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Hybrid incompatibilities in interspecific crosses between tetraploid wheat and its wild diploid relative *Aegilops umbellulata*

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Abstract

Aegilops umbellulata Zhuk., a diploid goatgrass species with a UU genome, has been utilized as a genetic resource for wheat breeding. Here, we examine the reproductive barriers between tetraploid wheat cultivar Langdon (Ldn) and various *Ae. umbellulata* accessions by conducting interspecific crossings. Through systematic cross experiments, three types of hybrid incompatibilities were found: seed production failure in crosses, hybrid growth abnormalities and sterility in the ABU hybrids. Hybrid incompatibilities were widely distributed over the entire range of the natural species, and in about 50% of the cross combinations between tetraploid Ldn and *Ae. umbellulata* accessions, ABU F₁ hybrids showed one of two abnormal growth phenotypes: severe growth abortion (SGA) or grass-clump dwarfism. Expression of the shoot meristem maintenance-related and cell cycle-related genes was markedly repressed in crown tissues of hybrids showing SGA, suggesting dysfunction of mitotic cell division in the shoot apices. The grass-clump dwarf phenotype may be explained by down-regulation of wheat *APETALA1*-like MADS box genes, which act as flowering promoters, and altered expression in crown tissues of the miR156/*SPLs* module, which controls tiller number and branching. These gene expression changes in growth abnormalities were well conserved between the Ldn/*Ae. umbellulata* plants and interspecific hybrids from crosses of Ldn and wheat D-genome progenitor *Ae. tauschii*.

Keywords: allopolyploid evolution, interspecific cross, microRNA, natural variation, reproductive isolation, transcriptome, wheat

Introduction

Allopolyploid speciation is one of the major evolutionary processes in higher plants (Rieseberg and Willis 2007; Matsuoka et al. 2014). For novel allopolyploid speciation through allopolyploidization to succeed, normal growth and fertility of the interspecific hybrids are essential. However, hybrid plants frequently fail to produce the next generation due to lethality and sterility. Such hybrid incompatibilities are generally one of the significant phenomena for postzygotic reproductive barriers (Bomblies and Weigel 2007). Hybrid necrosis, one typical symptom of hybrid weakness and lethality, can be triggered by autoimmune responses in hybrid plants (Bomblies et al. 2007; Alcázar et al. 2009, 2010; Jeuken et al. 2009; Todesco et al. 2014), and hybrid sterility is at least partly caused by reciprocal gene loss occurring between paralogs of an essential duplicated gene (Bikard et al. 2009; Mizuta et al. 2010).

To obtain common wheat (*Triticum aestivum* L.; genome constitution AABBDD), normal triploid hybrids with the ABD genome are produced from interspecific crosses between tetraploid wheat (*T. turgidum* L.; AABB genome) and diploid wild wheat *Ae. tauschii* Coss. (DD genome). Synthetic wheat hexaploids can be artificially generated through ABD triploids, but some ABD hybrids show abnormal growth phenotypes, such as germination failure, hybrid necrosis and sterility in the triploid hybrids (Nishikawa 1960; Matsuoka et al. 2007). The growth abnormalities in the ABD hybrids could act as postzygotic hybridization barriers to prevent the formation of common wheat, indicating that hybridization barriers act negatively in allopolyploid speciation. A tetraploid wheat cultivar Langdon (Ldn) is known to be an efficient female parent to produce synthetic wheat hexaploids (Matsuoka and Nasuda 2004), but four types of the abnormal growth phenotypes are frequently observed in the ABD hybrids between Ldn and diverse *Ae. tauschii* accessions (Mizuno et al. 2010): two types with hybrid necrosis (type II and type III), hybrid chlorosis and severe growth abortion (SGA). *Ae. tauschii* accessions are divided into two major lineages, TauL1 and TauL2 (Matsuoka et al. 2015), having each independently developed postzygotic hybridization barriers after divergence (Mizuno et al. 2010). These observations suggest that *Ae. tauschii* lacking growth

abnormalities in the ABD hybrids may have been the D-genome donor for present-day hexaploid wheat.

In type II hybrid necrosis, necrotic cell death occurs only under low temperature conditions (Mizuno et al. 2011). At normal growth temperatures, hybrids with type II necrosis exhibit grass-clump dwarfism with no necrotic symptoms, but with excess tillers, severe dwarfism and delayed flowering (Takumi and Mizuno 2011; Matsuda et al. 2017). Temperature-dependent phenotypic plasticity is associated with alteration of some microRNA (miRNA) accumulation levels in the ABD hybrids, especially the appearance of altered expression levels of miR156/*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)* modules that are at least partly related to grass-clump dwarfism at normal temperatures (Matsuda et al. 2017). Grass-clump dwarfism is sometimes found in intra- and interspecific crosses of wheat and its relatives (Hermesen 1967; Moore 1969). SGA shows severe lethality because SGA-exhibiting ABD triploids cease development and growth after expansion of the second or third leaves (Nishikawa 1960). Transcript accumulation levels of cell cycle-related genes were markedly reduced in crown tissues of SGA plants, and the reduction of meristematic activity might be extensive in the shoot apical meristem (SAM) of SGA plants (Hatano et al. 2012). In both type II necrosis/grass-clump dwarfism and SGA, critical alteration of the transcriptome might occur in the crown tissues, including shoot apices.

Synthetic hexaploid wheat lines can be generated through hybrid genome-doubling in ABD F₁ hybrids. Allohexaploidization is tightly related to spontaneous occurrence of unreduced gamete formation in both male and female gametes, and the first division restitution results in non-reductional meiosis and unreduced gamete formation in ABD hybrids (Matsuoka and Nasuda 2004; Matsuoka et al. 2013). Systematic cross experiments between Ldn and diverse *Ae. tauschii* accessions show wide natural variation in the hybrid genome-doubling frequency, and the genome-doubling frequency can be evaluated by the selfed-seedset rate in ABD hybrids (Matsuoka et al. 2007, 2013). Therefore, the selfed-seedset rate data in interspecific hybrids is important for studying allopolyploid speciation.

Synthetic wheat hexaploids with AABBDD genome have potential as genetic resources

for bread wheat breeding to increase D-genome diversity (Mujeeb-Kazi et al. 1996; Jones et al. 2013). Synthetic hexaploid wheat lines have also been exploited through interspecific crosses between tetraploid wheat and diploid *Aegilops* species in order to introduce disease- and insect-resistance genes of *Aegilops* species into the wheat genome (Schneider et al. 2008). One wheat relative, *Aegilops umbellulata* Zhuk., is a diploid goatgrass species with UU genome and distributed from Greece to Iraq. *Ae. umbellulata* has been utilized as a genetic resource for wheat breeding (Kimber 1967; Dai et al. 2015), mainly as a donor of disease-resistance genes (Sears 1956; Friebe et al. 1996; Özgen et al. 2004). As hybrid incompatibilities were observed between tetraploid wheat and *Ae. tauschii*, reproductive barriers might underlie tetraploid wheat and *Ae. umbellulata*, thus inhibiting hybrid plant production and allopolyploid generation. The aims of the present study were (1) to clarify the reproductive isolation underlying the AB and U genomes in the future utilization of genetic variations in *Ae. umbellulata* for wheat breeding and (2) to characterize two types of hybrid incompatibilities observed in the systematic cross experiments. We therefore performed interspecific crossings between Ldn and the various *Ae. umbellulata* accessions and conducted transcriptome profiling of the abnormal growth-showing hybrids. Moreover, we compared the gene expression patterns of the two hybrid incompatibilities between the ABU and ABD hybrids to consider the molecular mechanisms inducing the growth abnormalities.

Materials and methods

Plant materials and interspecific crosses

To generate interspecific hybrids with the ABU genome, a tetraploid wheat variety, *Triticum turgidum* ssp. *durum* (Desf.) Husn. cv. Langdon (Ldn), and 58 accessions of *Aegilops umbellulata* Zhuk. were used. The *Ae. umbellulata* seeds were supplied by the National BioResource Project (NBRP)-Wheat, Japan (<https://www.nbrp.jp>), and characteristics of these accessions are listed in Supplemental Table 1.

Seeds of Ldn and each of the *Ae. umbellulata* accessions covering the species' entire natural distribution range were sown in November 2013, and plants were grown in a field at

Kobe University (34°43'N, 135°13'E). Anthers were manually removed from the immature florets of Ldn, and interspecific crossings were made with Ldn as the female parent and each of the *Ae. umbellulata* accessions as the pollen parent. The F₁ hybrids were grown in the 2014-2015 season and selfed to produce synthetics (herein designated the F₂ generation) in a glasshouse at Kobe University without any artificial control of photoperiod or temperature. Selfed-seedset rate of each F₁ hybrid was evaluated as previously reported in Ldn-*Ae. tauschii* triploid hybrids (Matsuoka et al. 2007). Tilling number and plant height were compared between normal growth (wild type; WT) and grass-clump dwarf hybrids of Ldn and *Ae. umbellulata* in a growth chamber with a 16 h photoperiod (182 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 24°C.

Phylogenetic analysis

Total DNA was extracted from leaves of 58 *Ae. umbellulata* accessions. Three single-copy genes putatively assigned to different chromosomes of *Ae. umbellulata* were amplified with the following three region-specific PCR primer sets designed for this study: 5'-CGCCACTGCATGTACCAA-3' and 5'-TGGCACTTCCACTACCACTG-3' for *Ppd-1*, 5'-CACACACTTGGAAGGTCACG-3' and 5'-GTCCAACGAGAATCCCTTCA-3' for *APX*, and 5'-GAATCTTGCAATTTGGTGCTG-3' and 5'-CAGAGTGTTTGGCGCAATTG-3' for *e-LCY*. *Ppd-1* is well known as a flowering time-controlling gene on wheat homoeologous group 2 chromosomes related to photoperiodic responses (Beales et al. 2007). *APX* on group 6 chromosomes and *e-LCY* on group 3 chromosomes encode an ascorbate peroxidase and a carotenoid biosynthetic cyclase in wheat, respectively (Danna et al. 2003; Howitt et al. 2009). The regions amplified by ExTaq polymerase (Takara Bio, Shiga, Japan) were sequenced using a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems).

Multiple sequences were aligned using CLUSTAL W (Thompson et al. 1994) and manually revised. Population genetic analyses for estimating within-species diversities were conducted with the DnaSP program (Rozas et al. 2003). Values of Tajima's *D* statistic (Tajima 1989) and Fu and Li's *D** statistic (Fu and Li 1993) were also estimated with DnaSP ver. 5.10.

A neighbor-joining (NJ) tree and a maximum likelihood (ML) tree were constructed based on the merged nucleotide sequences of *Ppd-1*, *APX* and *e-LCY* using MEGA ver. 5.2 (Tamura et al. 2011). Nucleotide positions with indels were not included in the tree constructions. Bootstrap probability was calculated for 500 replications. The Kimura 2-parameter model was used as the distance model for the NJ tree. The general time reversible model and the nearest-neighbor-interchange method were used as the substitution model and the tree inference, respectively, for the MP tree.

mRNA transcriptome analysis

Four F₁ ABU hybrids between Ldn and four *Ae. umbellulata* accessions, Ldn/KU-4103 and Ldn/KU-5948 for WT, Ldn/KU-5928 for SGA and Ldn/KU-4052 for grass-clump dwarf were used in microarray analysis. Total RNA of Ldn/KU-4103 and Ldn/KU-4052 was extracted using an RNeasy Plant Mini kit (Qiagen, Hilden, Germany) from crown tissues of seedlings grown at normal temperature (24°C) for 50 days under 16-h day length (182 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Similarly, total RNA of Ldn/KU-5948 and Ldn/KU-5928 were isolated from crown tissues of 7-day-old seedlings. A KOMUGI 38k oligonucleotide DNA microarray, which is commercially available (Agilent Technologies, Santa Clara, CA), was supplied through NBRP-Wheat for analysis. Detailed information on the 38k microarray platform can be found in Kawaura et al. (2008) and the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information (NCBI) website under platform GPL9805. Cyanine 3-labeled cRNA was hybridized to the microarray, washed, and image scanning was performed according to our previous report (Takamatsu et al. 2015). Scanned images were analyzed with Feature Extraction Software, Version 9.5 (Agilent Technologies) using default parameters to obtain background-subtracted and spatially detrended processed signal intensities. For normalization, a trimmed mean excluding 2% of the highest and lowest values was determined, and the processed signal intensities of each probe were multiplied by the scaling factor (2500 divided by trimmed mean value). Two independent experiments were conducted for each sample. All microarray data were deposited as series GSE93943 and

GSE93969 in the NCBI GEO database (<http://www.ncbi.nlm.nih.gov/geo/>), along with supplementary files registered as GSM2465475 through GSM2465478 for SGA and GSM2465831 through GSM2465834 for grass-clump dwarf.

The functions of probes and genes were predicted by BLASTx searches (E value $< 1e^{-10}$) against the NCBI non-redundant protein database. Gene ontology (GO) annotation was performed using Blast2GO (Conesa et al. 2005). Annotations were further refined using ANNEX (Myhre et al. 2006) and mapped to plant-specific GO-Slim terms, both of which are available in Blast2GO. GO terms of differentially expressed (mean of fold changes ≥ 3 or $\leq 1/3$, determined by Student's t -test [$P < 0.05$]) probes were extracted and compared. Based on GO term enrichment analysis (Fisher's exact test, FDR < 0.05), differentially encountered probes were input for each biological process, molecular function and cellular component category.

For comparison of microarray data for SGA and grass-clump dwarf with previously reported data for growth abnormalities in ABD triploids from crosses between Ldn and *Ae. tauschii*, data sets including GSE33357 and GSE78784 in the NCBI GEO were respectively used for SGA and grass-clump dwarf. RNA samples were extracted from crown tissues of ABD triploids grown at 24°C for plants with SGA and grass-clump dwarf phenotypes (Hatano et al. 2012; Matsuda et al. 2017).

Quantitative reverse transcription (qRT)-PCR analysis

Quantitative reverse transcription (qRT)-PCR analysis was performed using RNA isolated from crown tissues of WT, SGA and grass-clump dwarf plants. Each plant was grown at 24°C under 16 h day length ($182 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), and total RNA extraction, first-strand cDNA synthesis and detection of transcript accumulation levels were performed according methods reported previously (Takamatsu et al. 2015). Some of the examined genes were selected to validate their altered transcription levels in the SGA- and grass-clump dwarf-exhibiting ABU hybrids based on the microarray data. Others were chosen according to the data of SGA- and grass-clump dwarf-exhibiting ABD plants from interspecific crosses between Ldn and *Ae. tauschii* in our previous studies (Hatano et al. 2012; Matsuda et al.

2017). The gene-specific primer sets for qRT-PCR are listed in Supplemental Table 2. The *Actin* gene was used as an internal control, and relative expression was calculated as $2^{-\Delta\Delta C_t}$ (Livak and Schmittgen 2001), representing the value relative to the transcript levels in crown tissues of WT (Ldn/KU-4103 or Ldn/KU-5948). Mean values were calculated based on three technical replicates.

For qRT-PCR of miRNA, the accumulation of each miRNA was detected by qRT-PCR using first-strand cDNA samples synthesized by a Mir-X miRNA First-Strand Synthesis kit (BD Biosciences Clontech, Tokyo, Japan). miRNA qRT-PCR was conducted using SYBR Advantage premix (BD Biosciences Clontech) with an miRNA-specific primer and an mRQ3' adapter primer. The miRNAs examined in the present study were selected according to the data of grass-clump dwarf-exhibiting ABD plants from interspecific crosses between Ldn and *Ae. tauschii* in our previous study (Matsuda et al. 2017). The miRNA-specific primers for qRT-PCR are listed in Supplemental Table 3. 18S rRNA was used as an internal control. The relative expression level was calculated as $2^{-\Delta\Delta C_t}$ of three technical replicates.

Results

Intraspecific variation in *Ae. umbellulata*

Three single-copy genomic sequences for *Ppd-1*, *APX* and *e-LCY* were determined in 58 *Ae. umbellulata* accessions that covered the entire species' natural distribution range. In total, 56 polymorphisms were found in the three genomic regions (Table 1). Average nucleotide diversity (π) for the three genomic regions was 0.0036, which was intermediate among values from 167 species classified in 14 phyla (Leffler et al. 2012). Values of Tajima's *D* statistic (Tajima 1989) and Fu and Li's *D** statistic (Fu and Li 1993) tests were used to examine evolutionary neutrality at the three loci. The *D* test statistics of neutrality were negative in all regions examined, although the results were not significant.

Considering the three genomic regions as being evolutionarily neutral, sequences of the 58 *Ae. umbellulata* accessions were used to construct a NJ tree and a ML tree for determining the phylogenetic relationship of these accessions (Fig. 1A, B). Nucleotide sequences of the

three genomic regions from *Triticum urartu* (A genome), *Ae. tauschii* (D genome) and *T. aestivum* (A, B and D genomes) obtained from draft genome information were used as outgroups (Jia et al. 2013; Ling et al. 2013; The International Wheat Genome Sequencing Consortium 2014). In both the NJ and ML trees, *Ae. umbellulata* was clearly differentiated from the A, B and D genomes. Genetic distances between *Ae. umbellulata* and the AB genomes were similar to those between the D and AB genomes (Supplemental Table 4). The tested accessions of *Ae. umbellulata* were classified into three major groups (G1, G2 and G3) with over 85% bootstrap probability supporting the groupings. No apparent differences were found in morphological traits and heading time among the major groups. The biggest group G1 contains at least 9 subgroups, but with low bootstrap probabilities. These subgroups did not seem to have a random distribution over the Middle East (Fig. 1C), as the violet subgroup had a relatively longer internode distance and was mainly located in central Turkey. Most of the accessions belonging to the brown subgroup were distributed over the northeastern part of Iraq, and some accessions were found in Syria, Turkey, Iran and Azerbaijan. The internode distance of the brown subgroup was zero, suggesting a recent expansion of this subgroup.

Crossability with tetraploid wheat

In total, the 56 accessions of *Ae. umbellulata* (excluding KU-4041 and KU-4043) were crossed to Ldn with more than 60 florets of Ldn crossed with pollen of the *Ae. umbellulata* accessions. Seed production rates from these crosses varied from 0% to 68.3% with a mean of $21.2 \pm 18.8\%$ production rate among the 56 cross combinations (Fig. 2A). Most of the crosses resulted in less than 20% production of the seeds, and eight *Ae. umbellulata* accessions showed high seed production rates (>50%). Hybrid seeds were obtained from all cross combinations between Ldn and *Ae. umbellulata* accessions, and seed production rates showed no geographical or phylogenetic associations (Fig. 2B, C).

Phenotypes of interspecific hybrids

Two types of growth abnormalities, SGA and grass-clump dwarfism, were clearly observed in

interspecific hybrids grown in a glasshouse (Fig. 3). Hybrid plants with the SGA phenotype extended two or three leaves without tillering until spring when they died (Fig. 3C). Therefore, no offspring were obtained from any hybrid plants displaying SGA. On the other hand, the grass-clump dwarf plants (Ldn/KU-4052) showed a marked increase in tiller number and a reduction in plant height compared to the WT plant (Ldn/KU-4103) in the growth chamber (Fig. 3D, E). In the grass-clump dwarf hybrids, flowering time was much more delayed (>2 weeks) than that in WT, and a few selfed seeds could be obtained.

Most of the crossed seed sets between Ldn and 58 *Ae. umbellulata* accessions (including KU-4041 and KU-4043) could germinate, and only seeds from three crosses with *Ae. umbellulata* accessions, KU-2752, KU-2760 and KU-12180, failed to germinate (Fig. 4A). For 31 of the remaining 55 cross combinations, ABU hybrids with normal growth phenotype were produced. Abnormal growth hybrids with SGA and grass-clump dwarf phenotypes were observed in 10 and 14 cross combinations, respectively. The ABU hybrids among *Ae. umbellulata* accessions with WT and grass-clump dwarf phenotype were widely distributed over the entire natural range of the species, whereas the accessions showing SGA were mainly found in western Turkey (Fig. 4B). Distribution of the three ABU hybrid phenotypes, WT, SGA and grass-clump dwarfism, showed no geographical or phylogenetic associations (Fig. 4C).

Of the ABD hybrids between Ldn and *Ae. tauschii*, selfed seeds with an allohexaploid genome were obtained through unreduced gamete formation (Matsuoka and Nasuda 2004; Matsuoka et al. 2013). Selfed-seedset rate was examined in interspecific hybrids from 31 cross combinations between Ldn and *Ae. umbellulata*, including 25 WT and 6 grass-clump dwarf phenotype-exhibiting combinations. In other cross combinations, the rates could not be evaluated due to the hybrid lethality and the small number of the crossed seeds. Selfed-seedset rate ranged from 0 to 38.9% with a mean of $11.5 \pm 0.10\%$ (Fig. 5A). The ABU hybrids from four *Ae. umbellulata* accessions, Ldn/KU-2932, Ldn/KU-4052, Ldn/KU-5910 and Ldn/KU-5949, produced no selfed progeny. Three of the four *Ae. umbellulata* accessions belonged to grass-clump dwarf phenotype-exhibiting cross combinations, and significant

differences were observed in selfed-seedset rate between WT and grass-clump dwarfism (Fig. 5B). Grass-clump dwarf hybrids produced much fewer selfed seeds than the WT hybrids, and Ldn/KU-12198, in particular, showed the highest selfed-seedset rate. *Ae. umbellulata* accessions with selfed-seedset rate lower than 20% were widely distributed over the entire range of the natural species, and no high fertility (>20%) accessions were found at the eastern range (Fig. 5C). Selfed-seedset rate showed no apparent phylogenetic associations (Fig. 5D).

Microarray analysis of the hybrid with SGA phenotype

To comprehensively compare gene expression profiles in crown tissues between WT (Ldn/KU-5948) and SGA (Ldn/KU-5928) hybrids under normal temperature, we analyzed transcriptomes using a wheat-specific 38k oligo DNA microarray (Kawaura et al. 2008). At least a threefold difference ($FDR < 0.05$) in signal intensity for RNA samples hybridized to probes compared to WT was defined as either up- or down-regulation for the genes. Of the 37,826 probes on the wheat microarray, 251 (0.66%) probes were regarded as indicating up-regulated genes in the crown tissues of the SGA hybrid compared to WT plants, and 213 (0.56%) were regarded as indicating down-regulated genes.

Probes targeting up- and down-regulated genes that were analyzed by GO term enrichment analysis and used in a homology search of the wheat EST database were found to belong to some groups having specific biological processes, molecular functions and cellular components (Fig. 6A, 6B). Up-regulated biotic and abiotic stress response, cell death and carboxy-lyase activity-related genes were frequently encountered in the crown tissues of Ldn/KU-5928. In contrast, many photosynthesis and plastid-related genes were down-regulated in Ldn/KU-5928. The up-regulated genes in Ldn/KU-5928 included defense-related genes such as peroxidase and defensin-encoding genes and abiotic stress responsive genes including dehydrin and late-embryogenesis abundant protein genes (Supplemental Table 5). The genes down-regulated in the crown tissues of Ldn/KU-5928 included photosynthesis-related genes such as the genes for chlorophyll a/b binding protein, and F-box protein-encoding and lipid and sterol biosynthesis-related genes were dramatically

repressed in the crown tissues of Ldn/KU-5928 (Supplemental Table 6).

Correlation of the log₂ ratios of gene expression levels was examined in crown tissues of the two SGA hybrids from interspecific crosses between Ldn and *Ae. tauschii* and between Ldn and *Ae. umbellulata* based on mRNA microarray data produced with 37,826 probes. The microarray data of the crown tissues in Ldn/IG47182 (*Ae. tauschii*) were reported previously (Hatano et al. 2012). Of the 37,826 probes in the wheat microarray, 67 were more than 3-fold up-regulated in both the Ldn/IG47182 and Ldn/KU-5928 hybrids, and 46 were less than 1/3-fold down-regulated (Fig. 7A). Differences in signal intensity of all 37,826 probes relative to WT were compared between the Ldn/IG47182 and Ldn/KU-5928 hybrids, and a significant positive correlation ($R = 0.285$, $P < 0.001$) was observed (Fig. 7B).

Microarray analysis in the grass-clump dwarf hybrid

For comprehensive comparison of gene expression profiles in crown tissues between WT (Ldn/KU-4103) and grass-clump dwarf (Ldn/KU-4052) hybrids under normal temperature, probes of the wheat microarray having an at least a three-fold difference ($FDR < 0.05$) in signal intensity compared to WT were defined. Of the 37,826 microarray probes, 331 (0.88%) probes were regarded as indicating up-regulated genes, and 194 (0.51%) indicated down-regulated genes in the crown tissues of Ldn/KU-4052 compared with WT plants.

GO term enrichment analysis for up-regulated genes in Ldn/KU-4052 showed significant changes in all categories of biological processes, molecular function and cellular components (Fig. 6C). Of the up-regulated genes, metabolic and catabolic process-related genes for cell wall-associated molecules were frequently encountered in crown tissues of Ldn/KU-4052. Among the down-regulated genes, many more genes related to responses to inorganic substances, acidic chemicals and water were encountered in Ldn/KU-4052 (Fig. 6D). In Ldn/KU-4052, peroxidase and P450-encoding and nodulin-related genes were up-regulated (Supplemental Table 7). The genes that were down-regulated in the crown tissues of Ldn/KU-4052 included photosynthesis-related genes such as the genes for NADPH and photosystem II stability assembly factor (Supplemental Table 8). Several transcription factor

genes that encode WRKY family proteins were highly up-regulated, whereas some transcription factor genes, one of which is a MADS-box gene, were markedly down-regulated in the crown tissues of Ldn/KU-4052 (Supplemental Tables 7 and 8).

The log₂ ratios of gene expression levels were compared in crown tissues of the two grass-clump dwarf hybrids from interspecific crosses between Ldn and *Ae. tauschii* (KU-2025) and between Ldn and *Ae. umbellulata*, as described in our previous study on the Ldn/KU-2025 (Matsuda et al. 2017). Of the 37,826 probes in the wheat microarray, 20 were more than 3-fold up-regulated for both the Ldn/KU-2025 and Ldn/KU-4052 hybrids, whereas no gene was down-regulated to less than 1/3-fold in both (Fig. 8A). For the differences in signal intensity of all microarray probes relative to WT, no significant positive correlation was observed between the Ldn/KU-2025 and Ldn/KU-4052 (Fig. 8B).

Expression analyses of selected genes in SGA

To validate microarray data in SGA hybrids, qRT-PCR was conducted for three photosynthesis-related genes, *PSII*, *PsbB* and *ATP synthase CF1*. In the crown tissues of three ABU hybrids derived from three independent crosses between Ldn and three *Ae. umbellulata* accessions, KU-5911, KU-5924 and KU-5928, these photosynthesis-related genes were significantly down-regulated compared to the WT hybrid, Ldn/KU-5948 (Fig. 9). Next, four genes, which expression levels were repressed in the crown tissues of SGA-exhibiting ABD plants (Hatano et al. 2012), were selected to compare their transcript accumulation levels between the SGA and WT ABU hybrids. A wheat *KN1*-type homeobox gene, *Wknox1*, was reported to play an important role in maintenance of SAM indeterminacy in wheat, similar to that of maize *KN1* and rice *OSH1* (Morimoto et al. 2005). Significant differences were observed in *Wknox1* expression levels between WT and the SGA hybrids of Ldn and *Ae. umbellulata*. Moreover, transcript accumulation levels of three cell cycle-related genes, *Histone H4*, *Cyclin B1* and putative *B-type cyclin*, were significantly reduced in SGA hybrids of Ldn and *Ae. umbellulata* (Ldn/KU-5911, Ldn/KU-5924 and Ldn/KU-5928) compared to WT (Ldn/KU-5948) (Fig. 9).

Expression analyses of the selected genes in grass-clump dwarfism

To validate the microarray data in the grass-clump dwarf plants from crosses between Ldn and *Ae. umbellulata*, qRT-PCR was conducted for three defense-related genes, *PR1*, *PR4* and *HR-related protein*, and two photosynthesis-related genes, *PSII* and *PsbB*. These defense-related genes were significantly up-regulated in the crown tissues of the three grass-clump dwarf hybrids, Ldn/KU-2770, Ldn/KU-4052 and Ldn/KU-5949, and the photosynthesis-related genes were down-regulated (Fig. 10). Alternative oxidase (AOX) functions in the suppression of reactive oxygen species (ROS) generation, initiating putative necrotic cell death, while the transcript accumulation level of wheat AOX gene, *Waox1a*, is sensitive to ROS generation and necrotic symptoms (Sugie et al. 2007; Mizuno et al. 2010). *Waox1a* expression levels were significantly reduced in the crown tissues of the three grass-clump dwarf hybrids of Ldn and *Ae. umbellulata* (Fig. 10), indicating that the ROS generation was not confirmed in the grass-clump synthetic hexaploids showing dwarfism.

Vrn-1, a homolog of *Arabidopsis APETALA1 (AP1)/FRUITFULL (FUL)*, controls the vernalization requirement and acts as a flowering promoter (Yan et al. 2003; Murai et al. 2003). In the grass-clump dwarf-showing ABD hybrids, expression levels of *Vrn-1* and its homologs are greatly reduced (Matsuda et al. 2017). The parental tetraploid cultivar Ldn contained a dominant *Vrn-A1* allele for spring habit (Fu et al. 2005) and, therefore, the *Vrn-A1* allele was transmitted to all synthetic hexaploid wheat lines among the crosses between Ldn and *Ae. umbellulata*. To compare the gene expression levels of *Vrn-1* and its homologs in the crown tissues between the ABU and ABD hybrids exhibiting grass-clump dwarfism, we conducted qRT-PCR analyses of the three MADS-box genes. qRT-PCR analysis showed that *Vrn-A1* was significantly down-regulated in the crown tissues of the three grass-clump dwarf hybrids of Ldn and *Ae. umbellulata* compared with the WT plant Ldn/KU-5948 (Fig. 10). Two wheat *Vrn-1* homologs, *TaAP1-2* and *TaAP1-3*, are respectively orthologous to *OsMADS18* and *OsMADS15*, and the rice orthologs play important roles in the promotion of flowering (Fornara et al. 2004; Wang et al. 2010). Transcript accumulation levels of *TaAP1-2*

and *TaAP1-3* were markedly decreased in the crown tissues of the three grass-clump dwarf hybrids (Fig. 10). The results corresponded to the three MADS-box gene expression patterns in the crown tissues of grass-clump dwarf plants of Ldn and *Ae. tauschii* under normal temperature (Matsuda et al. 2017).

Previously, deep sequencing of small RNAs in the crown tissues of synthetic hexaploids from crosses of Ldn and *Ae. tauschii* revealed alteration of some miRNA accumulation levels in the grass-clump dwarf hybrid under normal temperature (Matsuda et al. 2017). Expression levels of the four miRNAs, miR159, miR168, miR396 and miR5048, were found to be differentially expressed in the crown tissues of three grass-clump dwarf ABU hybrids of Ldn and *Ae. umbellulata*, Ldn/KU-2770, Ldn/KU-4052 and Ldn/KU-5949. The three grass-clump dwarf hybrids showed more abundant accumulation of the four miRNAs than did Ldn/KU-5948, a WT line (Fig. 11). miR156 is associated with the regulation of tillering and branching through interactions with *SPL* mRNAs in maize, rice and *Arabidopsis* (Schwab et al. 2005; Xie et al. 2006; Chuck et al. 2007), and recent reports confirmed conservation of the regulatory relationship between miR156 and *SPLs* in wheat (Xin et al. 2010; Pandey et al. 2014). Three of the wheat miR156 molecules, tae-miR156a, tae-miR156b and tae-miR156c, showed significantly more abundant accumulation in the crown tissues of the three grass-clump dwarf hybrids compared to that in Ldn/KU-5948 (Fig. 11). Expression levels of four *TaSPLs*, in which target sites of the tae-miR156 molecules were present (Matsuda et al. 2017), were examined in the three grass-clump dwarf hybrids of Ldn and *Ae. umbellulata*. Transcript accumulation levels of the four *TaSPLs* were significantly lower in the crown tissues of Ldn/KU-2770, Ldn/KU-4052 and Ldn/KU-5949 than in those of Ldn/KU-5948 (Fig. 11). The alteration of tae-miR156s and *TaSPLs* expression in the three grass-clump dwarf hybrids is consistent with the expression patterns of tae-miR156/*TaSPL* modules in the grass-clump dwarf hybrid of Ldn and *Ae. tauschii* under normal temperature (Matsuda et al. 2017).

Discussion

Reproductive barriers between tetraploid wheat and *Ae. umbellulata*

One of the diploid wheat relatives, *Ae. umbellulata*, has been a useful genetic resource for wheat breeding (Schneider et al. 2008). In an effort to better utilize *Ae. umbellulata* populations for wheat breeding, large-scale production of ABD hybrids has allowed us to better understand postzygotic reproductive barriers underlying tetraploid wheat and *Ae. umbellulata*. In the present study, three types of hybrid incompatibilities were observed between a tetraploid wheat cultivar Ldn and 58 *Ae. umbellulata* accessions (Figs. 2-5): failure of crossed seed production, hybrid growth abnormalities and sterility in the ABU hybrids. Of the hybrid incompatibilities, crossability ranged from 0 to 0.83 with a mean of 0.29 between Ldn and *Ae. tauschii* with about 90% of the cross combinations producing seeds (Matsuoka et al. 2007). Crossability between Ldn and *Ae. umbellulata* was 0.21, indicating that ABU F₁ hybrids can be obtained at a similar level as for ABD hybrids. Two types of growth abnormalities, SGA and grass-clump dwarfism, were widely present in F₁ hybrids between Ldn and *Ae. umbellulata* (Figs. 3 and 4). Four typical growth abnormalities, including two types of hybrid necrosis, hybrid chlorosis and SGA, were observed between Ldn and *Ae. tauschii*, and hybrid necrosis was most widely distributed in *Ae. tauschii* (Mizuno et al. 2010). In the present study, however, no cross combinations produced F₁ plants showing hybrid necrosis or hybrid chlorosis. In about 50% cross combinations between Ldn and *Ae. umbellulata*, hybrids failed to grow normally and showed either SGA or grass-clump dwarfism. The frequency of cross combinations with hybrid growth abnormalities was higher in *Ae. umbellulata* than in *Ae. tauschii*, which had normal growth phenotype in more than 60% of the cross combinations with Ldn (Mizuno et al. 2010). Selfed seeds with the allohexaploid genome had a high occurrence in ABD hybrids between Ldn and *Ae. tauschii*, and the hybrid genome doubling frequencies ranged from roughly 0.1 to 0.6 in both two major lineages of *Ae. tauschii* accessions (Matsuoka et al. 2013). Selfed-seedset rates in the ABU hybrids of Ldn and *Ae. umbellulata* were apparently lower than in the ABD hybrids (Fig. 5). Our results indicate that it is much more difficult to artificially produce allohexaploid plants through the use of *Ae. umbellulata* than *Ae. tauschii* as a pollen parent into tetraploid

wheat. Thus, a greater postzygotic reproductive isolation underlies the AB and U genomes than the AB and D genomes, although the genetic distances between AB and U genomes were similar to those between the AB and D genomes (Supplemental Table 4).

Our results indicate that *Ae. umbellulata* provides the ability to produce allohexaploids through interspecific crosses with tetraploid wheat and contains natural variation in the ability to express postzygotic reproductive phenotypes in the allopolyploid. This observation suggests that *Ae. umbellulata* could be a third genome donor for allohexaploid wheat speciation instead of *Ae. tauschii*. The large postzygotic reproductive isolation between the AB and U genome might play a critical role to inhibit allohexaploid speciation under actual field conditions. Our systematic crossing experiment allowed comparisons of the features of interspecific hybrid incompatibilities for two *Aegilops* species with distinct genomes.

Two major lineages were apparently differentiated in *Ae. tauschii*, and genealogical and geographical clines are clearly present for hybrid growth abnormalities in the ABD hybrids (Matsuoka et al. 2007; Mizuno et al. 2010). Our phylogenetic analysis using three genomic sequences showed that most of the *Ae. umbellulata* accessions belonged to one genetic lineage G1 (Fig. 1). G1 accessions covered the entire species' distribution range and the G1 accessions showed various responses in crossability, growth abnormality and hybrid fertility during ABU hybrid production (Figs. 2, 4 and 5). Therefore, unlike *Ae. tauschii*, no apparent clines for the hybrid incompatibilities with tetraploid wheat are observed in *Ae. umbellulata*. These observations indicate that intraspecific variations in hybrid incompatibilities are unique in diploid wheat relatives.

Similarity of growth abnormalities between Ldn/*Ae. umbellulata* and Ldn/*Ae. tauschii*

Many of the ABU F₁ hybrids showed one of two abnormal growth phenotypes, SGA or grass-clump dwarfism. These abnormalities have also been reported in ABD F₁ hybrids from crosses between tetraploid wheat and *Ae. tauschii* (Nishikawa et al. 1960; Matsuoka et al. 2007; Hatano et al. 2012; Matsuda et al. 2017). Our transcriptome analyses of crown tissues revealed distinct features of the two hybrid growth abnormalities in the ABU hybrids. The

transcriptome alteration in ABU hybrids showing SGA generally resembled alterations in ABD hybrids (Fig. 7), whereas no significant correlation was observed in the comprehensive changes of transcriptional profiles of SGA plants produced in Ldn/*Ae. umbellulata* and Ldn/*Ae. tauschii* crosses (Fig. 8). Thus, gene regulatory processes that express the SGA phenotype in ABU hybrids were closely related to those in ABD hybrids. On the other hand, although abnormal phenotypes of the grass-clump dwarfism showed much overlap, at least some signal pathways expressed grass-clump dwarfism that may be different between the Ldn/*Ae. umbellulata* and Ldn/*Ae. tauschii* hybrids.

Plants with SGA showed complete lethality in the ABU hybrids and produced no progeny, which is similar to outcomes in ABD hybrids. Transcriptome analysis revealed up-regulation of cell death-related genes and down-regulation of photosynthesis-related genes in ABU hybrids showing SGA (Fig. 6). These transcriptional changes in plants with SGA were also observed in ABD hybrids (Hatano et al. 2012). Dysfunction of mitotic cell division occurred in the SAM of ABD hybrids showing SGA, which is accompanied with extreme repression of cell cycle-related gene expression (Hatano et al. 2012). Similar changes in SAM maintenance and cell cycle-related genes, including *Wknx1*, *histone H4* and *B-type cyclin* genes, were also observed in crown tissues of the ABU hybrids showing SGA (Fig. 9). Therefore, global changes of transcriptomes in ABU hybrids showing SGA might be tightly associated with the dysfunction of mitotic cell division at shoot apices, similar to those in ABD hybrids showing SGA (Hatano et al. 2012). These observations suggest that molecular mechanisms regulating the expression of the SGA phenotype are well conserved between ABU and ABD F₁ hybrids.

In ABD F₁ hybrids and Ldn/*Ae. tauschii* synthetic hexaploid lines, grass-clump dwarfism is dramatically affected by growth temperature, and under the low temperature condition, the growth phenotype changes from grass-clump dwarf to type II necrosis with repression of stem elongation and delayed new-leaf expansion (Mizuno et al. 2011; Takumi and Mizuno 2011). The growth temperature-dependent phenotypic plasticity is not observed in the grass-clump dwarfism of Ldn/*Ae. umbellulata* hybrids, and no necrotic cell death appears, even under low temperature. The temperature-independence of grass-clump dwarfism in Ldn/*Ae. umbellulata*

might result in differences in global expression profiles from those in Ldn/*Ae. tauschii*. In Ldn/*Ae. umbellulata* hybrids exhibiting the grass-clump dwarfism, selfed-seedset rates were lower compared with those in the WT-showing hybrids (Fig. 5B). Previous reports demonstrated that the selfed-seedset rates were dependent on growth temperature in intraspecific wheat hybrids with grass-clump dwarfism (Manon and Cavin 1972a, 1972b). Therefore, optimization of growth conditions would increase in the unreduced gamete formation and selfed-seedset rates in the grass-clump dwarfism-showing Ldn/*Ae. umbellulata* hybrids. On the other hand, alterations of some gene expression levels were well-conserved in the grass-clump dwarf hybrids of Ldn/*Ae. umbellulata* and Ldn/*Ae. tauschii*. Three *AP1/FUL*-type MADS-box genes were dramatically down-regulated in crown tissues of the grass-clump dwarf hybrids of Ldn/*Ae. umbellulata* (Fig. 10). The repression of the three *AP1/FUL*-type MADS-box genes could explain the delayed flowering phenotype in the grass-clump dwarf hybrids, and similar results were found in grass-clump dwarf hybrids of Ldn/*Ae. tauschii* under normal temperature (Matsuda et al. 2017). Altered patterns of the five miRNAs, miR156, miR159, miR168, miR396 and miR5048, were at least partly overlapped in the grass-clump dwarfism hybrids of Ldn/*Ae. umbellulata* and Ldn/*Ae. tauschii*. Especially, miR156 overexpression induces morphological changes, including dwarfism, increased tiller number and late flowering through down-regulation of *SPLs* in maize, switchgrass and rice (Chuck et al. 2007; Fu et al. 2012; Hayashi-Tsugane et al. 2015). Phenotypic characters of transformants overexpressing miR156 closely resembled grass-clump dwarfism of Ldn/*Ae. umbellulata* (Fig. 3), and four *TaSPL* mRNA levels were significantly reduced in crown tissues of the grass-clump dwarf hybrids (Fig. 11). Therefore, unusual expression of the miR156/*SPLs* module could contribute to excess tiller number and dwarfism in the grass-clump dwarf hybrids of Ldn/*Ae. umbellulata*, as was postulated for Ldn/*Ae. tauschii* (Matsuda et al. 2017).

In the grass-clump dwarf hybrids of Ldn/*Ae. tauschii*, defense-related genes are up-regulated under low temperature, and their expression levels are reduced under normal temperature (Matsuda et al. 2017). It is assumed that the reduced expression of

defense-related genes is partly associated with the absence of a necrosis phenotype under normal temperature. Up-regulation of defense-related genes and down-regulation of photosynthesis-related genes are typical characteristics of hybrid necrosis, which is triggered by autoimmune responses (Bomblies et al. 2007; Alcázar et al. 2009, 2010; Jeuken et al. 2009). Although similar expression patterns of defense- and photosynthesis-related genes were found, no necrotic symptoms appeared in the grass-clump dwarf hybrids of *Ldn/Ae. umbellulata*. ROS is likely to be generated prior to the appearance of the necrotic symptoms in hybrid plants showing necrosis, including the hybrid necrosis lines of *Ldn/Ae. tauschii* (Mizuno et al. 2010, 2011). Expression of *Waox1a*, a marker gene for the ROS generation (Sugie et al. 2007; Mizuno et al. 2010), was markedly repressed in the grass-clump dwarf hybrids of *Ldn/Ae. umbellulata*, resulting in little generation of ROS. Thus, in the grass-clump dwarf hybrids of *Ldn/Ae. umbellulata*, up-regulation of defense-related genes appears to not to be linked with ROS generation.

As reported in ABD F₁ hybrids, incompatibilities are postulated to occur due to epistatic interaction between the AB and U genomes. Some of the epistatic interactions, inducing hybrid incompatibilities, are generally dependent on a phenomenon described by the Dobzhansky-Muller model (Bomblies and Weigel 2007), and at least two causal genes should participate in each hybrid growth abnormality of ABU hybrids; one should be assigned to a chromosome of the AB genome and the other to a U genome chromosome. As well as the transcriptional profiling, detail characterization of the growth abnormality of ABU hybrids should be required at the tissue and cellular levels in future studies to understand the effects of epistatic interactions of the causal genes. The hybrid incompatibilities underlying the AB and U genomes inhibit allohexaploid production through ABU hybrids and introgression of useful genes of *Ae. umbellulata* through synthetic hexaploids with the AABB₁UU genome. To overcome postzygotic barriers, therefore, the causal genes should be identified and mapped on the *Ae. umbellulata* genome in future studies.

Acknowledgments

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References

- Alcázar R, Garcia AV, Parker JE, Reymond M (2009) Incremental steps toward incompatibility revealed by *Arabidopsis* epistatic interactions modulating salicylic acid pathway activation. *Proc Natl Acad Sci USA* 106:334–339.
- Alcázar R, Garcia AV, Kronholm I, de Meaux J, Koornneef M, Parker JE, Reymond M (2010) Natural variation at Strubbelig Receptor Kinase 3 derives immune-triggered incompatibilities between *Arabidopsis thaliana* accessions. *Nat Genet* 42:1135–1139.
- Beales J, Turner A, Griffiths S, Snape JW, Laurie DA (2007) A *pseudo-response regulator* is misexpressed in the photoperiod insensitive *Ppd-D1a* mutant of wheat (*Triticum aestivum* L.). *Theor Appl Genet* 115:721–733.
- Bikard D, Patel D, Le Metté, Giorgi V, Camilleri C, Bennett MJ, Loudet O (2009) Divergent evolution of duplicate genes leads to genetic incompatibilities within *A. thaliana*. *Science* 323:623–626.
- Bomblies K, Weigel D (2007) Hybrid necrosis: autoimmunity as a potential gene-flow barrier in plant species. *Nat Rev Genet* 8:382–393.
- Bomblies K, Lempe J, Epple P, Warthmann N, Lanz C, Dangl JL, Weigel D (2007) Autoimmune response as a mechanism for Dobzhansky-Muller-type incompatibility syndrome in plants. *PLoS Biol* 5:e236.
- Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21:3674–3676.
- Chuck G, Cigan AM, Hake S (2007) The heterochronic maize mutant *Corngrass1* results

- from overexpression of a tandem microRNA. *Nat Genet* 39:544–549.
- Dai S, Zhao L, Xue X, Jia Y, Liu D, Pu Z, Zheng Y, Yan Z (2015) Analysis of high-molecular-weight glutenin subunits in five amphidiploids and their parental diploid species *Aegilops umbellulata* and *Aegilops uniaristata*. *Plant Genet Resources* 13:186–189.
- Danna CH, Bartoli CG, Sacco F, Ingala LR, Santa-María GE, Guiamet JJ, Ugalde RA (2003) Thylakoid-bound ascorbate peroxidase mutant exhibits impaired electron transport and photosynthetic activity. *Plant Physiol* 132:2116–2125.
- Fornara F, Parenicová L, Falasca G, Pelucchi N, Masiero S, Ciannamea S, Lopez-Dee Z, Altamura MM, Colombo L, Kater MM (2004) Functional characterization of *OsMADS18*, a member of the *AP1/SQUA* subfamily of MADS box genes. *Plant Physiol* 135:2207–2219.
- Friebe B, Jiang J, Raupp WJ, McIntosh RA, Gill BS (1996) Characterization of wheat-alien translocations conferring resistance to disease and pests: current status. *Euphytica* 91:59–87.
- Fu C, Sunkar R, Zhou C, Shen H, Zhang JY, Matts J, Wolf J, Mann DGJ, Stewart Jr CN, Tang Y, Wang ZY (2012) Overexpression of miR156 in switchgrass (*Panicum virgatum* L.) results in various morphological alterations and leads to improved biomass production. *Plant Biotech J* 10:443–452.
- Fu D, Szucs P, Yan L, Heluera M, Skinner JS, von Zitzewitz J, Hayes PM, Dubcovsky J (2005) Large deletions within the first intron in *VRN-1* are associated with spring growth habit in barley and wheat. *Mol Genet Genomics* 273:54–65.
- Fu YX, Li WH (1993) Statistical tests of neutrality of mutations. *Genetics* 133:693–709.
- Hatano H, Mizuno N, Matsuda R, Shitsukawa N, Park P, Takumi S (2012) Dysfunction of mitotic cell division at shoot apices triggered severe growth abortion in interspecific hybrids between tetraploid wheat and *Aegilops tauschii*. *New Phytologist* 194:1143–1154.
- Hayashi-Tsugane M, Maekawa M, Tsugane K (2015) A gain-of-function *Bushy dwarf tiller 1*

- mutation in rice microRNA gene *miR156d* caused by insertion of the DNA transposon *nDart1*. *Sci Rep* 5:14357.
- Hermesen JGTH (1967) Hybrid dwarfness in wheat. *Euphytica* 16:134–162.
- Howitt CA, Cavanagh CR, Bowerman AF, Cazzonelli C, Rampling L, Mimica JL, Pogson BJ (2009) Alternative splicing, activation of cryptic exons and amino acid substitutions in carotenoid biosynthetic genes are associated with lutein accumulation in wheat endosperm. *Func Integr Genomics* 9:363–376.
- Jeuken MJW, Zhang NW, McHale LK, Pelgrom K, den Boer E, Lindhout P, Michelmore RW, Visser RGF, Niks RE (2009) *Rin4* causes hybrid necrosis and race-specific resistance in an interspecific lettuce hybrid. *Plant Cell* 21:3368–3378.
- Jia J, Zhao S, Kong X, Li Y, Zhao G, He W, Appels R, Pfeifer M, Tao Y, Zhang X, Jing R, Zhang C, Ma Y, Gao L, Gao C, Spannagl M, Mayer KFX, Li D, Pan S, Zheng F, Hu Q, Xia X, Li J, Liang Q, Chen J, Wicker T, Gou C, Kuang H, He G, Luo Y, Keller B, Xia Q, Lu P, Wang J, Zou H, Zhang R, Gao J, Middleton C, Quan Z, Liu G, Wang J, International Wheat Genome Sequencing Consortium, Yang H, Liu X, He Z, Mao L, Wang J (2013) *Aegilops tauschii* draft genome sequence reveals a gene repertoire for wheat adaptation. *Nature* 496:91–95.
- Jones H, Gosman N, Horsnell R, Rose GA, Everst LA, Bentley AR, Tha S, Uauy C, Kowalski A, Novoselovic D, Simek R, Kobiljski B, Kondic-Spika A, Brbaklic L, Mitrofanova O, Chesnokov Y, Bonnett D, Greenland A (2013) Strategy for exploiting exotic germplasm using genetic, morphological, and environmental diversity: the *Aegilops tauschii* Coss. example. *Theor Appl Genet* 126:1793–1808.
- Kawaura K, Mochida K, Ogihara Y (2008) Genome-wide analysis for identification of salt-responsive genes in common wheat. *Funct Integr Genomics* 8:277–286.
- Kimber G (1967) The addition of the chromosomes of *Aegilops umbellulata* to *Triticum aestivum* (var. Chinese Spring). *Genet Res* 9:111–114.
- Leffler EM1, Bullaughey K, Matute DR, Meyer WK, Séguirel L, Venkat A, Andolfatto P, Przeworski M (2012) Revisiting an old riddle: what determines genetic diversity levels

within species? PLoS Biol 10:e1001388.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta \Delta CT}$ method. Methods 25:402–408.

Ling HQ, Zhao S, Liu D, Wang J, Sun H, Zhang C, Fan H, Li D, Dong L, Tao Y, Gao C, Wu H, Li Y, Cui Y, Guo X, Zheng S, Wang B, Yu K, Liang Q, Yang W, Lou X, Chen J, Feng M, Jian J, Zhang X, Luo G, Jiang Y, Liu J, Wang Z, Sha Y, Zhang B, Wu H, Tang D, Shen Q, Xue P, Zou S, Wang X, Liu X, Wang F, Yang Y, An X, Dong Z, Zhang K, Zhang X, Luo MC, Dvorak J, Tong Y, Wang J, Yang H, Li Z, Wang D, Zhang A, Wang J (2013) Draft genome of the wheat A-genome progenitor *Triticum urartu*. Nature 496:87–90.

Manon JD, Cavin DT (1972a) Low temperature effects on growth and physiology specific to a ‘grass-clump’ dwarf wheat hybrid. Can J Bot 50:253–261.

Manon JD, Cavin DT (1972b) Cytological and histological changes induced by cold temperature in the young shoots of Marquillo x Kenya Farmer wheat dwarf 1. Can J Bot 50:403–408.

Matsuda R, Iehisa JCM, Sakaguchi K, Ohno R, Yoshida K, Takumi S (2017) Global gene expression profiling related to temperature-sensitive growth abnormalities in interspecific crosses between tetraploid wheat and *Aegilops tauschii*. PLoS ONE 12:e0176497.

Matsuoka Y, Nasuda S (2004) Durum wheat as a candidate for the unknown female progenitor of bread wheat: an empirical study with a highly fertile F₁ hybrid with *Aegilops tauschii* Coss. Theor Appl Genet 109:1710–1717.

Matsuoka Y, Takumi S, Kawahara T (2007) Natural variation for triploid F₁ hybrid formation in allohexaploid wheat speciation. Theor Appl Genet 115:509–518.

Matsuoka Y, Nasuda S, Ashida Y, Nitta M, Tsujimoto H, Takumi S, Kawahara T (2013) Genetic basis for spontaneous hybrid genome doubling during allopolyploid speciation of common wheat shown by natural variation analyses of the paternal species. PLoS ONE 8:e68310.

Matsuoka Y, Takumi S, Nasuda S (2014) Genetic mechanisms of allopolyploid speciation

- through hybrid genome doubling: novel insights from wheat (*Triticum* and *Aegilops*) studies. *Int Rev Cell Mol Biol* 309:199–258.
- Matsuoka Y, Takumi S, Kawahara T (2015) Intraspecific lineage divergence and its association with reproductive trait change during species range expansion in central Eurasian wild wheat *Aegilops tauschii* Coss. (Poaceae). *BMC Evol Biol* 15:213.
- Mizuno N, Hosogi N, Park P, Takumi S (2010) Hypersensitive response-like reaction is associated with hybrid necrosis in interspecific crosses between tetraploid wheat and *Aegilops tauschii* Coss. *PLoS ONE* 5:e11326.
- Mizuno N, Shitsukawa N, Hosogi N, Park P, Takumi S (2011) Autoimmune response and repression of mitotic cell division occur in inter-specific crosses between tetraploid wheat and *Aegilops tauschii* Coss. that show low temperature-induced hybrid necrosis. *Plant J* 68:114–128.
- Mizuta Y, Harushima Y, Kurata N (2010) Rice pollen hybrid incompatibility caused by reciprocal gene loss of duplicated genes. *Proc Natl Acad Sci USA* 107:20417–20422.
- Moore K (1969) The genetical control of the grass-dwarf phenotype in *Triticum aestivum* L. *Euphytica* 25:471–483.
- Morimoto R, Kosugi T, Nakamura C, Takumi S (2005) Intragenic diversity and functional conservation of the three homoeologous loci of the *KN1*-type homeobox gene *Wknox1* in common wheat. *Plant Mol Biol* 57:907–924.
- Mujeeb-Kazi A, Rosas V, Roldan S (1996) Conservation of the genetic variation of *Triticum tauschii* (Coss.) Schmalh. (*Aegilops squarrosa* auct. non L.) in synthetic hexaploid wheats (*Triticum turgidum* L. s.lat. x *T. tauschii*; 2n = 6x = 42, AABBDD) and its potential utilization for wheat improvement. *Genet Resource Crop Evol* 43:129–134.
- Murai K, Miyamae M, Kato H, Takumi S, Ogihara Y (2003) *WAP1*, a wheat *APETALA1* homolog, plays a central role in the phase transition from vegetative to reproductive growth. *Plant Cell Physiol* 44:1255–1265.
- Myhre S, Tveit H, Mollestad T, Laegreid A (2006) Additional gene ontology structure for improved biological reasoning. *Bioinformatics* 22:2020–2027.

- Nishikawa K (1960) Hybrid lethality in crosses between Emmer wheats and *Aegilops squarrosa*, I. Vitality of F₁ hybrids between emmer wheats and *Ae. squarrosa* var. *typica*. Seiken Zihō 11:21–28.
- Özgen M, Yildiz M, Ulkan H, Koyuncu N (2004) Association of gliadin protein pattern and rust resistance derived from *Aegilops umbellulata* Zhuk. in winter *Triticum durum* Desf. Breed Sci 54:287–290.
- Pandey R, Joshi G, Bhardwaj AR, Agarwal M, Katiyar-Agarwal S (2014) A comprehensive genome-wide study on tissue-specific and abiotic stress-specific miRNAs in *Triticum aestivum*. PLoS ONE 9:e95800.
- Rieseberg LH, Willis JH (2007) Plant speciation. Science 317:910–914.
- Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19:2496–2497.
- Schneider A, Molnár I, Molnár-Láng M (2008) Utilisation of *Aegilops* (goatgrass) species to widen the genetic diversity of cultivated wheat. Euphytica 163:1–19.
- Schwab R, Palatnik JF, Riester M, Schommer C, Schmid M, Weigel D (2005) Specific effects of MicroRNAs on the plant transcriptome. Dev Cell 8:517–527.
- Sears ER (1956) The transfer of leaf rust resistance from *Aegilops umbellulata* to wheat. Brookhaven Symp Biol 9:1–22.
- Sugie A, Murai K, Takumi S (2007) Alteration of respiration capacity and transcript accumulation level of alternative oxidase genes in necrosis lines of common wheat. Genes Genet Syst 82:231–239.
- Tajima F (1989) Statistical test for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595.
- Takamatsu K, Iehisa JCM, Nishijima R, Takumi S (2015) Comparison of gene expression profiles and responses to zinc chloride among inter- and intraspecific hybrids with growth abnormalities in wheat and its relatives. Plant Mol Biol 88:487–502.
- Takumi S, Mizuno N (2011) Low temperature-induced necrosis shows phenotypic plasticity in wheat triploid hybrids. Plant Signal Behav 6:1431–1433.

- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739.
- The International Wheat Genome Sequencing Consortium (IWGSC) (2014) A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. *Science* 345:1251788.
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl Acids Res* 22:4763–4680.
- Todesco M, Kim ST, Chae E, Bomblies K, Zaidem M, Smith LM, Weigel D, Laitinen RAE (2014) Activation of the *Arabidopsis thaliana* immune system by combinations of common *ACD6* alleles. *PLoS Genet* 10:e1004459.
- Wang K, Tang D, Hong L, Xu W, Huang J, Li M, Gu M, Xue Y, Cheng Z (2010) *DEP* and *AFO* regulate reproductive habit in rice. *PLoS Genet* 6:e1000818.
- Xie K, Wu C, Xiong L (2006) Genomic organization, differential expression, and interaction of *SQUAMOSA promoter-binding-like* transcription factors and microRNA156 in rice. *Plant Physiol* 142:280–293.
- Xin M, Wang Y, Yao Y, Xie C, Peng H, Ni Z, Sun Q (2010) Diverse set of microRNAs are responsive to powdery mildew infection and heat stress in wheat. *BMC Plant Biol* 10:123.
- Yan L, Loukoianov A, Tranquilli G, Helguera M, Fashima T, Dubcovsky J (2003) Positional cloning of the wheat vernalization gene *VRN1*. *Proc Natl Acad Sci USA* 100:6263–6268.

Table 1. Summary of nucleotide variation in the three genomic regions in *Ae. umbellulata* population

Gene		Entire region	Exon	Synonymous	Nonsynonymous	Intron
<i>APX</i>	# of sites	648	177	37.67	139.33	465
	# of variable sites	44	4	4	0	41
	π	0.00785	0.00627	0.02946	0.00000	0.00908
	θ	0.01546	0.00488	0.02294	0.00000	0.02003
	Tajima's <i>D</i>	-1.66 NS	0.62 NS	0.62 NS	-	-1.83 NS
	Fu and Li's <i>D</i> *	-2.64 NS	-0.14 NS	-0.14 NS	-	-2.51 NS
<i>e-LCY</i>	# of sites	676	308	74.68	321.32	368
	# of variable sites	6	3	2	1	3
	π	0.00083	0.00096	0.00305	0.00029	0.00071
	θ	0.00192	0.00210	0.00579	0.00093	0.00176
	Tajima's <i>D</i>	-1.41 NS	-1.09 NS	-0.81 NS	-0.89 NS	-1.19 NS
	Fu and Li's <i>D</i> *	0.28 NS	0.88 NS	-0.73 NS	0.53 NS	-0.45 NS
<i>Ppd-1</i>	# of sites	737	358	64	233	346
	# of variable sites	6	4	2	1	3
	π	0.002	0.00201	0.00834	0.00015	0.00149
	θ	0.002	0.00288	0.00675	0.00093	0.00189
	Tajima's <i>D</i>	-0.41 NS	-0.67 NS	-0.40 NS	-1.09 NS	-0.42 NS
	Fu and Li's <i>D</i> *	-0.60 NS	-2.39 NS	-0.93 NS	-1.88 NS	0.88 NS

NS: not significant

Figure legends

Figure 1. Phylogenetic trees and geographic distribution of 58 accessions of *Ae. umbellulata*.

(A) A neighbor-joining tree and (B) a maximum likelihood tree of 58 *Ae. umbellulata* accessions based on the three genomic regions of *Ppd-1*, *APX*, and *e-LCY*. Bootstrap probabilities are shown on the branches. Three major groups G1, G2, and G3 are shown by red, orange, and yellow bars, respectively, to the right of the trees. Circles indicating groups or subgroups on the phylogenetic trees are mapped. (C) The geographic distribution of the 58 accessions corresponding to subgroups in the trees is shown on the map of the northwestern part of the Middle East.

Figure 2. Success rate of interspecific crosses between tetraploid Ldn wheat (florets) and *Ae. umbellulata* (pollen).

(A) Histogram of seed production rates in Ldn florets crossed with *Ae. umbellulata* pollen. (B) Geographic distribution of the *Ae. umbellulata* accessions crossed into Ldn. Colors of circles representing accessions are coded to seed production rates. (C) Phylogenetic relationship of *Ae. umbellulata* accessions crossed into Ldn with color coded circles representing seed production rates.

Figure 3. Growth abnormalities in the triploid F₁ hybrids between Ldn and *Ae. umbellulata*.

Photos of three typical growth phenotypes in the Ldn/*Ae. umbellulata* F₁ hybrids: (A) normal (WT), (B) grass-clump dwarf and (C) SGA. Phenotypic effects in grass-clump dwarfism compared against WT of (D) tiller number and (E) plant height.

Figure 4. Distribution of growth abnormalities in the triploid F₁ hybrids between Ldn and *Ae. umbellulata*.

(A) Histogram of hybrid growth abnormality type in *Ae. umbellulata* accessions crossed into Ldn. (B) Geographic distribution of the *Ae. umbellulata* accessions showing abnormal growth in the ABU triploids. Colors of circles representing accessions are coded to growth abnormalities in the ABU triploids. (C) Phylogenetic relationship of *Ae. umbellulata* accessions showing abnormal growth in the ABU triploids

with color coded circles representing hybrid growth abnormalities.

Figure 5. Hybrid fertility in the triploid F₁ hybrids between Ldn and *Ae. umbellulata*. (A) Histogram of selfed seedset rates in the ABU hybrids derived from examined *Ae. umbellulata* accessions. (B) Box plots of selfed seedset rates in ABU triploids showing WT and grass-clump dwarf phenotypes. (C) Geographic distribution of the hybrid-fertilizing *Ae. umbellulata* accessions. Colors of circles representing accessions are coded to selfed seedset rates in the ABU hybrids. (D) Phylogenetic relationship of hybrid-fertilizing *Ae. umbellulata* accessions with color-coded circles representing selfed seedset rates in ABU hybrids.

Figure 6. GO terms enriched among differentially expressed genes. Black bars indicate percentages of differentially encountered probes by microarray analysis (test set), and gray bars represent percentages of probes on the used array (reference set). (A, B) Comparison of transcriptomes between the SGA and WT triploid hybrids. (A) Up-regulated and (B) down-regulated genes in the SGA hybrid (Ldn/KU-5928) relative to the WT hybrid (Ldn/KU-5948) were categorized into functional groups. (C, D) Comparison of transcriptomes between the grass-clump dwarf and WT hybrids of synthetic hexaploid. (C) Up-regulated and (D) down-regulated genes in the grass-clump dwarf hybrid (Ldn/KU-4052) relative to the WT hybrid (Ldn/KU-4103) categorized into functional groups. The GO term enrichment analysis was performed using Fisher's exact test with FDR < 0.05, and the differentially encountered probes were categorized.

Figure 7. Comparison of microarray data for crown tissues of triploid F₁ hybrids showing SGA. (A) Venn diagram of differentially expressed up- and down-regulated genes of the SGA hybrid (Ldn/KU-5928) relative to the WT hybrid (Ldn/KU-5948) compared with up- and down-regulated genes of the SGA hybrid of Ldn and *Ae. tauschii*. (B) Scatter plot of differential rates of signal intensities in crown tissues of hybrids showing SGA from

crosses between Ldn and *Ae. umbellulata* (KU-5928) and between Ldn and *Ae. tauschii* (IG47182). All of the probes on the 38k wheat microarray are plotted along with a regression line. The correlation was statistically significant ($P < 0.001$).

Figure 8. Comparison of microarray data for crown tissues of the interspecific hybrids showing grass-clump dwarfism. (A) Venn diagram of differentially expressed up- and down-regulated genes of the grass-clump dwarf hybrid (Ldn/KU-4052) relative to the WT hybrid (Ldn/KU-4103) compared with up- and down-regulated genes of the grass-clump dwarf plant of Ldn and *Ae. tauschii*. (B) Scatter plot of differential rates of signal intensities in crown tissues of hybrids showing grass-clump dwarfism from crosses between Ldn and *Ae. umbellulata* (KU-4052) and between Ldn and *Ae. tauschii* (KU-2025). All of the probes on the 38k wheat microarray are plotted along with a regression line.

Figure 9. Comparison of transcript accumulation of the selected genes in crown tissues of the WT and SGA F₁ hybrids. Transcript accumulation levels of photosynthesis, SAM and cell cycle-related genes were analyzed by qRT-PCR. The transcript levels are shown as values relative to those in crown tissues of Ldn/KU-5948. Means $\pm$ SD were calculated from data obtained in three technical replicates. The *Actin* gene was used as an internal control. Student's *t*-test was used to test for statistical significance (** $P < 0.01$, *** $P < 0.001$) between WT and SGA hybrids. For each ABU hybrid, crown tissues of at least three independent plants were pooled for RNA extraction.

Figure 10. Comparison of transcript accumulation of the selected genes in crown tissues of the WT and grass-clump dwarf hybrids. Transcript accumulation levels of defense and photosynthesis-related, AOX and MADS-box genes were analyzed by qRT-PCR. The transcript levels are shown as values relative to those in crown tissues of Ldn/KU-5948. Means $\pm$ SD were calculated from data obtained in three experiments. The *Actin* gene

was used as an internal control. Student's *t*-test was used to test for statistical significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) between WT and grass-clump dwarf hybrids. For each ABU hybrid, crown tissues of at least three independent plants were pooled for RNA extraction.

Figure 11. Comparison of transcript accumulation of the selected wheat miRNAs and *SPLs* in the WT and grass-clump dwarf hybrids. Total RNA extracted from the crown tissues of at least three independent plants per each ABU hybrid was pooled for qRT-PCR. The transcript levels are shown as values relative to those in crown tissues of Ldn/KU-5948. Means $\pm$ SD were calculated from data obtained in three experiments. 18S rRNA and the *Actin* gene were used as an internal control for miRNA and *SPLs*, respectively. Student's *t*-test was used to test for statistical significance (** $P < 0.01$, *** $P < 0.001$) between WT and grass-clump dwarf hybrids.

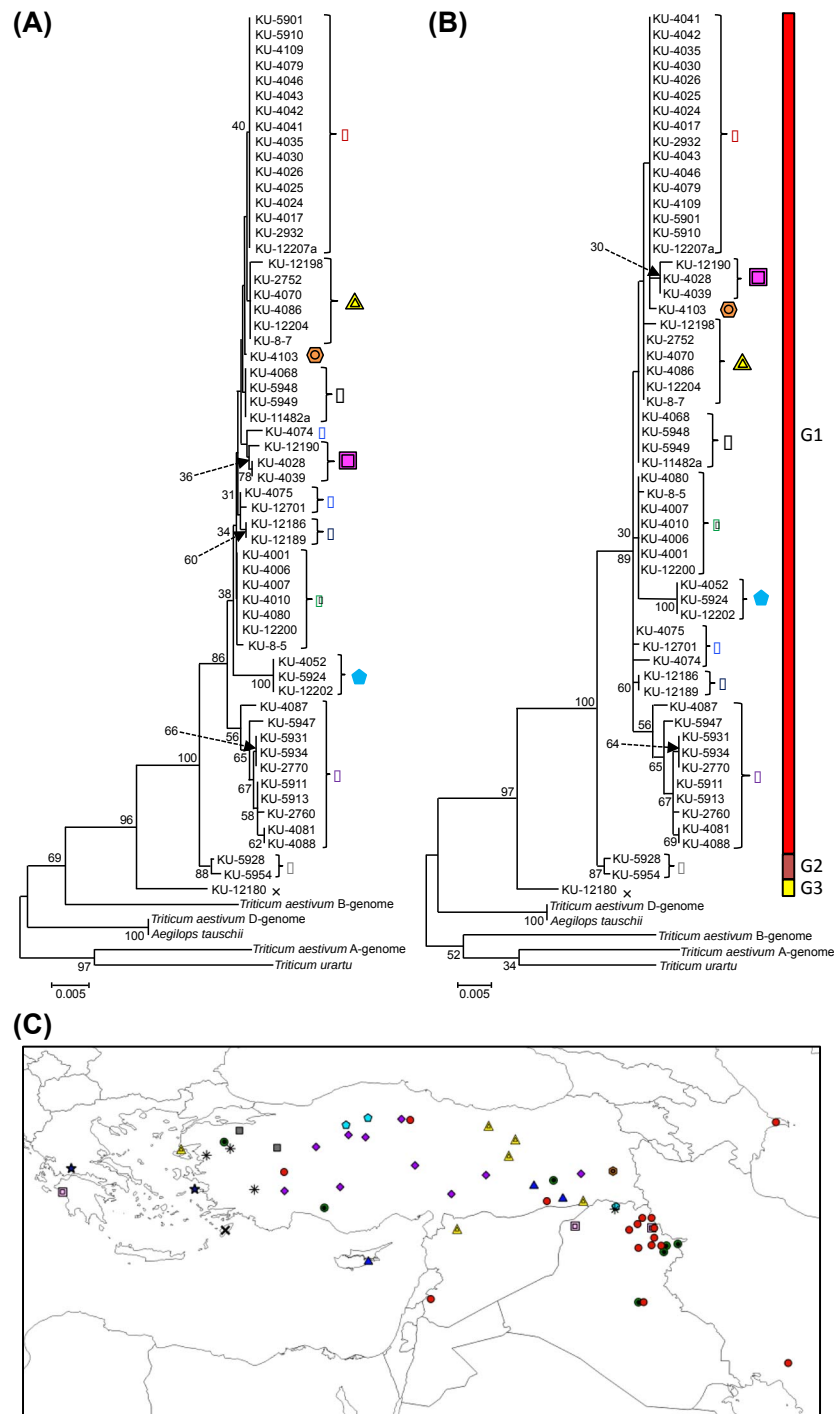


Fig. 1 (Okada et al.)

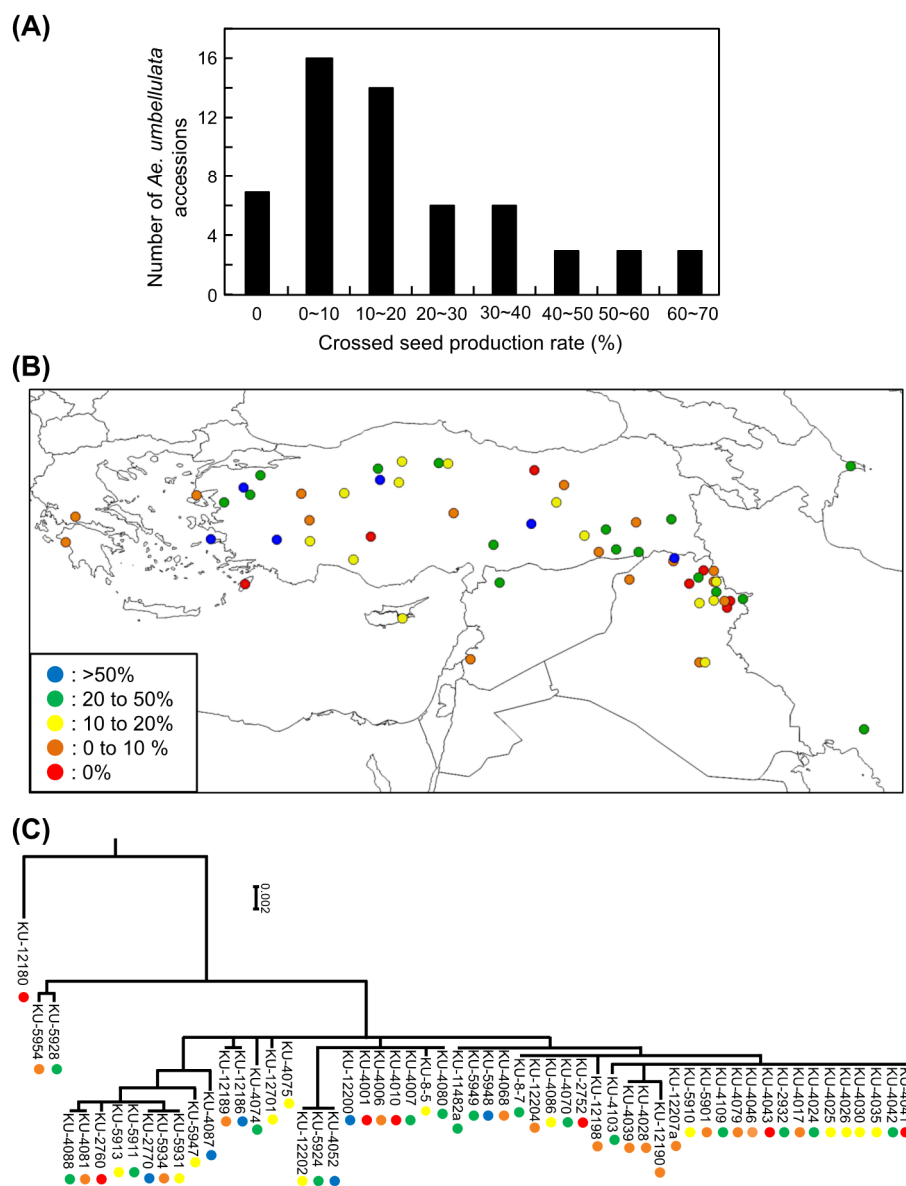


Fig. 2 (Okada et al.)

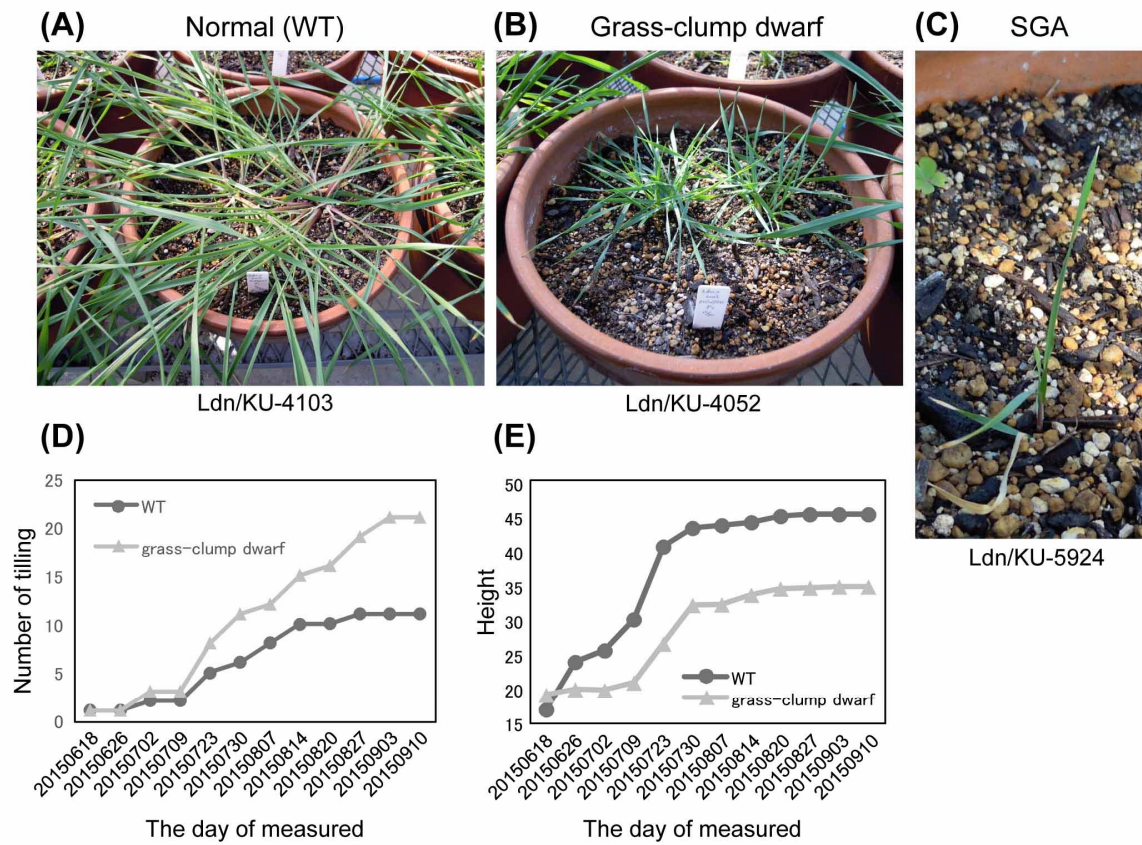


Fig. 3 (Okada et al.)

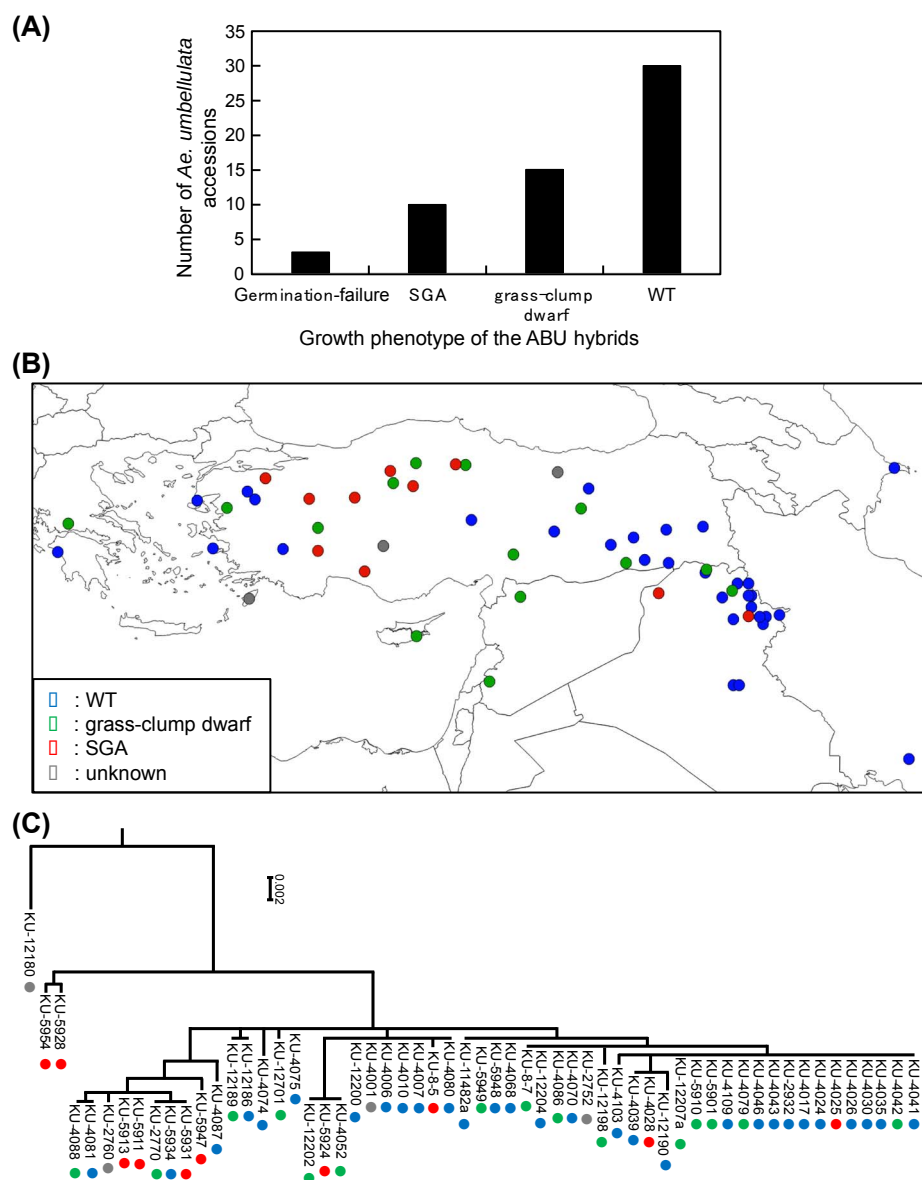


Fig. 4 (Okada et al.)

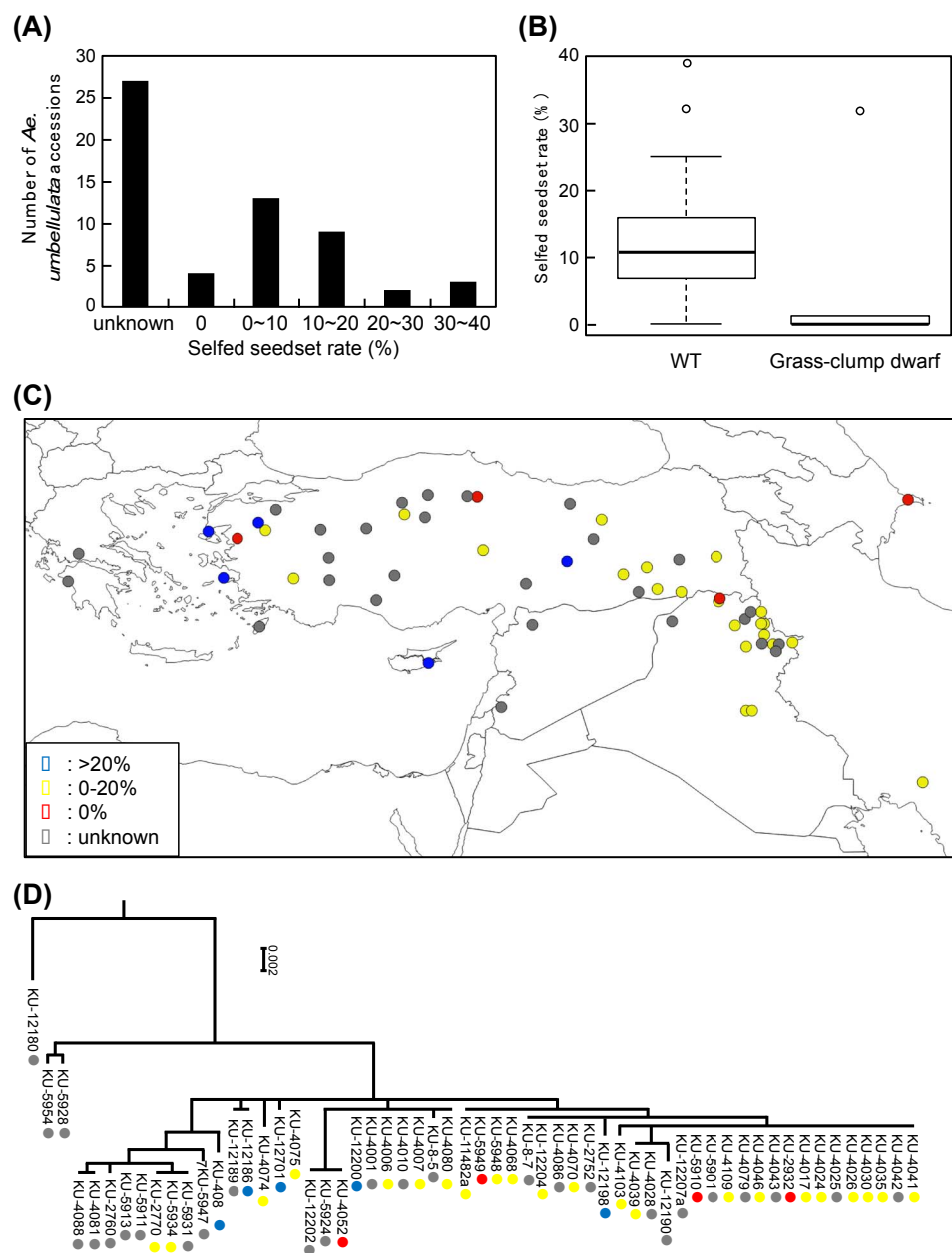


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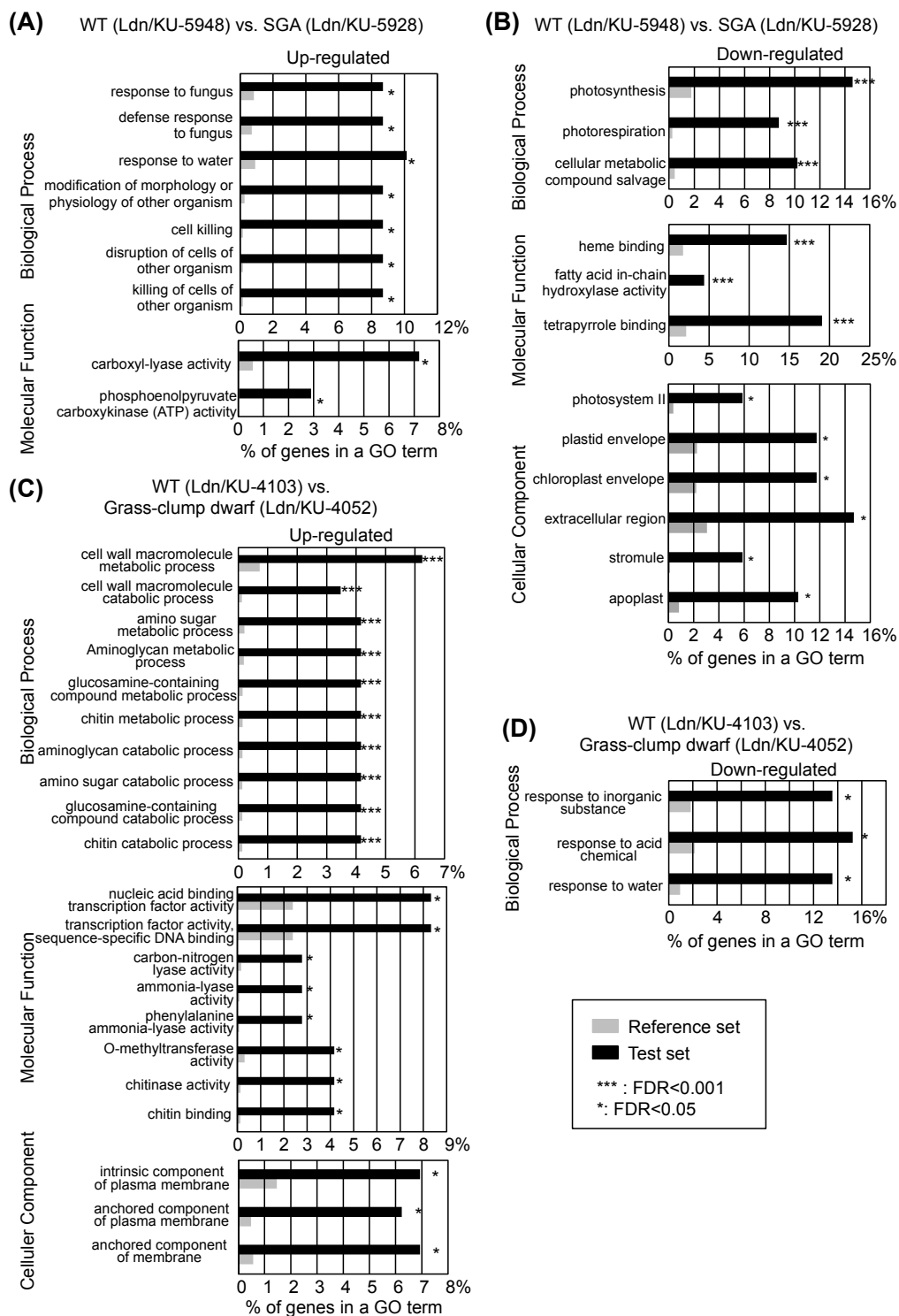


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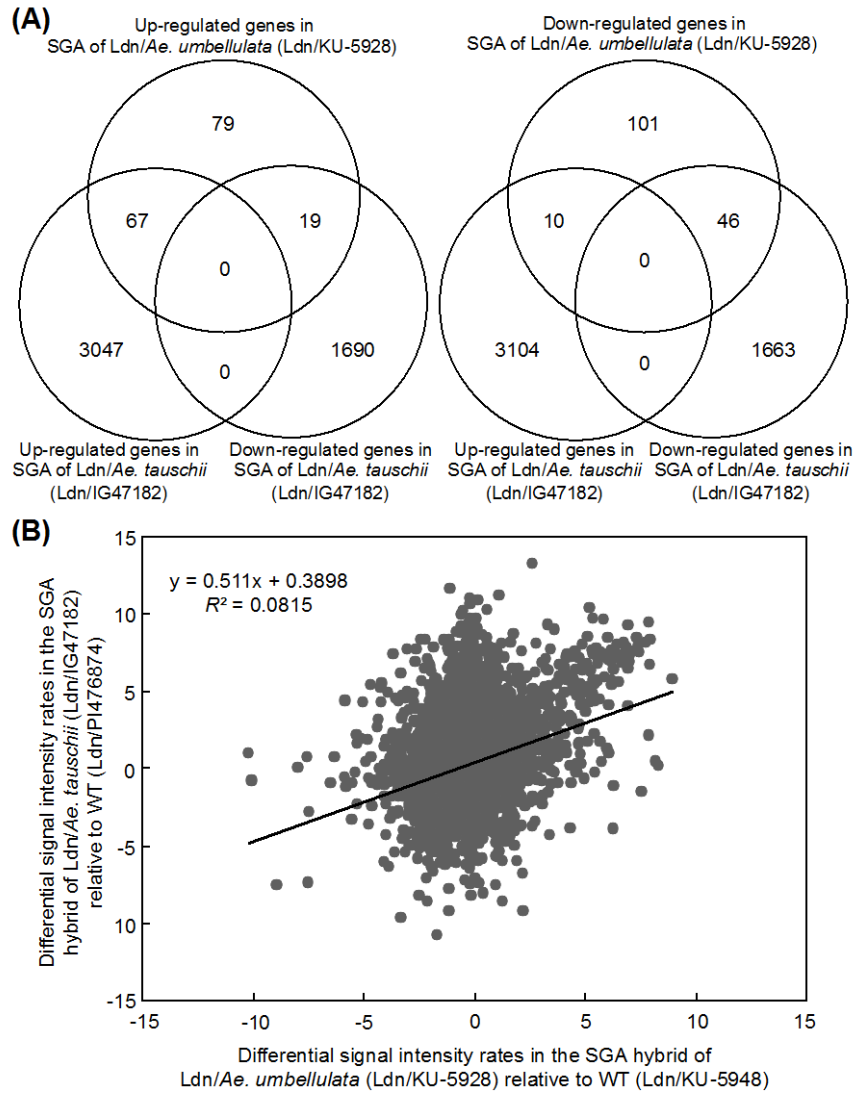


Fig. 7 (Okada et al.)

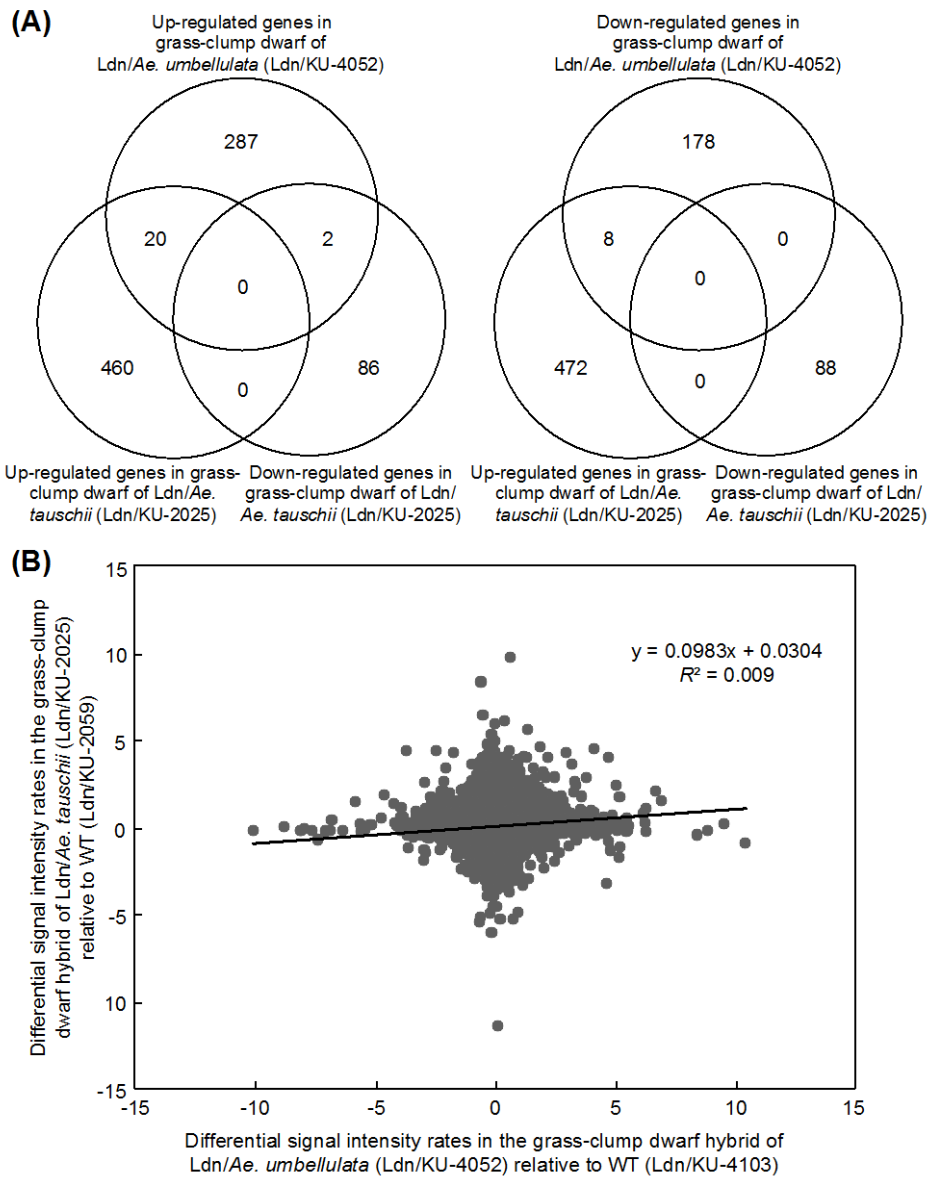
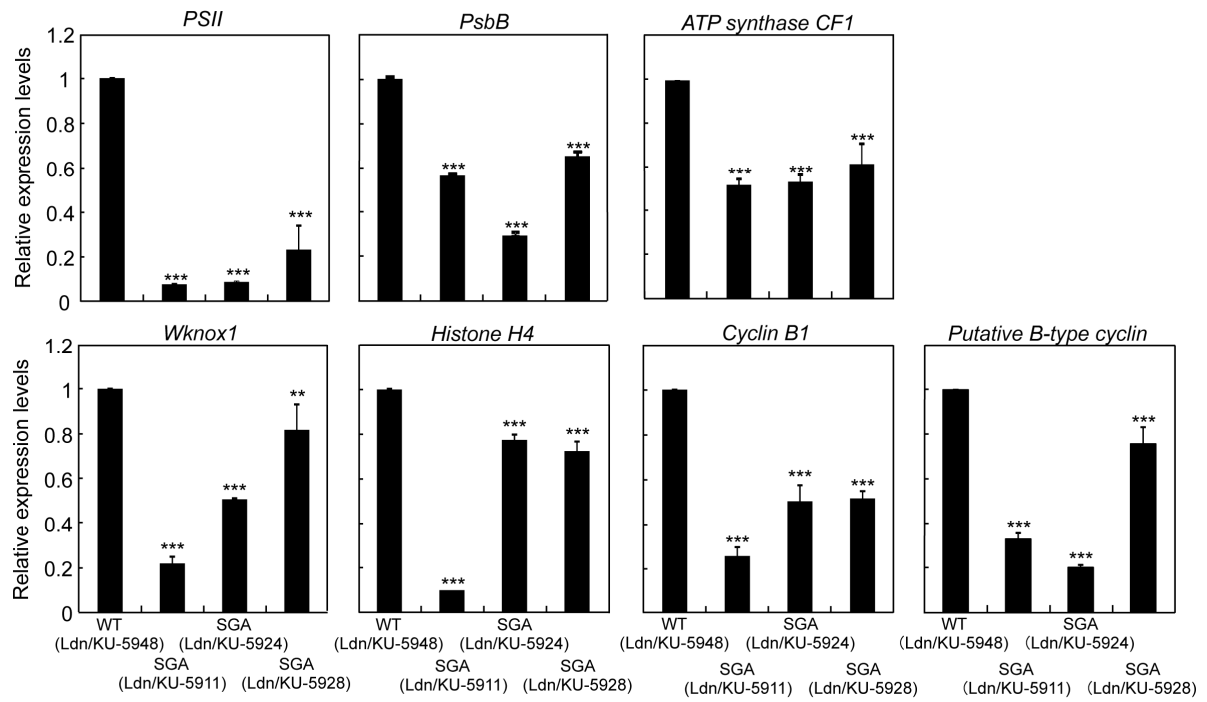
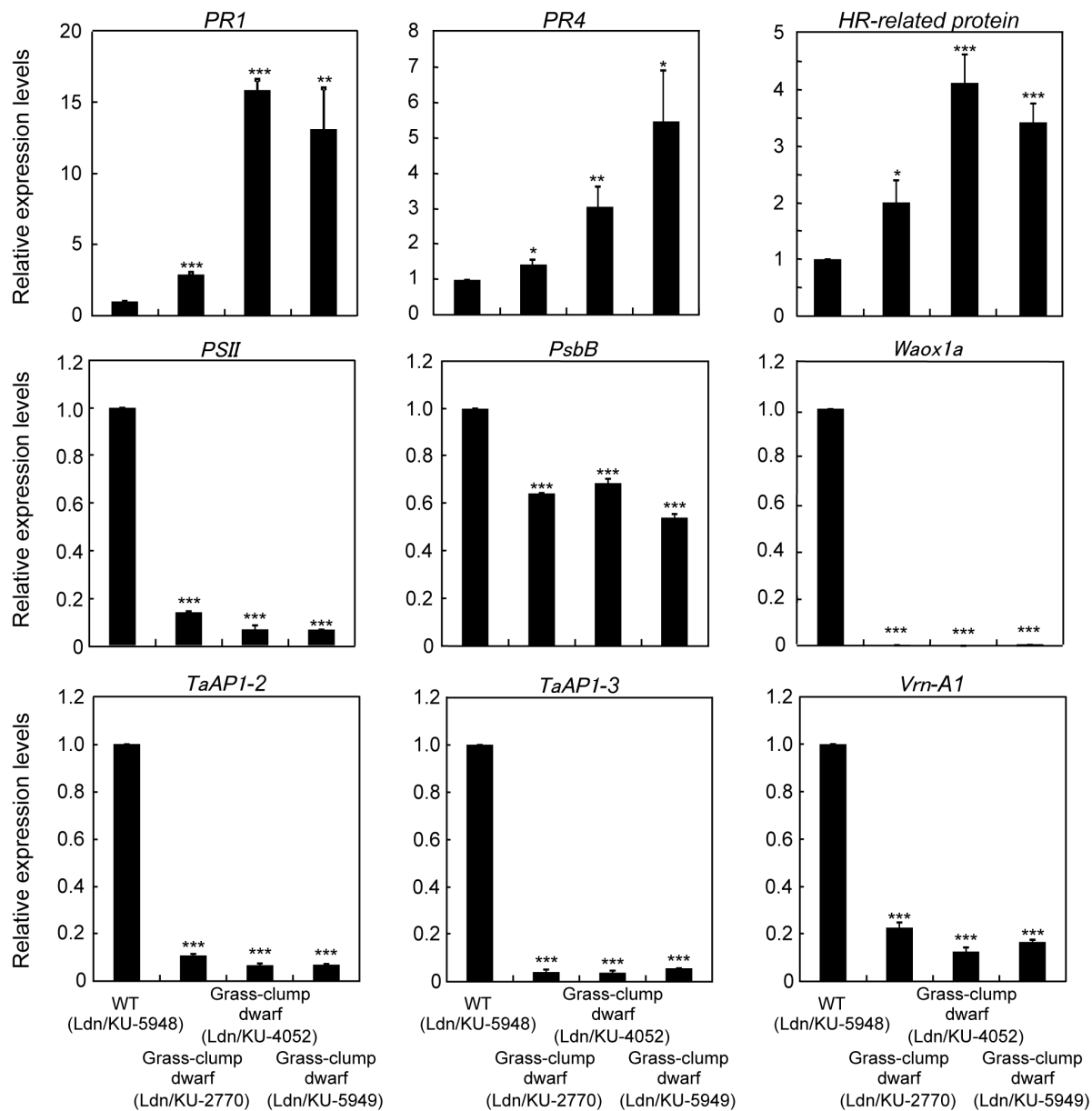
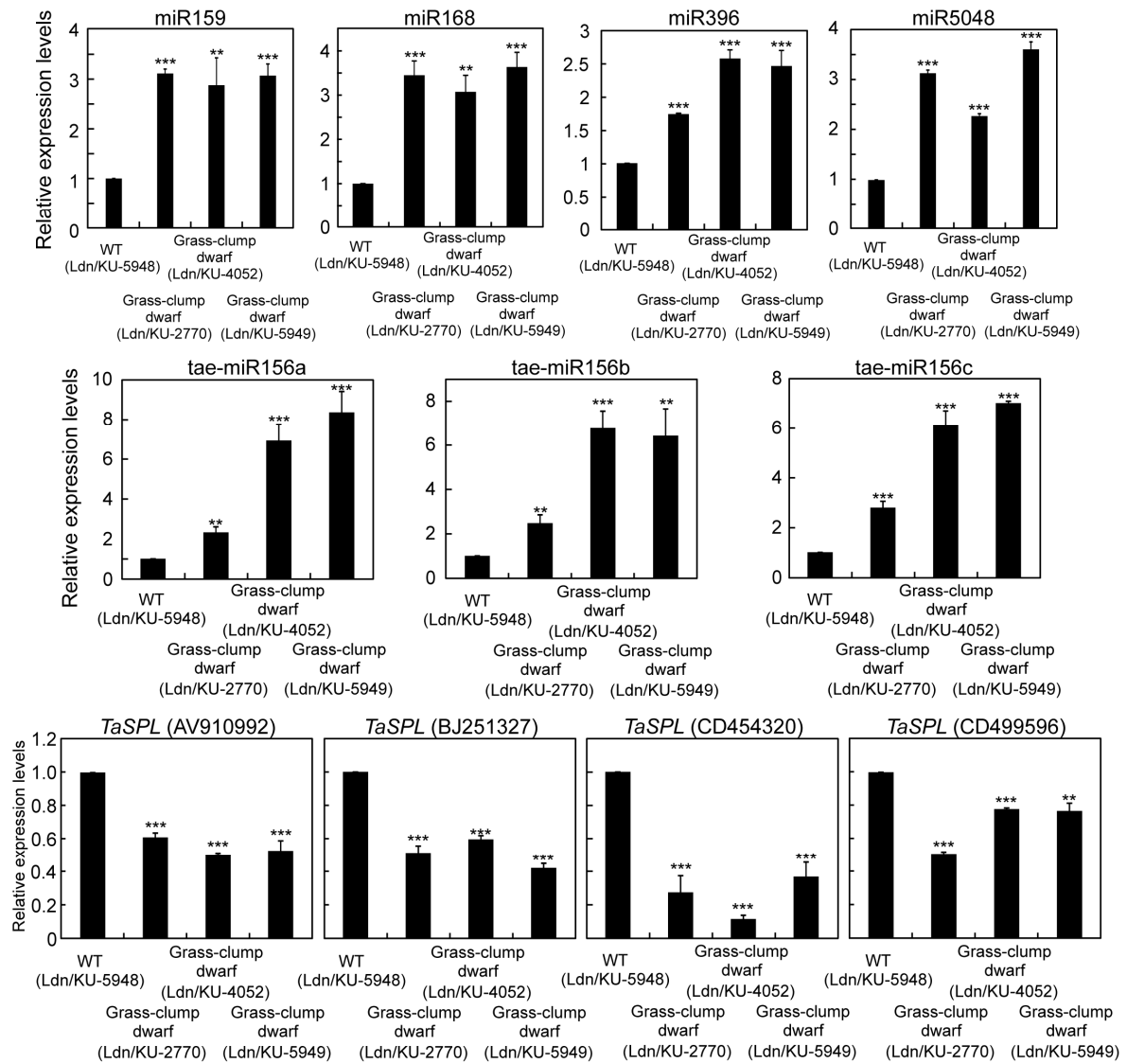


Fig. 8 (Okada et al.)







Supplemental Table 1. List of 58 *Ae. umbellulata* accessions used in this study

Strain No.	Variety	Locality	Country
KU-2932	<i>typica</i> Zhuk.	Collection of Dr. Mustafav	Azerbaijan
KU-12701	-	7 km N of Kophinou, Larnaca (Roadside embarkment)	Cyprus
KU-12180	-	5.1 km W from Platania to Laerma, Rhodes	Greece
KU-12186	-	12 km W from Samos to Karovasi, Samos	Greece
KU-12189	-	10 km S from Platanos to Pirkos, Samos	Greece
KU-12190	-	12 km S from Platanos to Pirkos, Samos	Greece
KU-12198	-	5.4 km E from Mithymna to Madamados, Lesbos	Greece
KU-4109	<i>typica</i> Zhuk.	37.7 km W from Sanandaj to Dezh Shahpur	Iran
KU-4001	<i>pilosa</i> Eig.	110.8 km NNE from Al Migdadiyah to Sulavmaniyah	Iraq
KU-4006	<i>typica</i> Zhuk.	29.8 km SSE from Arbat to Baghdad	Iraq
KU-4007	<i>typica</i> Zhuk.	51.3 km ENE from Arbat to Panjwin	Iraq
KU-4010	<i>typica</i> Zhuk.	13.2 km S from Sulaymaniyah to Qara Dagħ	Iraq
KU-4017	<i>typica</i> Zhuk.	18.8 km NNE from Sulaymaniyah to Chuarta	Iraq
KU-4024	<i>typica</i> Zhuk.	52.4 km NW from Sulaymaniyah to Surdash	Iraq
KU-4025	<i>typica</i> Zhuk.	13.8 km E from Chamchamal to Jarmo	Iraq
KU-4026	<i>typica</i> Zhuk.	25.9 km S from Kirkuk to Baghdad	Iraq
KU-4028	<i>typica</i> Zhuk.	South slope of Jabal Sinjar, North Sinjar	Iraq
KU-4030	<i>pilosa</i> Eig.	37.7 km S from Arbil to Kirkuk	Iraq
KU-4035	<i>pilosa</i> Eig.	5.5 km ENE from Koi Sanjak to Ranya	Iraq
KU-4039	<i>pilosa</i> Eig.	11.4 km ENE from Koi Sanjak to Ranya	Iraq
KU-4041	<i>typica</i> Zhuk.	19.1 km W from Shaqlawa to Arbil, SW slope of Pirman Dagħ	Iraq
KU-4042	<i>typica</i> Zhuk.	33.2 km W from Rowanduz to Shaqlawa	Iraq
KU-4043	<i>typica</i> Zhuk.	SSW of Rowanduz	Iraq
KU-4046	<i>typica</i> Zhuk.	ca. 2 km S from the position of 38.9 km from Rowanduz to Rayat	Iraq
KU-4052	<i>pilosa</i> Eig.	4.4 km NW from Amadiyah, Mazorka Gorge	Iraq
KU-4068	<i>pilosa</i> Eig.	27.6 km WSW from Amadiyah to Dohuk	Iraq
KU-2752	<i>typica</i> Zhuk.	8 km NE of Sebinkarahisar (Giresun – Sebinkarahisar)	Turkey
KU-2760	<i>typica</i> Zhuk.	220 km SE of Konya (Mersin – Konya)	Turkey
KU-2770	<i>typica</i> Zhuk.	Collection of Ankara University (Ankara)	Turkey
KU-4070	<i>typica</i> Zhuk.	28.9 km ESE from Midyat to Cizre	Turkey
KU-4074	<i>pilosa</i> Eig.	29.4 km NE from Mardin to Midyat	Turkey
KU-4075	<i>typica</i> Zhuk.	25.6 km NNW from Mardin to Diyarbakir	Turkey
KU-4079	<i>pilosa</i> Eig.	11.4 km SE from Diyarbakir to Mardin	Turkey
KU-4080	<i>pilosa</i> Eig.	38.6 km E from Diyarbakir to Silvan	Turkey
KU-4081	<i>typica</i> Zhuk.	17.3 km E from Silvan to Bitlis	Turkey
KU-4086	<i>typica</i> Zhuk.	24.8 km N from Elazig to Hozat	Turkey
KU-4087	<i>pilosa</i> Eig.	53.1 km SW from Elazig to Malatya	Turkey
KU-4088	<i>pilosa</i> Eig.	26.4 km SW from Malatya to Maras	Turkey

KU-4103	<i>typica</i> Zhuk.	North of Van	Turkey
KU-5910	<i>typica</i> Zhuk.	20 km E of Mecitoezue (Amasya – Corum)	Turkey
KU-5911	<i>typica</i> Zhuk.	14 km NE of Corum (Corum – Alicik)	Turkey
KU-5913	<i>typica</i> Zhuk.	27 km NW of Kirikkale (Ankara – Kirikkale)	Turkey
KU-5924	<i>typica</i> Zhuk.	10 km S of Pazar (Bolu – Ankara)	Turkey
KU-5928	<i>typica</i> Zhuk.	22 km W of Ayas	Turkey
KU-5931	<i>typica</i> Zhuk.	10 km W of Sivrihisar (Ankara – Eskisehir)	Turkey
KU-5934	<i>typica</i> Zhuk.	Suburbs of Kayseri	Turkey
KU-5947	<i>pilosa</i> Eig.	Suburbs of Burdur	Turkey
KU-5948	<i>typica</i> Zhuk.	Denizli	Turkey
KU-5949	<i>typica</i> Zhuk.	Suburbs of Bergama	Turkey
KU-5954	<i>typica</i> Zhuk.	Suburbs of Kutahia	Turkey
KU-12200	-	10.4 km N from Bigadic to Balikesir	Turkey
KU-12202	-	9.0 km N from Kalecik to Cankiri	Turkey
KU-12204	-	44.2 km W from Tercan to Erzincan	Turkey
KU-12207a	-	10.9 km W from Afyon to Sandikli	Turkey
KU-11482a	-	The pass of south hill of Bagadic	Turkey
KU-8-7	<i>typica</i> Zhuk.	Suburbs of Burdur	Turkey
KU-5901	<i>typica</i> Zhuk.	6 km W of Qatana (Damascus – Mt. Hermon)	Syria
KU-8-5	<i>typica</i> Zhuk.	6 km W of Qatana (Damascus – Mt. Hermon)	Syria

KU accessions were provided by Plant Germ-Plasm Institute, Kyoto University.

Supplemental Table 2. Primer sets used for mRNA qRT-PCR analysis

Gene	Forward primer sequence (5' - 3')	Reverse primer sequence (5' - 3')
<i>PsbB</i>	GTTTTGGTAGATGAGGAGGG	AGCAAAGCAAAGGTAGCAT
<i>PSII</i>	GAAGGGTAGCCAAGGGGAAG	ATTTTGGTTTGCCGCACAG
<i>ATP synthase CF1</i>	TAGGAAATGATGCGGAAC	CCTCTCCCTTTTTCTTTGT
<i>Wknox1</i>	TCTCCGTCACGACCAAC	CTCACGACTGCCAAGCT
<i>putative B-type cyclin</i>	TGCTCGTGTA CTGCCATCC	CCTTCTGCTTGCTGCTCCC
<i>Histone H4</i>	CACCACCAGCAACAAAGCCA	TGGTGATGCCCTGGATGTTG
<i>TaSPL</i> (AV910992)	AGAGTGCATCTGGGGACTTG	ATCATGGTTTCGATGGGTTC
<i>TaSPL</i> (BJ251327)	CAGAGCCCCACCGATGAT	TACCCTTCCAGGTGTCCAAG
<i>TaSPL</i> (CD454320)	AGGCATTGCCAAACCATCCG	AGCTCCCAAAGCCTTCCTAC
<i>TaSPL</i> (CD499596)	TGGCTAGCACCAAGTGAATTG	TGCTGTTCCAGCACTGAGTC
<i>PR1</i>	GCAGAACTCGCCTCAGGACT	GCCGAGGTTATTGTTGCAGA
<i>PR4</i>	CGCGGTGAGCGCCTACTG	CCGATCCCGTTGGTG
<i>HR-related protein</i>	ACTTAGGCTTAGGCAAATGGA	TAGCCATAAGCAAACATAGCC
<i>Waox1a</i>	TGCTCGACAAGATCGCCTACT	GGAGGCGCCAGTAGTCGAT
<i>Vrn-A1</i> (wheat0130contig14238)	ATCAGACTCAGCCTCAAACA	TAGAGACGGGTATCATGGAA
<i>TaAP1-2</i> (rwhfl48m16)	TGGAGCAACAACAAGCTCAC	CAGCCTTCATCTGTTGCTGA
<i>TaAP1-3</i> (wheat0130contig14728)	ATCTACCACCGTGGATGCTC	CAACCACAAGATCGTGCTCA
<i>Actin</i>	GCCGTGCTTTCCCTCTATG	GCTTCTCCTTGATGTCCCTTA

Supplemental Table 3. Primers used for miRNA qRT-PCR analysis

miRNA	Primer sequence (5' – 3')
miR159	TTTGGATTGAAGGGAGCTCTA
miR168	TCGCTTGGTGCAGATCGGGAC
miR396	TCCACAGGCTTTCTTGAAGT
miR5048	TATGTTTGCAGGTTTTAGGTCT
tae-miR156a	TGACAGAAGAGAGTGAGCAC
tae-miR156b	CGACAGAAGAGAGTGAGCAC
tae-miR156c	TGACAGAAGAGAGCGAGCAC
18S rRNA	AGGCCTTCACCAAGTATGCTCTGA and TGGGCGATAACACGGACAACAGTA

Supplemental Table 4. Genetic distance between each genome of *Triticum aestivum* (A, B and D genomes), *T. urartu* (A genome), *Aegilops tauschii* (D genome) and *Ae. umbellulata* (U genome) based on nucleotide variations in the three genomic regions of *Ppd-1*, *APX* and *e-LCY*.

		A		B	D	
		<i>T. aestivum</i>	<i>T. urartu</i>	<i>T. aestivum</i>	<i>T. aestivum</i>	<i>Ae. tauschii</i>
A	<i>T. aestivum</i>					
	<i>T. urartu</i>	0.03283				
B	<i>T. aestivum</i>	0.05790	0.05675			
D	<i>T. aestivum</i>	0.04978	0.05198	0.04282		
	<i>Ae. tauschii</i>	0.05275	0.05119	0.04759	0.00000	
U	<i>Ae. umbellulata</i>	0.05817	0.06485	0.04365	0.03788	0.04126

Supplementary Table 5. Genes identified by microarray analysis as up-regulated in crown tissues of SGA plants (Ldn/KU-5928) compared with those of WT (Ldn/KU-5948)

Supplementary Table 6. Genes identified by microarray analysis as down-regulated in crown tissues of SGA plants (Ldn/KU-5928) compared with those of WT (Ldn/KU-5948)

Supplementary Table 7. Genes identified by microarray analysis as up-regulated in crown tissues of grass-clump dwarf plants (Ldn/KU-4052) compared with those of WT (Ldn/KU-4103)

Supplementary Table 8. Genes identified by microarray analysis as down-regulated in crown tissues of grass-clump dwarf plants (Ldn/KU-4052) compared with those of WT (Ldn/KU-4103)