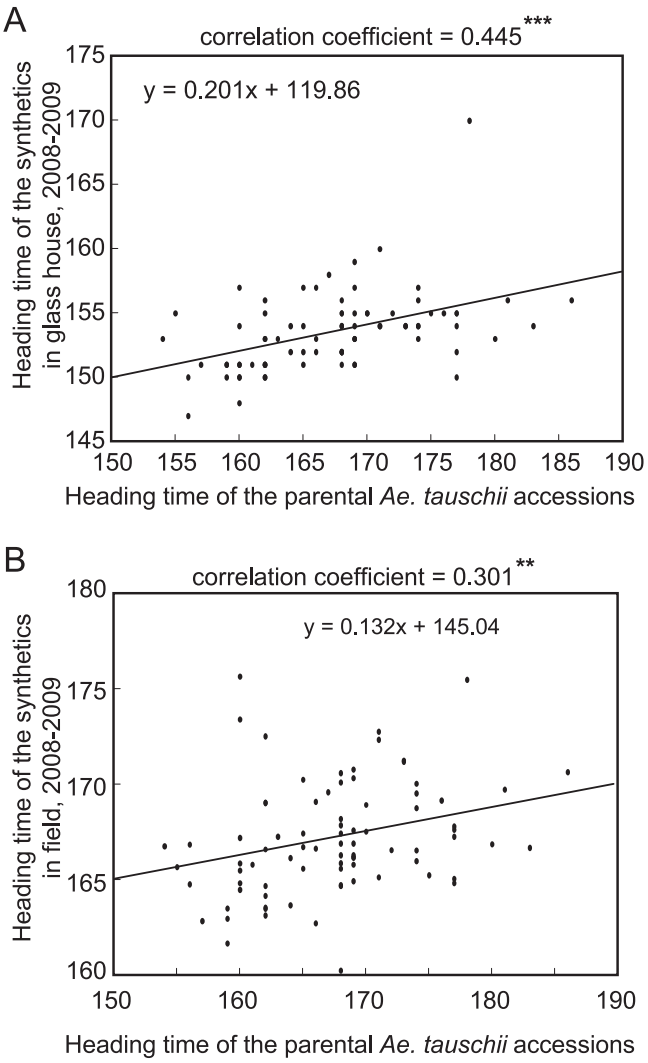

Fig. 2. Comparison of heading time between wheat synthetics and parental *Ae. tauschii*. The regression line for each plot is indicated. The correlation coefficient is represented at the top of each plot. Significant correlation coefficients are indicated by asterisks (**$P < 0.01$, ***$P < 0.001$).

negative correlation between grain-filling period and flowering time indicated that earlier-flowering lines of the wheat synthetics required a longer period for grain maturation, and that the grain-filling period in the late-flowering synthetics tended to be shorter.

Comparison of gene expression patterns between two lines different in grain-filling periods

On the whole, no significant correlations were observed in grain-filling periods between the two growing seasons and under the two conditions, implying that variation in grain-filling period seems to be not genetically determined. However, some combinations of synthetic wheat lines having similar flowering time showed stable differences in grain-filling period under all conditions examined (Fig. 4). For two synthetic lines derived from crosses between Ldn and KU-2093 and between Ldn and KU-2152, the grain-filling period of the former was consistently 1 to 5 days shorter than the latter, although the flowering time was almost identical. Grain water content in the former wheat decreased more rapidly than in the latter one (Fig. 5A). A similar relationship of grain-filling period was observed for other combinations such as the synthetics from crosses between Ldn and KU-2126 and between Ldn and KU-2097. However, no significant difference in grain water content was found between the two synthetic hexaploids (Ldn × KU-2126 and Ldn × KU-2097).

To compare gene expression profiles during the grain-filling period, quantitative RT-PCR analysis was conducted using total RNA samples isolated from developing seeds and flag leaves after flowering in the two synthetic lines derived from crosses between Ldn and KU-2093 and between Ldn and KU-2152. Previous study showed that *TaSAG6* transcripts gradually accumulated in maturing and ripening seeds of the common wheat cultivar Chinese Spring (CS) (Kajimura *et al.* 2010). *TaSAG6* transcript linearly increased in the developing seeds of the two wheat synthetics (Fig. 5A). The increase in the *TaSAG6* transcript in the short grain-filling period line (Ldn × KU-2093) started at the 24 days after flowering, which was earlier than in the long grain-filling period line (Ldn × KU-2152). *TaSAG5* transcript gradually increased in the flag leaf of CS (Kajimura *et al.* 2010). The *TaSAG5* expression pattern in the flag leaves of the long grain-filling period line was similar to that of CS (Fig. 5B). The *TaSAG5* transcripts continued to accumulate to higher levels in the flag leaves of the short grain-filling period line than in those of the long grain-filling period line. Thus, the expression profiles of the two senescence-associated genes, *TaSAG5* and *TaSAG6*, would correspond to the difference in grain-filling period between the two synthetic lines (Ldn × KU-2093 and Ldn × KU-2152).

Discussion

Geographical and genealogical distribution of the synthetics' parental accessions

The wheat synthetic lines established in this study were derived through endoreduplication forming triploid gametes in interspecific ABD hybrids obtained by crossing Ldn with 69 *Ae. tauschii* accessions. Thus, the synthetic hexaploids putatively shared identical A and B genomes derived from Ldn and contained the diverse D genomes originating from the *Ae. tauschii* pollen parents. The 69 *Ae. tauschii* accessions used as pollen parents were distributed from western habitats, Syria and Turkey, to eastern habitats, Afghanistan and China, covering almost entire natural habitat, and only *Ae. tauschii* accessions from Dagestan and Uzbekistan were missing in the parental accessions.

Genealogical analysis of the distribution of the parental *Ae. tauschii* accessions showed that the D genomes of the synthetic lines established were derived from all three lineages, L1, L2 and HGL17, of *Ae. tauschii*. However, no

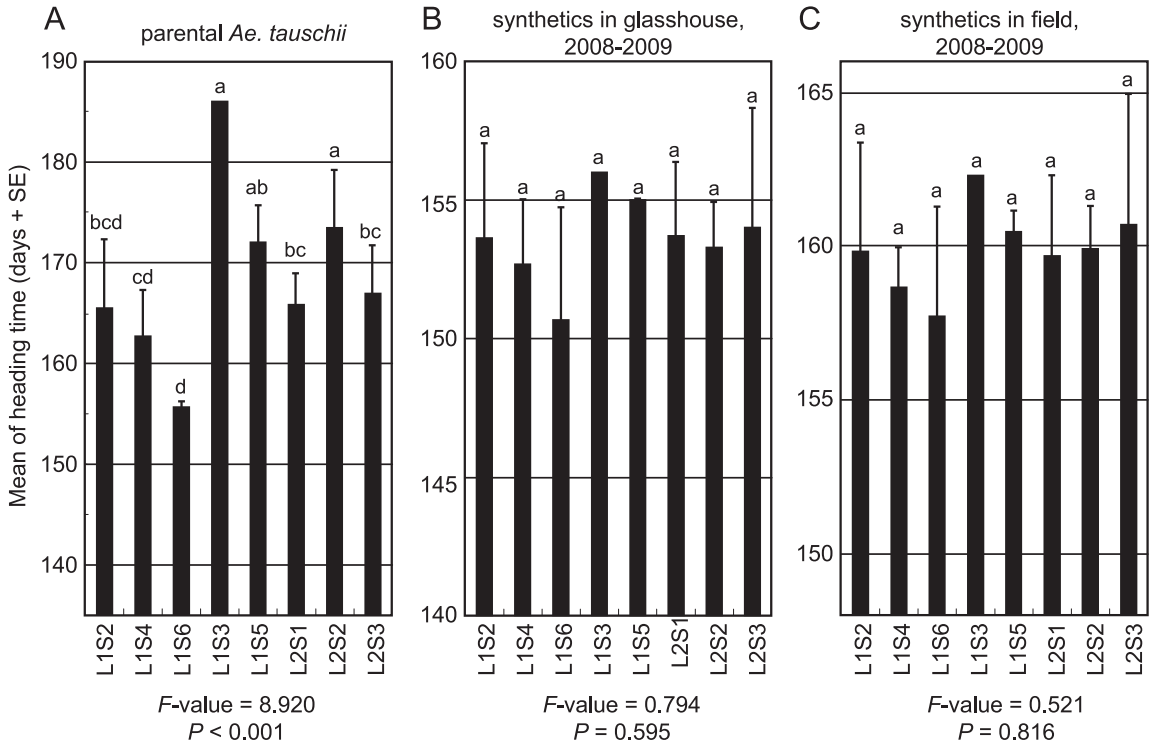

Fig. 3. Relationship between heading time and the sublineage differentiation of the D genome. Statistical significance of the D-genome effect among sublineages was estimated by analysis of variance; the *F*-values are presented. Means ± standard errors with the same letters were not significantly different ($P > 0.05$) (Tukey-Kramer's HSD test).

Table 3. *P* values for significance of the line difference in each examined trait in the two independently developed synthetic lines

Parental *Ae. tauschii* accession	2007–2008								2008–2009							
	glasshouse				field				glasshouse				field			
	HT	FT	MT	GFP	HT	FT	MT	GFP	HT	FT	MT	GFP	HT	FT	MT	GFP
KU-2126	<0.01	<0.01	0.735	<0.001	<0.001	<0.001	0.239	0.169	<0.01	<0.001	0.289	<0.001	<0.05	<0.05	0.536	<0.001
KU-2158	0.91	0.285	<0.05	<0.01	<0.01	<0.01	0.413	0.197	<0.05	0.069	<0.001	<0.001	<0.001	<0.001	<0.001	<0.05
PI476874	<0.001	<0.001	<0.001	0.829	<0.001	<0.001	1	<0.05	0.147	0.861	0.866	1	0.106	0.145	0.641	0.233
KU-2090	<0.001	<0.001	<0.001	0.097	<0.001	<0.001	0.271	<0.001	<0.001	<0.001	0.122	<0.001	<0.01	<0.001	<0.001	0.727
IG47259	0.05	<0.05	0.887	<0.01	0.637	0.155	<0.001	<0.001	0.324	0.657	0.082	0.111	<0.05	<0.001	<0.001	0.514
KU-2069	<0.001	<0.001	<0.001	0.476	0.313	0.529	0.766	0.511	<0.001	<0.001	0.29	<0.001	<0.01	<0.01	0.76	<0.01
KU-2078	0.149	0.455	0.11	<0.01	0.151	0.073	<0.05	0.689	0.573	0.711	0.391	0.691	0.07	0.091	<0.001	<0.001
KU-2091	<0.01	<0.05	0.506	<0.001	0.983	0.166	<0.001	0.145	0.061	<0.05	0.22	0.439	<0.001	<0.001	<0.01	<0.001
KU-2103	0.517	<0.05	<0.05	0.214	0.686	<0.01	0.549	<0.001	0.072	0.742	0.533	0.754	0.055	0.079	0.809	0.069
KU-2156	<0.001	<0.001	<0.01	<0.001	<0.05	<0.05	0.297	0.086	0.434	0.858	<0.05	0.237	0.655	0.226	0.051	0.188
KU-2124	<0.05	0.078	<0.001	<0.01	0.296	<0.05	0.133	0.752	<0.001	<0.001	<0.01	<0.001	0.236	0.594	<0.001	<0.001
AE454	0.512	0.121	<0.05	0.317	0.336	0.082	<0.05	<0.01	<0.001	<0.001	0.373	<0.001	<0.001	<0.001	<0.001	<0.05
KU-2105	0.764	0.915	0.378	0.306	0.142	0.271	0.051	0.681	<0.001	<0.001	<0.001	<0.05	0.679	0.601	<0.01	<0.001

HT; Heading time.
FT; Flowering time.
MT; Maturation time.
GFP; Grain-filling period.

Ae. tauschii accession belonging to sublineages 1-1 or 1-3 contributed to the synthetic lines established, almost certainly due to postzygotic hybridization barriers between Ldn and *Ae. tauschii* (Mizuno *et al.* 2010b). Types II and III necrosis frequently occur in triploid hybrids between Ldn and L1 accessions of *Ae. tauschii*, and hybrid necrosis inhibits the efficient production of wheat synthetics. The causal genes inducing types II and III necrosis are widely distributed in L1 but not in L2 (Mizuno *et al.* 2010b) and only four sublineages of L1 represent the eastern distribution (Mizuno *et al.* 2010a), which resulted in the limited production of wheat synthetics from the *Ae. tauschii* accessions in eastern

Table 4. Correlation coefficients among the traits examined in wheat synthetics grown in a glasshouse and a field in 2007–2008 (below the diagonal) and 2008–2009 (above the diagonal)

	Heading time in glasshouse	Flowering time in glasshouse	Maturation time in glasshouse	Grain-filling period in glasshouse	Heading time in field	Flowering time in field	Maturation time in field	Grain-filling period in field
Heading time in glasshouse		0.97***	0.91***	–0.21	0.66***	0.62***	0.67**	–0.10
Flowering time in glasshouse	0.98***		0.93***	–0.24*	0.66***	0.63***	0.70***	–0.07
Maturation time in glasshouse	0.43***	0.50***		0.13	0.65***	0.61***	0.69***	–0.05
Grain-filling period in glasshouse	–0.74***	–0.73***	–0.07		–0.09	–0.11	–0.10	0.08
Heading time in field	0.55***	0.54***	0.31*	–0.41**		0.92***	0.75***	–0.57***
Flowering time in field	0.54***	0.54***	0.33**	–0.40**	0.98***		0.74***	–0.57***
Maturation time in field	0.40**	0.41**	0.26*	–0.33**	0.77***	0.80***		0.12
Grain-filling period in field	–0.30*	–0.29*	–0.16	0.18	–0.49***	–0.47***	0.15	

Significant correlation coefficients are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

habitats such as Pakistan and Afghanistan. On the other hand, synthetic lines were efficiently produced from the L2 accessions of *Ae. tauschii*. Hybrid chlorosis and severe growth abortion were rarely observed and the hybrid plants might have developed only locally in Transcaucasia (Mizuno *et al.* 2010b), which thus did not prevent the L2 accessions from efficient production of wheat synthetics. In particular, more than 20 accessions of *Ae tauschii* in sublineage 2-3 contributed to synthetic wheat production, and most of the subspecies *strangulata* accessions belonged to the pollen parents from sublineage 2-3. The rate in the subspecies *strangulata* accessions for producing synthetic hexaploids was 100%, as was that of the variety *meyeri* accessions, which belonged to sublineages 2-2 and 2-3 (Mizuno *et al.* 2010a).

Distinct regulation of flowering time and grain-filling period

We conducted empirical studies on the four maturation time-related traits using the established 82 synthetic hexaploids. The wheat synthetics exhibited wide variations in the traits examined. Significant positive correlations were observed among the three traits of heading, flowering, and maturation time, implying that these three traits are controlled by the same genetic mechanisms. However, no correlation was found between grain-filling period and the other traits examined, indicating that grain-filling period is regulated by distinct mechanisms from other traits. The grain-filling period correlated negatively with flowering time. The grain-filling process progressed under higher temperatures in the late-flowering lines of synthetic wheat than in the early-flowering lines because the temperature gradually increased in the glasshouse and field in April and May (data not shown). Thus, the negative correlation between flowering time and grain-filling period might be due to an environmental effect rather than a genetic one. Although the grain-filling period was influenced by temperature in wheat synthetics, as previous reports had mentioned for hexaploid and tetraploid wheat (Knott and Gebeyehou 1987, Wiegand and Cuellar 1981), some combinations of two synthetic wheat lines with similar flowering time reproducibly exhibited a line difference in grain-filling period. This observation indicated the presence of genetic differences in grain-filling period between the paired wheat synthetics. In conclusion, two major genetic pathways seem to independently determine the wheat maturation time: one controls the heading and flowering times and the other regulates the grain-filling period.

The grain-filling period is a complex trait under the control of QTLs (Wang *et al.* 2009). The *Gpc-B1* locus on wheat chromosome 6B encodes a NAC domain-containing transcription factor and is involved in multiple processes regulating grain protein content, grain micronutrient concentrations, leaf senescence, and grain-filling duration (Uauy *et al.* 2006a, 2006b, Distelfeld *et al.* 2007). No previous report has mentioned any pleiotropic effect of *Gpc-B1* on flowering time. In this study, at least one pair of synthetic hexaploids (Ldn × KU-2093 and Ldn × KU-2152) showed a stable line difference in grain-filling period, and the difference corresponded to that of grain water content and the gene expression profiles of two developmental markers, *TaSAG5* and *TaSAG6*. In this pair of synthetic hexaploids, genetic factors determining the line difference in grain-filling period might affect at least grain water content and flag leaf senescence. On the other hand, no correlation of grain-filling period with grain water content or flag leaf senescence was found in

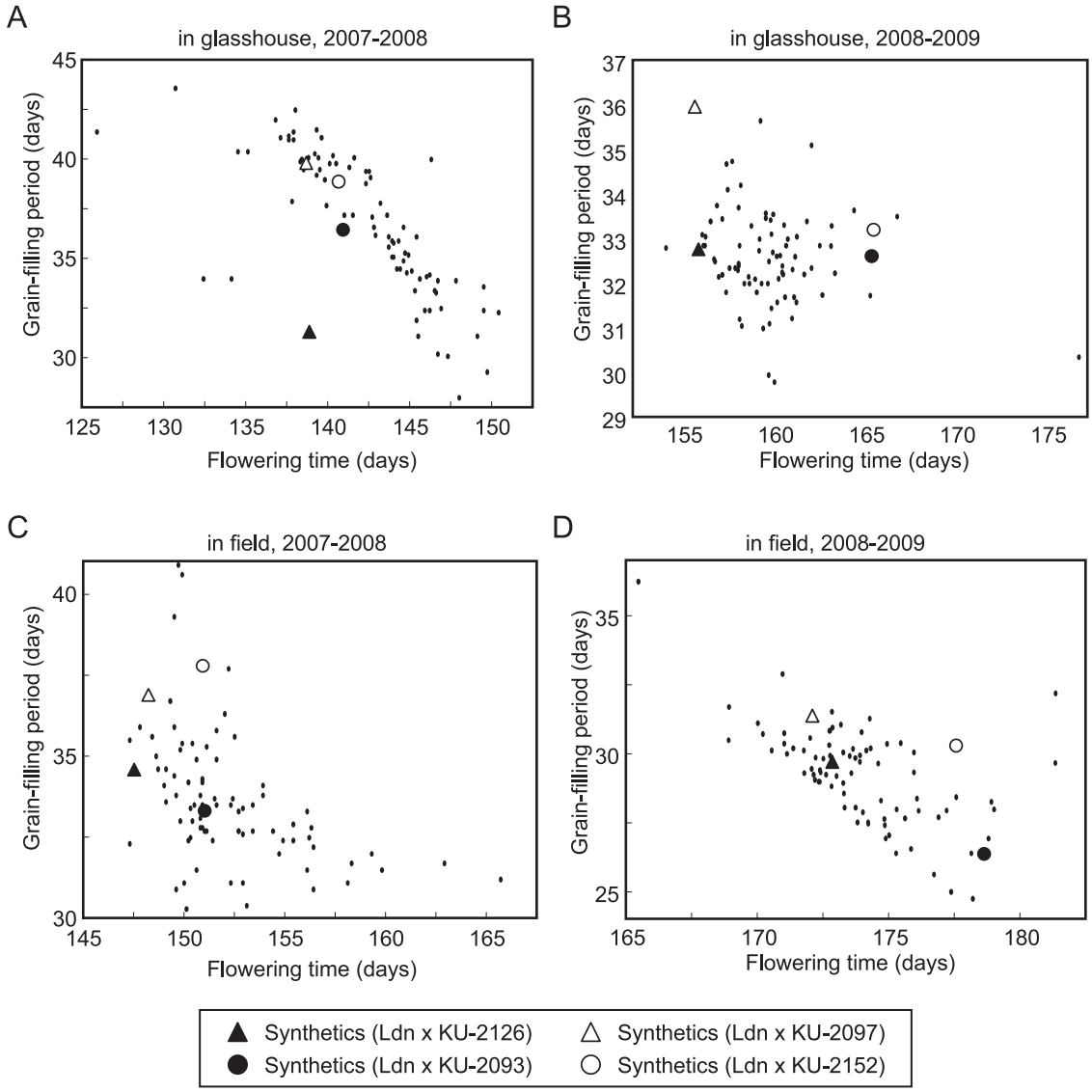

Fig. 4. Scatter plots of flowering time and grain-filling period in synthetic wheat lines.

another pair of synthetic hexaploids (Ldn × KU-2126 and Ldn × KU-2097). Thus, these observations indicate that different genetic pathways contribute to the variation of grain-filling period in synthetic wheat lines.

Expression of the Ae. tauschii flowering phenotype in the hexaploid background

In our previous study with 27 synthetic hexaploids, we failed to find any significant correlation of heading time between wheat synthetics and their parental *Ae. tauschii* accessions (Takumi *et al.* 2009b), which seemed to be due to the small number of accessions used for the empirical study. Using the 82 synthetic lines in the present study, a significant positive correlation was observed (Fig. 2), which implied that the wide variation observed in heading time in *Ae. tauschii* was expressed well in the hexaploid background of the synthetic wheat lines. Under photoperiod-regulating conditions with vernalization, the synthetic hexaploid from an early-flowering D-genome donor showed an early-flowering phenotype, whereas a synthetic hexaploid from a late-flowering D-genome donor was late-flowering (Fujiwara *et al.* 2010). Through hexaploidization, new interactions occur among A, B, and D genomes. In synthetic hexaploids, it has been reported that gene expression of homoeologous genes is nonadditively altered, and thus genome-wide gene expression is changed (Chagué *et al.* 2010, Pumphrey *et al.* 2009). In spite of the modification of gene expression profiles, the early-flowering phenotype in synthetic hexaploids could have been affected by the homoeolog for early-flowering in the D-genome donor. Therefore, the flowering genes of *Ae. tauschii* function in the hexaploid background and affect heading, flowering, and maturation time in wheat synthetics, implying that the flowering genes of *Ae. tauschii* would be useful in wheat breeding to improve maturation time. Future studies should identify the D-genome loci associated with early-flowering and early-maturing phenotypes in the wheat

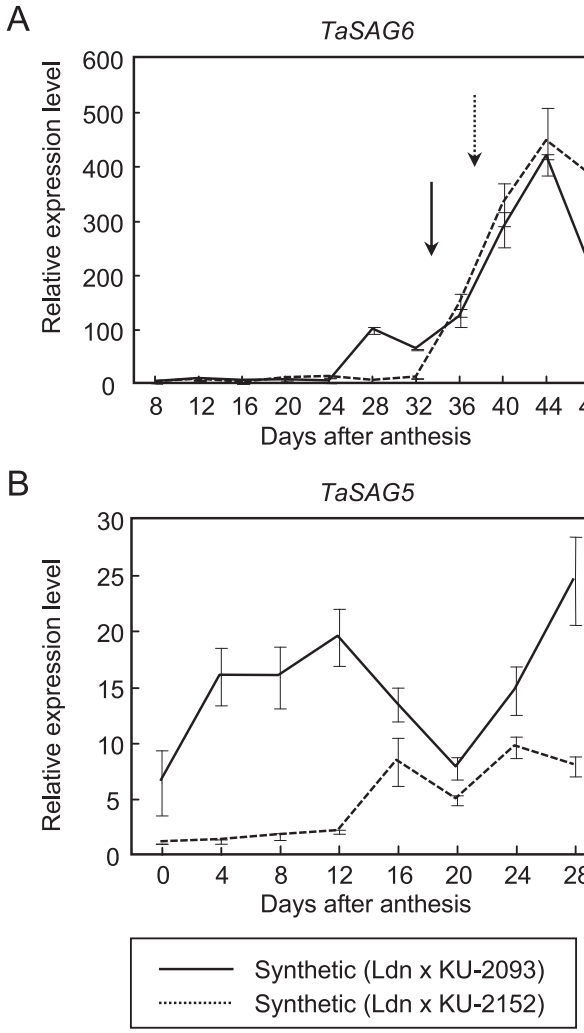

Fig. 5. Expression profiles of senescence associated gene transcripts in two synthetic wheat lines based on real-time RT-PCR analysis. (A) *TaSAG6* expression and water content in maturation and ripening seeds. Arrows indicate the days when grain water contents became lower than 30%. Total RNA was extracted from the seeds at days 8, 12, 16, 20, 24, 28, 32, 36, 40, 44 and 48 after flowering. The time course of transcript accumulation was quantified relative to that of the seeds on the 8th day after flowering in the synthetic line between Ldn and KU-2152 and was normalized to *Act* transcript; values are means with standard deviation. (B) *TaSAG5* expression in the flag leaves. Total RNA was extracted from the flag leaves at days 0, 4, 8, 12, 16, 20, 24 and 28 after flowering. The time course of transcript accumulation was quantified relative to that of the flag leaves on the day of flowering in the synthetic line between Ldn and KU-2152 and was normalized to *Act* transcript; values are means with standard deviation.

synthetic lines, and confirm their usefulness in the genetic backgrounds of elite cultivars.

Genetic and epigenetic modifications occur in allopolyploid wheat genomes (Kashkush *et al.* 2002, Ozkan *et al.* 2001, Shaked *et al.* 2001). Recent study showed that the genome stability of synthetic hexaploids depends on the particular genotypes of both AB and D genome donors (Mestiri *et al.* 2010). Mestiri *et al.* (2010) reported that aneuploidy represented the only structural change observed in their synthesized hexaploids, and that the frequency of aneuploids was higher in the first generation of the wheat synthetics than in the following generations. Ldn is a valuable female cultivar to produce ABD triploid hybrids crossed with various *Ae. tauschii* accessions (Matsuoka and Nasuda 2004), and euploid lines of synthetic hexaploids were efficiently established through unreduced gamete formation in the triploid hybrids. Our empirical analysis with the 13 pairs of synthetic hexaploids independently derived from the same parental combinations showed that no significant differences in the examined traits were observed in some pairs, whereas the other pairs exhibited significant differences. It still remains to be clarified whether the significant differences were genetically or epigenetically induced.

At least under field conditions, the heading time variation in the synthetics showed half the range of the parental *Ae. tauschii* accessions (Fig. 2B). A similar suppressive and buffering effect on expression of heading time variation was observed in a study on other morphological variations (Takumi *et al.* 2009b). Fujiwara *et al.* (2010) mentioned that early-heading or flowering in synthetic hexaploids results partly from a decreased vernalization requirement due to the effect of a dominant *Vrn-A1* allele of Ldn. Moreover, it was assumed that the early heading or flowering in synthetic hexaploids is also associated with shortened narrow-sense earliness caused by an additional D genome (Fujiwara *et al.* 2010). The epistatic interactions between the A, B and D genomes seem to at least partly result in masking the expression of the D-genome heading time variation. It has been reported that genome-wide expression profiles are stochastically and epigenetically changed during the generation of allopolyploid wheat (Chagué *et al.* 2010, Kashkush *et al.* 2002, Pumphrey *et al.* 2009). These previous studies on the transcriptome in allopolyploids used a limited number of artificially produced lines. Therefore, future systematic analyses will be needed to elucidate the molecular basis for modifications of D-genome variation patterns following hexaploidization.

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