

Variation in abscisic acid responsiveness of *Aegilops tauschii* and hexaploid wheat synthetics due to the D-genome diversity

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Common wheat (*Triticum aestivum* L.) is an allohexaploid that originated from natural hybridization between tetraploid wheat (*Triticum turgidum*) and diploid *Aegilops tauschii*. *Ae. tauschii* is considered one of the potential sources of new genetic variation in abiotic stress tolerance for improving common wheat. Abscisic acid (ABA) plays an important role in plant adaptation to environmental stresses. In this study, ABA responsiveness of 67 *Ae. tauschii* accessions and their synthetic hexaploid wheat lines, derived from crosses between *T. turgidum* cv. Langdon and the *Ae. tauschii* accessions, was evaluated based on growth inhibition by 20 μ M ABA. Wide variation was found in ABA responsiveness for both synthetic wheat lines and their parental *Ae. tauschii* accessions. The variations due to D-genome found at the diploid level were also expressed in a hexaploid genetic background. Two pairs of synthetic wheat lines differing in ABA responsiveness were then selected for gene expression analysis and to test abiotic stress tolerance, because their parental *Ae. tauschii* accessions similarly exhibited the differential response to ABA. Gene expression of ABA inducible transcription factor, *WABI5*, and the downstream *Cor/Lea* genes (*Wrab17*, *Wdhn13* and *Wrab18*) were analysed. In one pair, the highly responsive line exhibited higher induction of *Wrab17* by ABA treatment, but no significant difference in dehydration or salinity tolerance was observed between these lines. In contrast, in the second pair, the highly ABA-responsive line showed higher levels of *Wdhn13* expression and dehydration and salinity tolerance. In synthetic wheat lines, the difference in the ABA responsiveness of the lines appeared to be determined by the different sets of D-genome genes. Our findings suggest that highly ABA-responsive *Ae. tauschii* accessions should be valuable genetic resources for improving the abiotic stress tolerance of common wheat.

Key words: abscisic acid, heterosis, synthetic hexaploid wheat, abiotic stress, *Aegilops tauschii* Coss

INTRODUCTION

Higher plants have evolved complex mechanisms to rapidly sense and adapt to changing environmental conditions. ABA induces expression of the *Cor* (cold-responsive)/*Lea* (late embryogenesis abundant) gene family that promote stress tolerance by protecting cellular components from stress (Thomashow, 1999). A number of *Cor/Lea* genes contain both CRT (C-repeat)/DRE (dehydration-responsive element) and ABRE (ABA-responsive

element) motifs in their promoters, and these *cis*-acting elements are considered to function independently (Yamaguchi-Shinozaki and Shinozaki, 2006).

In common wheat, CRT/DRE, ABRE and other cold-responsive motifs have been identified in the promoter regions of many *Cor/Lea* genes such as *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19* (Kobayashi et al., 2008a). These *Cor/Lea* genes are responsive to low temperature, drought and ABA (Egawa et al., 2006; Kobayashi et al., 2004, 2006; Ohno et al., 2003). Some of the stress-responsive transcription factors, TaDREB1, WDREB2, WLIP19, TaOBF1 and WABI5, act through ABA signalling (Shen et al., 2003; Egawa et al., 2006; Kobayashi et al., 2008b, 2008c). *Trans*-activation analysis of these

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Cor/Lea gene promoters revealed that *Wdhn13*, *Wrab18* and *Wrab19* expression is induced by WABI5, WLIP19 and WDREB2, whereas *Wrab17* expression is activated by WLIP19 and WDREB2 but not by WABI5 (Kobayashi et al., 2008a, 2008b, 2008c).

Common wheat (*Triticum aestivum* L., AABBDD genome) arose by natural hybridization between tetraploid wheat (*Triticum turgidum* L., AABB genome), including emmer and durum wheat, and *Aegilops tauschii* Coss. (DD genome) (Kihara, 1944; McFadden and Sears, 1944). *Ae. tauschii*, a wild relative of common wheat, is widely distributed in Eurasia and shows abundant genetic variation (Dudnikov and Goncharov, 1993; Dvorak et al., 1998; Dudnikov and Kawahara, 2006; Matsuoka et al., 2007, 2008, 2009). Common wheat is said to originate from Transcaucasia and the region along the southern coast of the Caspian Sea (Tsunewaki, 1966; Dvorak et al., 1998). The narrow distribution range of the *Ae. tauschii* populations involved in the origin of common wheat, compared to the range of the entire species, suggests an expansive genetic diversity that is not represented in common wheat (Feldman, 2001; Mizuno et al., 2010a, 2010b). *Ae. tauschii* can be crossed into tetraploid wheat artificially to produce synthetic hexaploid wheat (Kihara and Lilienfeld, 1949; Matsuoka and Nasuda, 2004). Agronomically important traits can be transferred to cultivated wheat by crossing with the synthetics. Therefore, *Ae. tauschii* is considered one of the potential sources of new genetic variation for improving the abiotic stress tolerance of common wheat.

In our previous study, ABA responsiveness of synthetic

wheat lines could not be compared statistically with parental *Ae. tauschii* accessions because the sample number was limited (Kurahashi et al., 2009). The objectives of the present study are to elucidate natural variation in the ABA responsiveness encoded by the D-genome in the hexaploid genetic background, and to study relationships among ABA responsiveness, *Cor/Lea* gene expression and dehydration and salinity stress tolerance. Therefore, we first evaluated the ABA responsiveness of a large number of synthetic hexaploid wheat lines derived from the cross between *T. turgidum* ssp. *durum* cv. Langdon (Ldn) and *Ae. tauschii* accessions (Takumi et al., 2009a; Kajimura et al., 2011), and of their parental accessions. Furthermore, two pairs of synthetic wheat lines were selected, according to their ABA responsiveness, for comparison of gene expression pattern and abiotic stress tolerance.

MATERIALS AND METHODS

Plant materials In total, 67 synthetic wheat lines and their *Ae. tauschii* parental accessions were used in this study. The accession number and origin of 67 *Ae. tauschii* accessions are shown in Table 1. The passport data of *Ae. tauschii* accessions, including the geographical coordinates of the original collection sites, have been given in Matsuoka et al. (2007, 2008). The tetraploid wheat cultivar Langdon (Ldn) was used as the female parent and was crossed with each of the 67 *Ae. tauschii* accessions. The F₁ progeny were grown and selfed to produce synthetics (herein designated the F₂ generation) as previously

Table 1. Origins and accession numbers of *Ae. tauschii* accessions used in this study

Origin	Accession number
Afghanistan	KU-2022, KU-2059, PI476874
Armenia	KU-2810, KU-2811, KU-2814, KU-2816, KU-2824
Azerbaijan	IG47202
China	AT47, AT55, AT76, AT80, PI499262
Georgia	AE454, AE929
India	IG48042
Iran	KU-2069, KU-2074, KU-2075, KU-2076, KU-2078, KU-2079, KU-2080, KU-20-8, KU-20-9, KU-20-10, KU-2083, KU-2088, KU-2090, KU-2092, KU-2093, KU2096, KU-2097, KU-2098, KU-2100, KU-2101, KU-2102, KU-2104, KU-2105, KU-2106, KU-2108, KU-2109, KU-2111, KU-2118, KU-2124, KU-2126, KU-2144, KU-2145, KU-2152, KU-2155, KU-2156, KU-2157, KU-2158, KU-2159, KU-2160
Kazakhstan	AE1090
Kyrgyzstan	IG131606
Pakistan	IG46663, CGN10768, CGN10770
Syria	IG46623, IG47259
Tajikistan	IG48554
Turkey	KU-2132, KU-2136
Turkmenistan	IG126387

KU: Plant Germ-Plasm Institute, Faculty of Agriculture, Kyoto University, Japan.

PI: National Small Grains Research Facility, USDA-ARS, USA.

IG: International Centre for Agricultural Research in the Dry Areas (ICARDA), Syria.

CGN: Centre for Genetic Resources, The Netherlands.

AE: Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Germany.

AT: Faculty of Agriculture, Okayama University, Japan.

reported (Takumi et al., 2009a; Mizuno et al., 2010b). All 67 synthetics were independently generated through unreduced gamete formation in each of the triploid F₁ hybrids (Matsuoka and Nasuda, 2004). 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 F₁ hybrids between Ldn and *Ae. tauschii* show abnormal growth, such as hybrid necrosis, hybrid chlorosis and severe growth abortion (Matsuoka et al., 2007; Mizuno et al., 2010b). Hybrids showing necrosis, chlorosis and severely aborted growth were excluded from the 67 synthetics. The somatic chromosome number of 42 was previously confirmed using two F₃ seeds from one F₂ plant of each synthetic (Kajimura et al., 2011). In the present study, F₃ plants derived from one F₂ plant of each synthetic were used.

Bioassay for ABA responsiveness Bioassay for ABA responsiveness and abiotic stress tolerance were evaluated as in our previous report (Iehisa et al., 2011). Briefly, seeds were pre-germinated for 24 h for synthetic wheat or 48 h for *Ae. tauschii* at 23°C in darkness. Four or five synchronously germinated seeds were placed in a plastic petri dish containing two overlapping sheets of filter paper wetted with 6 mL of distilled water (control) or 20 µM (±)-ABA (Wako Pure Chemical Industries, Osaka, Japan) solution, then incubated at 23°C in the dark. After 2 days of treatment, the length of shoots and primary roots were recorded in three or four independent experiments. Relative growth inhibition was calculated as the difference between growth of the control group and the ABA-treated group, relative to the control. 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 correlation coefficient values.

Expression analysis of ABA-inducible genes Seeds were germinated under the same conditions as in the bioassay. After 48 h, seedlings were transferred to pots containing soil and incubated at 23°C under long day conditions (16/8 h light/darkness). Seedlings that were 7-d-old (for synthetic wheat lines) or 12-d-old (for *Ae. tauschii*) were treated with 20 µM ABA solution containing 0.1% (w/v) Tween 20 by foliar spray. Total RNA was extracted from leaves at various times after ABA treatment using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). First-strand cDNA was synthesized from DNase I-treated mRNA samples with oligo-dT primers using the high fidelity ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan).

The transcript accumulation of each gene was detected by quantitative RT-PCR using a Thermal Cycler Dice Real Time System (Takara-Bio, Ohtsu, Japan) with the

following gene-specific primer sets: 5'-GGCGAGAAGAAG-GGCGTCAT-3' and 5'-GTGGTGGCTGTGGTGGCAT-3' for *Wdhn13*, 5'-AGACGGGGAAGACCATTCAAGA-3' and 5'-CTCCTTGTTGGCATCGGCA-3' for *Wrab17*, 5'-TCGAT-TCATCCAAGCCAGAG-3' and 5'-ACCAAACGAGTAAAG-GAAGCAA-3' for *Wrab18*, 5'-CACCTCAGCGCCAAGAC-3' and 5'-CTCCCATACCAACTGCCCTC-3' for *WABI5*, and 5'-GCCGTGCTTTCCCTCTATG-3' and 5'-GCTTCTC-CTTGATGTCCCTTA-3' for *Actin*. These primers amplified the target genes derived from all A-, B- and D-genomes of common wheat. The *Actin* gene was used as an internal control. The rate of amplification was monitored using THUNDERBIRD SYBR qPCR mix (Toyobo) according to the manufacturer's protocol. The relative expression level was calculated as $2^{-\Delta\Delta C_t}$, where ΔC_t is the difference in number of PCR cycles required to reach the log phase of amplification of the target gene relative to *Actin*; representative values were expressed relative to the transcript levels in Ldn samples obtained at 0 h.

Bioassay for dehydration and salinity tolerance

Seven-d-old seedlings, grown under the same conditions as for expression analysis, were used for evaluation of abiotic stress tolerance. Plants were removed from the soil and the roots washed with tap water. Dehydration tolerance was evaluated as percentage of water loss from the whole plant ($n = 10-12$) kept in a growth chamber (23°C, 40–60% relative humidity). First, the difference between initial fresh weight and weight at the indicated time was calculated, which was then divided by the initial fresh weight. Two independent experiments were performed yielding similar results.

For salinity tolerance, plants were removed from soil and grown hydroponically in a solution containing 0.2% Hyponex (HYPONEX Japan, Osaka, Japan) with ($n = 12-13$) or without (control group, $n = 6-7$) 0.15 M NaCl. Fresh weight of plants was measured every day, and weight gain was calculated as the percentage of weight gain (weight at the indicated time - initial fresh weight) of the salt-treated group versus weight gain of the control group. The results were normalized by dividing the weight gain under salt stress by the weight gain in the control group at the same day. Two independent experiments were performed yielding similar results.

RESULTS

Natural variation in ABA responsiveness in *Ae. tauschii* accessions The ABA responsiveness of 67 *Ae. tauschii* accessions was evaluated based on growth inhibition by 20 µM ABA. Wide variations were found in the relative rates of root and shoot growth inhibition. The relative rates of root growth inhibition ranged from $7.11 \pm 11.90\%$ (KU-2156) to $54.73 \pm 9.68\%$ (KU-2111), with a mean of $32.47 \pm 10.34\%$ (Fig. 1A). The tetraploid wheat

cultivar Ldn showed higher ABA responsiveness ($66.38 \pm 3.41\%$) than any of the *Ae. tauschii* accessions. Relative rates of shoot growth inhibition varied from $52.68 \pm 6.07\%$ (KU-2157) to $87.14 \pm 1.20\%$ (IG48042), with an overall mean of $72.36 \pm 6.71\%$ (Supplementary Fig. S1A). Root growth inhibition was positively correlated with shoot growth inhibition ($r = 0.498$, $P < 0.001$), suggesting that inhibition of root and shoot growth is controlled by similar mechanisms.

Ae. tauschii accessions can be classified in two major genealogical lineages (Mizuno et al., 2010a). Of 67 accessions, 24 have been classified in lineage 1 (L1) and 36 in lineage 2 (L2). The ABA responsiveness, based on root growth inhibition, was significantly higher in accessions belonging to L2 ($P < 0.01$) (Supplementary Fig. S2A). In turn, L1 and L2 can be divided respectively in six and three sublineages (Mizuno et al., 2010a). ABA responsiveness of sublineages 2-3 ($38.23 \pm 6.46\%$) was significantly higher than that of sublineage 1-2 ($24.87 \pm 10.40\%$) (Supplementary Fig. S2B). Seven accessions were not included in this analysis. To test whether latitudinal and longitudinal clines can be recognized, we performed

multiple regression analysis using latitude and longitude as independent variables and ABA responsiveness as the response variable. However, no significant effect of latitude and longitude on ABA responsiveness was observed (Supplementary Fig. S2, C and D; Supplementary Table S1).

Comparison of ABA responsiveness among synthetic wheat lines To study whether the natural variation in ABA responsiveness of *Ae. tauschii* is expressed in the hexaploid genetic background, the ABA responsiveness of the synthetic wheat lines was evaluated. The relative rates of root growth inhibition varied widely, as observed in *Ae. tauschii*, and ranged from $44.59 \pm 0.36\%$ (Ldn $\times$ KU-2118) to $80.61 \pm 6.40\%$ (Ldn $\times$ KU-2160), with a mean value of $63.52 \pm 7.92\%$ (Fig. 1B). The relative rates of shoot growth inhibition varied from $68.44 \pm 2.60\%$ (Ldn $\times$ KU-20-8) to $86.52 \pm 2.27\%$ (Ldn $\times$ IG48042), with a mean of $80.21 \pm 3.69\%$ (Supplementary Fig. S1B). Since root growth inhibition showed broader variation than shoot growth inhibition, and a positive correlation was found between them in synthetic hexaploids ($r =$

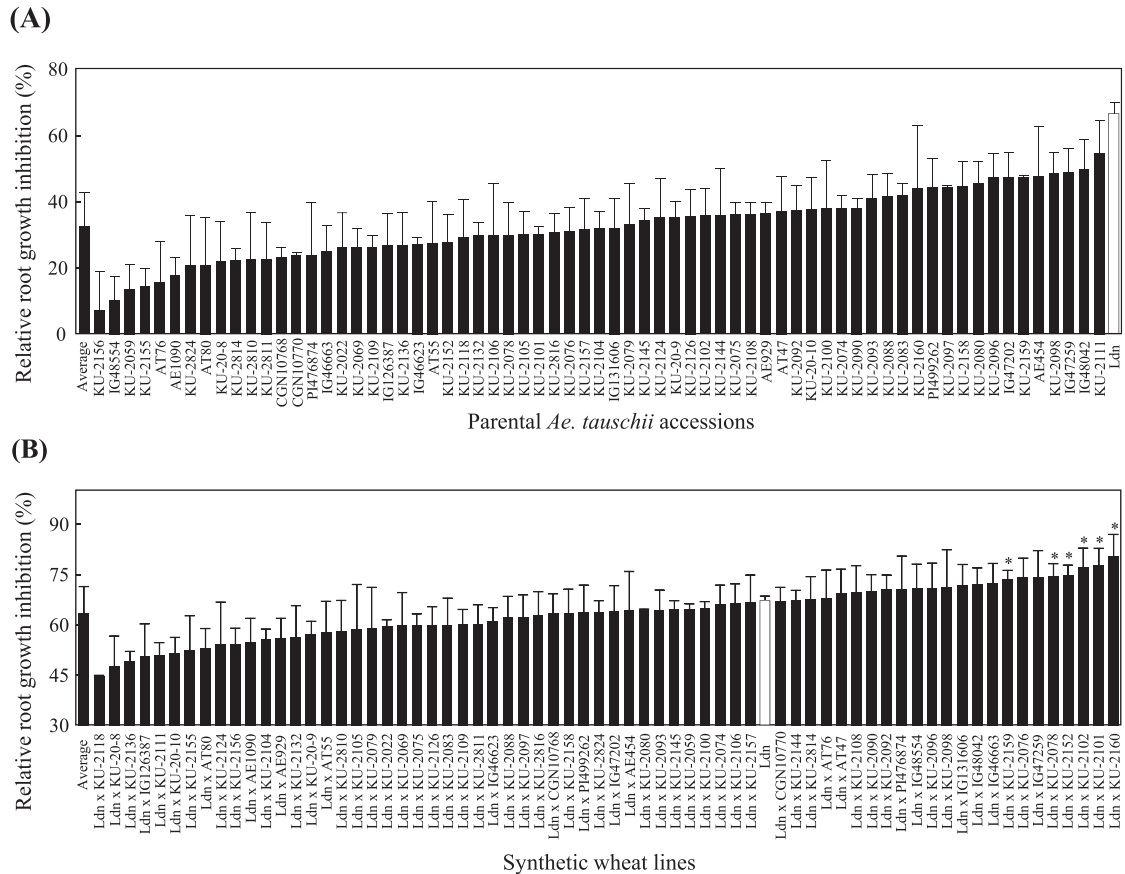


Fig. 1. Variation in root growth inhibition by 20 μ M ABA. (A) Relative root growth inhibition of 67 *Ae. tauschii* accessions. Data are means $\pm$ SD calculated from three or four independent experiments. Open bars: *T. turgidum* cv. Langdon. (B) Relative root growth inhibition of the synthetic wheat lines. Student's *t*-test was used to test for statistical significance (* $P < 0.05$).

0.507, $P < 0.01$) as well as parental *Ae. tauschii*, root growth inhibition is equated to ABA responsiveness hereafter.

Next, the correlation between ABA responsiveness of synthetic hexaploids and their parental accessions was studied. The correlation was positive, with $r = 0.248$ and $P < 0.05$ (Fig. 2A). Despite this positive correlation, in some cases the ABA responsiveness of *Ae. tauschii* is not reflected in synthetic wheat. For example, the *Ae. tauschii* accession KU-2111 is the highest responsive line but its derived synthetic showed a very low responsiveness. In contrast, IG48554 exhibited very low responsiveness but was relatively higher in hexaploid background. Synthetic wheat lines were classified into three groups according to their ABA responsiveness, and the mean value of their parental accessions was calculated for each group. This grouping was based on the total average and standard deviation (SD) of ABA responsiveness ($63.52 \pm 7.92\%$) in synthetic wheat. That is, low responsive group is composed by synthetics with ABA responsiveness less than

mean – SD (55.60%), synthetics with responsiveness higher than mean + SD (71.44%) in the highly responsive group, and the intermediate group ranging from 55.60% to 71.44%. Eleven synthetic lines were classified into a less responsive group, 45 lines into an intermediate group, and 11 lines into a highly responsive group. The mean ABA responsiveness of the parental *Ae. tauschii* accessions in the less responsive group was $26.61 \pm 12.87\%$, $32.92 \pm 9.52\%$ in the intermediate group and $36.50 \pm 9.21\%$ in the highly responsive group (Fig. 2B). The ABA responsiveness of parental *Ae. tauschii* accessions was significantly higher in the intermediate ($P < 0.05$) and highly responsive group ($P < 0.05$) than in the less responsive group. Of the 67 synthetic hexaploids, six lines that had parental *Ae. tauschii* accessions of KU-2159, KU-2078, KU-2152, KU-2102, KU-2101 and KU-2160 showed significantly higher ABA responsiveness than Ldn, 47 lines showed similar responsiveness, and 13 showed lower responsiveness. None of the synthetic wheat lines exhibited lower ABA responsiveness than their parental *Ae. tauschii* accessions.

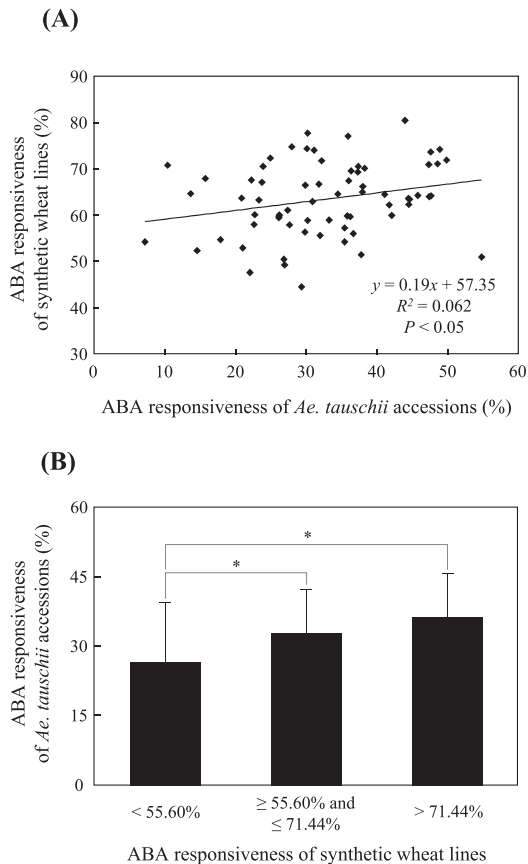


Fig. 2. Correlation of ABA responsiveness between wheat synthetics and their parental accessions. (A) Scatter plot of ABA responsiveness in the synthetic wheat lines and their parental *Ae. tauschii* accessions. (B) Comparison of ABA responsiveness of parental *Ae. tauschii* accessions among synthetic lines classified according to their growth inhibition rates. Data are mean $\pm$ SD. Student's *t*-test was used for statistical significance (* $P < 0.05$).

Expression analysis of ABA-inducible genes To compare gene expression patterns between less ABA-responsive and highly responsive lines, two pairs of synthetic hexaploids were randomly selected from the synthetic lines. Pair 1 included a low ABA-responsive line from Ldn $\times$ KU-2124 and a highly responsive line from Ldn $\times$ IG47259, and pair 2 included a low responsive line from Ldn $\times$ IG126387 and a highly responsive line from Ldn $\times$ KU-2159. These lines were selected because their parental *Ae. tauschii* accessions also exhibited similar response to ABA. In pair 2, ABA responsiveness of the parental *Ae. tauschii* accessions correlated with that of the synthetics, being high for KU-2159 and low for IG126387. The correspondence was similar in pair 1 except for the low ABA-responsive synthetic, for which its parental *Ae. tauschii* accession exhibited intermediate responsiveness.

In pair 1, the gene expression patterns of the *WABI5* transcription factor and three *Cor/Lea* genes, *Wrab17*, *Wrab18* and *Wdhn13*, were examined by real-time RT-PCR analysis. These genes were selected because they are known to express under low temperature, dehydration and ABA treatment (Tsuda et al., 2000; Ohno et al., 2003; Kobayashi et al., 2004, 2008c). Transcript accumulation of *WABI5* in the highly ABA-responsive synthetic wheat (Ldn $\times$ IG47259) reached a maximum at 5 h after ABA treatment (Fig. 3A). The expression of *WABI5* in the low responsive line (Ldn $\times$ KU-2124) was similar to that of the maximum value for the highly responsive line, but was reached more slowly (12 h after treatment). Both pair 1 synthetics showed higher *WABI5* expression than the tetraploid wheat Ldn. In the diploid *Ae. tauschii*, *WABI5* expression was lower in the low ABA-responsive accession (KU-2124) (Supplementary Fig. S3A). The

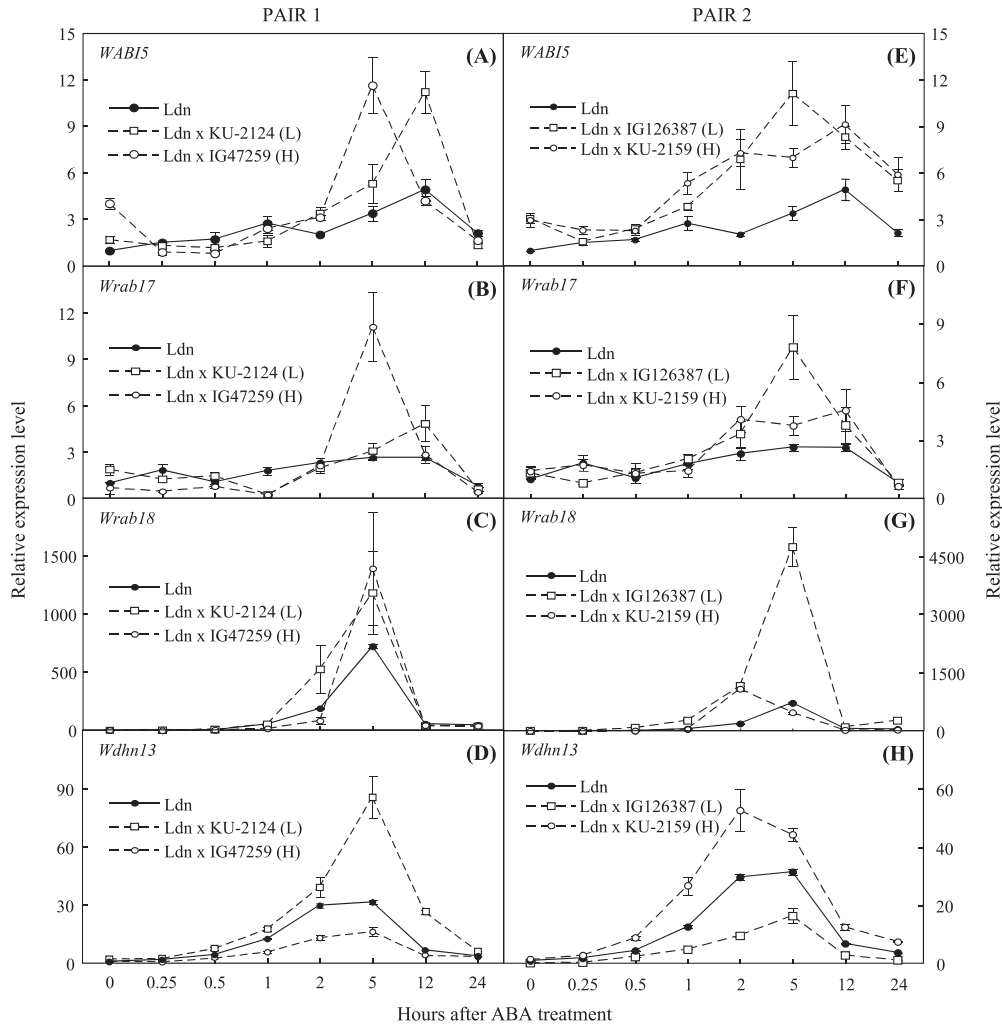


Fig. 3. Expression patterns of ABA-inducible genes in the synthetic lines of pairs 1 (A–D) and 2 (E–H). Transcripts of *WABI5* (A, E), *Wrab17* (B, F), *Wrab18* (C, G), and *Wdhn13* (D, H) were examined by real-time RT-PCR analysis, and represented as mean $\pm$ SD. Synthetic wheat lines are indicated by dashed lines, and Ldn by continuous lines. Highly and low ABA-responsive synthetic lines are indicated as (H) and (L), respectively. The *Actin* gene was used as an internal control. Each transcript level is represented as the value relative to that of Ldn at 0 h.

highly responsive accession (IG47259) showed similar expression to that of Ldn, except 12 h after treatment. The expression of *Wrab17* was higher in the highly responsive synthetic and its parental *Ae. tauschii* line (Fig. 3B, Supplementary Fig. S3B). Transcript accumulation of *Wrab18* was similar for the synthetics in pair 1 (Fig. 3C), but was higher in the low responsive *Ae. tauschii* than in the highly responsive accession (Supplementary Fig. S3C). The *Wdhn13* expression level was higher in the low responsive synthetic line of pair 1 (Fig. 3D).

In pair 2, expression of *WABI5* was slightly higher in the intermediate responsive *Ae. tauschii* (IG126387) and its derived (low responsive) synthetic wheat; Ldn showed the lowest expression (Fig. 3E, Supplementary Fig. S3D). The transcript accumulation of *Wrab17* was higher in IG126387 and its synthetic (Fig. 3F, Supplementary Fig. S3E). As in pair 1, *Ae. tauschii* accessions showed

Wrab17 expression that was lower than or similar to Ldn, but their derived hexaploids showed higher expression. *Wrab18* was also expressed at a higher level in IG126387 and its derived synthetic line (Fig. 3G, Supplementary Fig. S3F). In contrast, expression of *Wdhn13* was higher in the highly ABA-responsive synthetic line (Fig. 3H).

To test whether these ABA-inducible genes are expressed additively in the pair 1 synthetics, a mid-parent value (MPV) was calculated as a 2:1 mixture of Ldn and the parental *Ae. tauschii* accession based on an AB-to-D genome ratio of 2:1 (Pumphrey et al., 2009). For example, the highest expression level of *WABI5* in Ldn $\times$ KU-2124 was reached at 12 h after ABA treatment. The MPV was calculated as the sum of Ldn expression level multiplied by two (4.95×2) and the parental *Ae. tauschii* expression (1.96), both at 12 h after treatment, and then dividing by three ($11.86/3 = 3.95$). The estimated MPVs

Table 2. Altered expression levels of four ABA-inducible genes in synthetic wheat lines

Gene	Synthetic wheat	MPV (2:1) ^a	Observed value ^b
Pair 1			
<i>WABI5</i>	Ldn × KU-2124 (L)	3.95 ± 0.43	11.24 ± 1.37***
	Ldn × IG47259 (H)	3.37 ± 0.31	11.67 ± 1.79**
<i>Wrab17</i>	Ldn × KU-2124 (L)	1.94 ± 0.12	4.86 ± 1.18*
	Ldn × IG47259 (H)	2.75 ± 0.35	11.10 ± 2.21**
<i>Wrab18</i>	Ldn × KU-2124 (L)	621.20 ± 47.70	1523.67 ± 816.51 ^{ns}
	Ldn × IG47259 (H)	525.05 ± 14.14	1424.92 ± 895.57*
<i>Wdhn13</i>	Ldn × KU-2124 (L)	24.61 ± 0.87	85.39 ± 10.90***
	Ldn × IG47259 (H)	21.28 ± 0.74	16.17 ± 2.09*
Pair 2			
<i>WABI5</i>	Ldn × IG126387 (L)	4.19 ± 0.56	11.15 ± 2.07**
	Ldn × KU-2159 (H)	5.30 ± 0.22	9.14 ± 1.21**
<i>Wrab17</i>	Ldn × IG126387 (L)	4.37 ± 0.67	7.82 ± 1.67*
	Ldn × KU-2159 (H)	1.86 ± 0.13	4.56 ± 1.09*
<i>Wrab18</i>	Ldn × IG126387 (L)	147.28 ± 2.91	4755.83 ± 500.00***
	Ldn × KU-2159 (H)	1393.32 ± 185.29	1085.93 ± 57.80***
<i>Wdhn13</i>	Ldn × IG126387 (L)	21.11 ± 0.73	16.37 ± 2.57*
	Ldn × KU-2159 (H)	23.07 ± 0.77	52.75 ± 7.13**

^a MPVs (mean ± SD) were calculated using the expression level of parental Ldn and *Ae. tauschii* accessions at the time point at which each synthetic wheat line reached a maximum.

^b Student's *t*-test was used for statistical significance (ns; not significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001).

(L); low responsive line, (H); highly responsive line.

for *WABI5* and *Wrab17* at their maximal expression were lower than the observed expression level in both IG47259- and KU-2124-derived synthetic hexaploids (Table 2). For *Wrab18*, the observed expression level was significantly higher than the MPV in the IG47259-derived synthetic line, but no difference was observed in the low responsive synthetic line. For *Wdhn13*, both synthetic lines exhibited non-additive expression, however, the observed expression level of the highly responsive line was lower than the MPV. These results indicate that these ABA-inducible genes are non-additively expressed in the pair 1 synthetic lines, except for *Wrab18* in the KU-2124-derived synthetic line. These three genes also showed non-additive expression in both pair 2 synthetic lines, similarly to the pair 1 synthetics.

Water loss and salinity tolerance in pairs 1 and 2

Since the expression analyses of ABA inducible genes showed no clear difference between higher and lower responsive lines, the relationship between ABA responsiveness and abiotic stress tolerance in wheat synthetics was studied. Dehydration and salt stress tolerance was compared in the two selected pairs of synthetic wheat lines. Dehydration tolerance was evaluated as water loss rate, whereas salt stress tolerance was estimated based on growth rate under hydroponic conditions.

In pair 1, the water loss rate was similar between the highly (Ldn × IG47259) and low (Ldn × KU-2124) ABA-responsive lines (Fig. 4A). However, in pair 2, the KU-2159-derived synthetic (a higher ABA-responsive line)

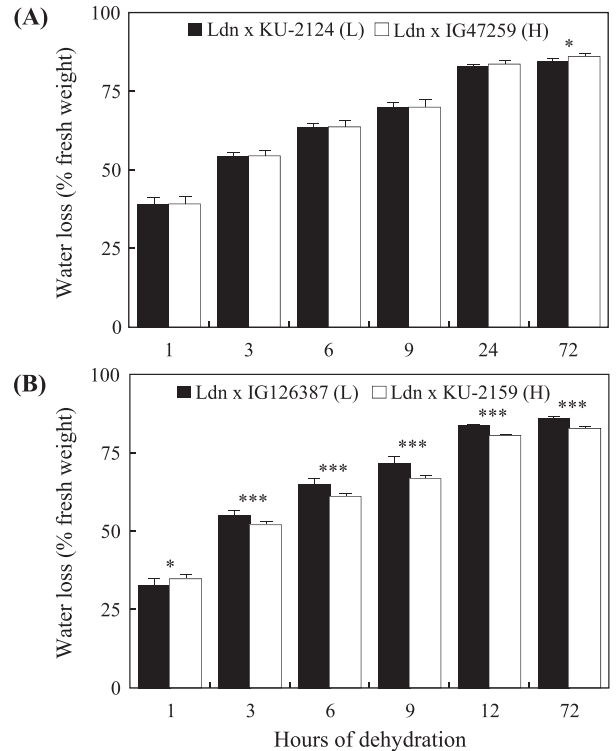


Fig. 4. Comparison of stomatal response under dehydration in synthetic wheat lines. Water loss from whole plants was determined in pair 1 (A) and pair 2 (B), and represented as mean ± SD. Black and open bars represent the percentage of water loss in the low (L) and highly (H) ABA-responsive lines, respectively. Student's *t*-test was used for statistical significance (**P* < 0.05, ****P* < 0.001).

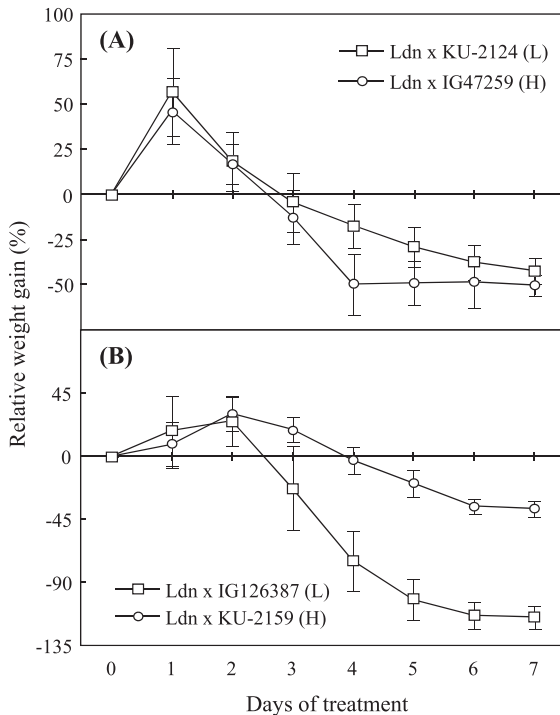


Fig. 5. Comparison of salt stress tolerance in the synthetic wheat lines. Weight gain was determined in 7-d-old seedlings grown in 0.2% Hyponex solution containing 0.15 M NaCl. Percentage of weight gain was determined in pair 1 (A) and pair 2 (B). Low (L) and highly (H) ABA-responsive lines are indicated by open squares and circles, respectively. Data are mean $\pm$ SD.

lost water more slowly than the low ABA-responsive IG126387-derived line (Fig. 4B).

Growth rate under the hydroponic condition, implying salt tolerance, was slightly different among the synthetic wheat lines. In pair 1, no significant difference in growth rate was observed between the synthetic wheat lines (Fig. 5A). In pair 2, both synthetic lines showed a similar weight gain between the first and second day of treatment. From the third day, the low responsive line (Ldn $\times$ IG126387) lost weight more rapidly than the highly responsive line (KU-2159-derived synthetic) (Fig. 5B).

DISCUSSION

Analysis of natural variation has contributed to understanding of many aspects of plant biology (Tonsor et al., 2005; Alonso-Blanco et al., 2009). *Ae. tauschii*, the D-genome progenitor of common wheat, is widely distributed in central Eurasia, ranging from northern Syria and Turkey to western China, and shows abundant natural variation in various traits including flowering time, morphological traits and fertile triploid F_1 formation (Matsuoka et al., 2007, 2008, 2009; Takumi et al., 2009b). Similarly, this study showed that the *Ae. tauschii* population pro-

vides wide natural variation in ABA responsiveness, based on rates of shoot and root growth inhibition by exogenous ABA treatment.

The 67 synthetic wheat lines used in the present study were generated through crossing Ldn with 67 different *Ae. tauschii* accessions. These diploid accessions exhibited wide natural variation in ABA responsiveness, and it was higher in the accessions belonging to L2 than in L1. A wide variation in ABA responsiveness was also observed in the 67 synthetic hexaploid lines. Although the contribution of AB-genomes on ABA responsiveness was relatively high, the natural variation of *Ae. tauschii* in ABA responsiveness is expressed well in the hexaploid genetic background.

In our previous study, a positive correlation between ABA responsiveness of 17 synthetics and their parental accessions was also found, but was not statistically significant (Kurahashi et al., 2009), likely due to the small population size. In the present study using a larger population size of both *Ae. tauschii* and synthetic wheat, the correlation was significant. In our previous studies, a significant correlation of heading time was observed between 82 wheat synthetics and their parental *Ae. tauschii* accessions (Kajimura et al., 2011), but not using 27 hexaploid synthetics (Takumi et al., 2009a). In spikelet morphology-related traits, on the other hand, positive correlations were observed using 27 hexaploid synthetics and their parental *Ae. tauschii* accessions (Takumi et al., 2009a). The set of 67 synthetic wheat lines is thus a valuable resource to study expression of D-genome variation in various traits in a hexaploid genetic background.

Correlation between ABA responsiveness and expression of abiotic stress-inducible genes has been reported in common wheat cultivars (Kobayashi et al., 2006, 2010). In this study, two pairs of synthetic wheat lines were selected based on differences in ABA responsiveness, and gene expression patterns and abiotic stress tolerance were compared using the highly and low ABA-responsive synthetic lines of each pair. In the highly ABA-responsive lines, not all the *Cor/Lea* genes were necessarily highly expressed, suggesting that a complex ABA signaling network regulates the expression of *Cor/Lea* genes and growth inhibition by ABA. In pair 2, only *Wdhn13* expression was correlated with ABA responsiveness and abiotic stress tolerance, suggesting that ABA-dependent development of abiotic stress tolerance might be tightly associated with *Wdhn13* expression in the synthetic wheat lines. On the other hand, the difference in ABA responsiveness was not reflected in the tolerance to dehydration and salinity in pair 1. One explanation could be due to the higher expression of *Wdhn13* in the low responsive line of pair 1, and the high *Wdhn13* expression led in a dehydration and high salinity tolerance similar to the highly responsive line, supporting the correlation between this *Cor/Lea* gene and abiotic stress tolerance.

Another explanation is the difference among synthetic wheat lines in the endogenous ABA concentration, since this phytohormone is known to increase under dehydration and high salinity conditions. Further studies are needed to investigate the variation among synthetic wheat lines (and their parental accessions) in the endogenous ABA level. In *Arabidopsis thaliana*, a quantitative trait locus (QTL) for salt tolerance and ABA sensitivity has been reported (Ren et al., 2010). Kobayashi et al. (2006, 2008d) found a significant association between ABA responsiveness and freezing tolerance in common wheat. However, the association between QTL for ABA responsiveness and other abiotic stress tolerance have not been reported to our knowledge. Since higher accumulation of ABA in spikes causes pollen sterility and grain loss (Ji et al., 2011), it is important to select QTLs that do not decrease grain yield under abiotic stress. Therefore, identification of the QTLs that determine the difference in ABA responsiveness between the synthetic wheat lines is needed to assess the use of highly ABA-responsive *Ae. tauschii* accessions as genetic resources to improve the abiotic stress tolerance of common wheat.

Hybrid vigor or heterosis is the phenotypic superiority of hybrid offspring over their parents, generally referring to a superior level of biomass, growth rate, fertility and yield. In allopolyploids, permanent fixation of heterozygosity and hybrid vigor occur due to the whole genome duplication of interspecific hybrids (Chen, 2010). Here, permanent fixation of heterosis in ABA responsiveness was observed in some synthetic hexaploid wheat lines. ABA responsiveness in most of the synthetic wheat lines surely ranged between the ABA responsiveness of their parents, which were Ldn and the respective *Ae. tauschii* accessions. However, in the six synthetic wheat lines, ABA responsiveness was significantly higher than in their parental *Ae. tauschii* accessions or Ldn, showing phenotypic superiority (heterosis) for ABA responsiveness over their parents. It was brought about through the allopolyploidization between Ldn and the respective *Ae. tauschii* accessions. Epistatic gene interactions between the AB and D genomes could result in heterosis. However in some cases like KU-2111, the AB and D interaction could also lead in a decrease of ABA responsiveness.

At the gene expression level, most of the ABA-inducible genes examined in this study showed higher expression than the estimated MPV in the synthetic wheat lines. Allopolyploids are well known to undergo changes at the genetic, epigenetic and gene expression levels (Chen, 2010; Jackson and Chen, 2010). Many of the non-additively expressed genes were reported to be underexpressed in synthetic hexaploid wheat (Chague et al., 2010). In particular, phytohormone- and stress-related genes tend to be underexpressed in *Arabidopsis* allopolyploids grown under normal conditions (Jackson and Chen,

2010; Kim and Chen, 2011). However, these underexpressed phytohormone- and stress-related genes are highly expressed under stress conditions (Kim and Chen, 2011). In this study, many ABA-inducible genes were overexpressed (expression higher than MPV), and reaching expression level higher or similar to the sum of Ldn and parental *Ae. tauschii* level (AABB + DD, not the average). Kurahashi et al. (2009) reported that drought tolerance levels of synthetic wheat lines are generally higher than their D-genome parental accessions. ABA-inducible genes might be overexpressed under drought conditions, leading to higher tolerance in synthetics. Although non-additive expression was observed in all the synthetics analyzed in this study, heterosis for ABA responsiveness was commonly observed in only six lines. This indicates that not all epistatic interactions between the D- and AB-genomes necessarily lead to heterosis appearing in phenotypes, despite the non-additive expression of ABA-inducible genes. To understand the molecular basis of the heterosis, alterations of gene expression profiles after allopolyploidization should be analyzed in the six synthetic lines showing heterosis in ABA responsiveness.

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