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Okada, Moeko
Ikeda, Tatsuya M.
Yoshida, Kentaro
Takumi, Shigeo

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Moeko Okada[a], Tatsuya M. Ikeda[b], Kentaro Yoshida[a] and Shigeo Takumi[a,*]

[a] *Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan*

[b] *Western Region Agricultural Research Center, National Agriculture and Food Research Organization, 6-12-1 Nishifukatsu, Fukuyama, Hiroshima 721-8514, Japan*

* Corresponding author: Shigeo Takumi

Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai, Nada-ku, Kobe 657-8501, Japan

Tel. and Fax: +81 78-803-5860

E-mail: takumi@kobe-u.ac.jp

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Abbreviations: AS, grain area size; CS, circularity or grain roundness; GL, grain length; GW, grain width; Ha, hardness; Ldn, Langdon; LWR, length-width ratio; SKCS, single-kernel characterization system; PC, principal component; PIN, puroindoline; PL, perimeter length; RIN, RNA integrity number; SEM, scanning electron microscope; 2D, two-dimensional

Abstract

Grain hardness is an important trait for improvement of wheat grain quality, and is mainly controlled by two puroindoline genes, *Pina* and *Pinb*, on chromosome 5D. The presence of functional alleles for both PINA and PINB proteins results in a soft grain texture. Here, we report nucleotide sequence variation and novel alleles of *Pina* and *Pinb* in a diploid wild wheat relative, *Aegilops umbellulata* Zhuk. Despite the presence of various alleles, mature grains of all *Ae. umbellulata* accessions examined had a hard texture. The hard-textured grains could be due to nonsynonymous substitutions in the *Pina* and *Pinb* alleles or lack of either PINA or PINB protein accumulation. Synthetic hexaploids with the AABBUU genome, derived from interspecific crosses between durum wheat and the *Ae. umbellulata* accessions, showed hard-textured grain character due to absent transmission of functional PINA and PINB proteins from the U-genome donors. In addition, increased thickness of the cell wall in the endosperm might contribute to the hard-texture in synthetic hexaploids. The U-genome addition to cultivated tetraploid wheat generally generated hard grains, suggesting that the *Ae. umbellulata* variation in grain-related traits will be useful for enlargement of grain hardness diversity in hard-textured common wheat.

1. Introduction

Grain hardness is an important trait related to grain quality in wheat breeding. The *Hardness* (*Ha*) locus on the short arm of chromosome 5D mainly controls grain hardness variation in common wheat (Sourdille et al., 1996). The *Ha* locus contains two puroindoline protein-encoding genes, *Pina-D1* and *Pinb-D1*, and allelic differences in these genes distinguish the soft and hard types of common wheat cultivars (Giroux and Morris, 1998; Morris, 2002; Ikeda et al., 2005). The puroindolines are basic proteins with a tryptophan-rich hydrophobic domain showing affinity for polar lipids, and are related to grain softness based on their association with the surface of starch granules (Douliez et al., 2000). Tetraploid wheat species including durum wheat lack puroindolines, resulting in a very hard kernel texture (Gautier et al., 2000). Diploid progenitor species of common wheat preserve the homoeologous sequences of *Pina-D1* and *Pinb-D1*, and recurrent elimination of the *Pina-Pinb* region has occurred in evolutionary lineages of wheat polyploids (Chantret et al., 2005; Li et al., 2008). Thus, *Pina-D1* and *Pinb-D1* of common wheat were derived from the wheat D-genome donor *Aegilops tauschii* Coss., and their diverse alleles in modern wheat cultivars were largely generated after the birth of common wheat (Lillemo and Morris, 2000; Bhave and Morris, 2008a). Nucleotide polymorphisms for the *Pina* and *Pinb* sequences are abundantly accumulated in wheat relative species of *Triticum* and *Aegilops*, and this genetic variation could be useful in wheat breeding to extend the range of kernel textures (Gedye et al., 2004; Massa et al., 2004; Chen et al., 2005; Gazza et al., 2006; Bhave and Morris, 2008a; Li et al., 2008; Cuesta et al., 2013).

To introduce the genetic variation found in agriculturally important traits from *Ae. tauschii* to the common wheat genome, synthetic wheat hexaploids derived from interspecific crosses between tetraploid wheat and *Ae. tauschii* have been utilized (Jones et al., 2013). The alleles *Pina-D^{tau}1* and *Pinb-D^{tau}1* from *Ae. tauschii* are expressed in the hexaploid genetic background of synthetic wheat lines, and the D-genome addition to the tetraploid wheat genome changes the grain texture from hard to soft (Gedye et al., 2004). The observed change

in kernel texture is supported by scanning electron micrographs of transverse sections of mature grains of synthetic hexaploid wheat, in which smoothly rounded starch granules fill the inside of the endosperm, resembling the appearance of the soft type of common wheat (Okamoto et al., 2012). A significant amount of the variation in kernel texture could be assigned to both the tetraploid wheat and *Ae. tauschii* parental accessions in synthetic wheat lines (Gedye et al., 2004). No hard-type alleles of the *Ae. tauschii Pin* genes have been identified (Lillemo et al., 2002; Massa et al., 2004), whereas the *Ae. tauschii* allelic differences are immediately available for extension of variation in kernel texture in common wheat breeding through synthetic wheat lines (Gedye et al., 2004). Addition of chromosome 5H of *Hordeum* species to common wheat enhances grain softness, presumably due to expression of the *Hordeum* puroindoline homologs, hordoindolines, in the chromosome-addition lines (Yanaka et al., 2011). Moreover, *Pin* genes translocated from chromosome 5D of a soft wheat cultivar reduce the grain hardness in durum wheat (Heinze et al., 2016). These observations imply that additive *Pin* genes introduced from other relative species could alter the grain hardness depending on the genotype of the introduced *Pin* genes in polyploid wheat. As well as the allelic differences, transcript accumulation of *Pin* genes greatly affects wheat grain hardness. Silencing of the *Pin* genes increases grain hardness through reduction of the *Pina-D1* and *Pinb-D1* transcript levels in transgenic common wheat (Gasparis et al., 2011).

A wild wheat relative, *Aegilops umbellulata* Zhuk., is a diploid U-genome species, and has been used as a genetic resource for wheat breeding (Friebe et al., 1996). A previous study showed the presence of allelic diversity in the *Pina-U1* and *Pinb-U1* genes based on nucleotide sequence polymorphisms in *Ae. umbellulata*, with at least four alleles reported in *Pina-U1* and three in *Pinb-U1* (Cuesta et al., 2013). However, the effect of the allelic differences on grain hardness remains unresolved in *Ae. umbellulata*, although some allopolyploid *Aegilops* species with the U genome have the soft type of kernel texture (Chen et al., 2005). Hybrid growth abnormalities are frequently observed in interspecific crosses between tetraploid wheat and *Ae. umbellulata*, while selfed seeds can be obtained in ABU

triploid hybrids from some interspecific cross combinations (Okada et al., 2017). Here, we sequenced the *Pina-U1* and *Pinb-U1* genes in more than 40 accessions of *Ae. umbellulata* and produced synthetic allohexaploid lines from interspecific crosses between a durum wheat cultivar and various *Ae. umbellulata* accessions that contained different combinations of *Pina-U1* and *Pinb-U1* alleles. The aim of the study was to evaluate the phenotypic influence of *Pin* allelic differences on grain hardness in the hexaploid genetic background of newly produced synthetic wheat with the U genome.

2. Experimental

2.1. Plant materials

In this study, 58 *Ae. umbellulata* accessions, a tetraploid wheat (*Triticum turgidum* L. ssp. *durum*) cultivar, and six synthetic hexaploid lines were used (Table 1). For production of the synthetic hexaploids, the tetraploid wheat cultivar Langdon (Ldn) was used as the female parent and was crossed with each of the six *Ae. umbellulata* accessions. The F_1 progeny were grown and selfed to produce synthetics (herein designated the F_2 generation). All six synthetics (Ldn x *Ae. umbellulata*) were independently generated, and thus contained the A and B genomes from Ldn and the diverse U genomes originating from the *Ae. umbellulata* pollen parents. Some triploid F_1 hybrids between Ldn and *Ae. umbellulata* show abnormal growth, such as grass-clump dwarfness and severe growth abortion (Okada et al., 2017). Hybrids showing grass-clump dwarfness and severely aborted growth were excluded from selection of the six synthetics. A somatic chromosome number of 42 was confirmed using root tips of two F_3 seeds from one F_2 plant of each synthetic. F_3 grains and plants derived from one F_2 plant of each synthetic were used. The F_3 seeds of each synthetic were sown in November 2016, and the F_3 plants were grown in season 2016-2017 using pots arranged randomly in a glasshouse of Kobe University (34˚43’N, 135˚13’E). The temperature of the glasshouse was not regulated. In addition, five lines of the wheat synthetics, which were derived from interspecific crosses between Ldn and five accessions of the wheat D-genome donor species

Ae. tauschii Coss. (Kajimura et al., 2011), were used for evaluation of the grain-related characters. The F_4 plants of the synthetic wheat lines with the AABBDD genome were grown under the same conditions as the F_3 plants of the five synthetics with the AABBUU genome. The five parental accessions of the synthetic wheat lines were KU-2076, KU-2103, KU-2105, KU-2109, and IG47202. The common wheat cultivar Chinese Spring was used for expression analysis of *Pina* and *Pinb*. For protein electrophoresis, the common wheat cultivar Norin 61 was used as a positive control.

2.2. Sequencing of Pina *and* Pinb *genes*

The *Pina* and *Pinb* gene sequences were amplified with the region-specific PCR primer sets described in a previous report (Cuesta et al., 2013). The regions amplified by ExTaq polymerase (Takara Bio, Shiga, Japan) were sequenced using a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 3730*xl* DNA Analyzer (Applied Biosystems). Multiple sequence alignment and population genetic analyses for estimating within-species diversity were performed according to our previous study (Okada et al., 2017). The nucleotide sequences of *Pina* and *Pinb* in *Ae. umbellulata* were deposited in the DDBJ database under the following accession numbers: LC375775 to LC375780 for *Pina-U1* and LC375781 to LC375790 for *Pinb-U1*.

2.3. Measurement of grain-related traits

Grain size and shape were measured in each *Ae. umbellulata* accession and synthetic hexaploid line using *SmartGrain* software ver. 1.2, which was developed for high-throughput phenotyping of rice seeds (Tanabata et al., 2012). Six parameters for grain size and shape, namely, grain area size (AS), perimeter length (PL), grain length (GL), grain width (GW), length-width ratio (LWR), and circularity or grain roundness (CS), were recorded for at least 50 seeds of each accession and line according to the *SmartGrain* protocol. AS indicates the area within the perimeter of grain, and CS was calculated based on the AS and PL values (Tanabata et al., 2012).

Four grain-related traits, grain hardness, weight, diameter and moisture, were evaluated using a single-kernel characterization system (SKCS 4100, Perten, Stockholm, Sweden). The SKCS hardness index was obtained from crushing a sample of at least 50 kernels from Ldn, each *Ae. umbellulata* accession, and each synthetic hexaploid line.

The grain-related trait data from the *SmartGrain* and SKCS analyses were statistically analyzed and principal component (PC) analysis was conducted using Rstudio ver. 1.0.143 (http://www.rstudio.com) in R software ver. 3.3.2 (https://www.R-project.org).

2.4. Scanning electron microscopy and fluorescent microscope observation of grains

A transverse section of grain was observed by an S-3400N scanning electron microscope (Hitachi High-Technology, Tokyo, Japan) after the grain was snapped in the middle as previously described (Okamoto et al., 2012). Scanning electron microscopy (SEM) was performed without any pretreatment at an accelerating voltage of 8.00 kV under low vacuum conditions of 70 Pa at –25ºC according to previous reports (Araki et al., 2009; Kobayashi et al., 2010).

Transverse sections were stained by 0.01% fluorescent brightener 28 (FB28) and 0.1% acid fuchsin, and fluorescent images were captured with a JX71 fluorescent inverted microscope (Olympus, Tokyo, Japan). The blue fluorescence of FB28 excited by ultraviolet light indicates cell walls and the red fluorescence of acid fuchsin indicates stained proteins.

2.5. Analysis of puroindoline proteins

Two-dimensional (2D) gel electrophoresis (IEF/SDS-PAGE) was conducted to detect the puroindoline a and b proteins (PINA and PINB) according to previous reports (Ikeda et al., 2005; Yanaka et al., 2011). Grains of *Ae. umbellulata* accessions and synthetic hexaploid lines were crushed, and the proteins soluble in Triton X-114 were used to compare their puroindoline profiles.

3. Results

3.1. Nucleotide sequence polymorphisms in Pin *genes*

Pina and *Pinb*, which are intronless genes in *Ae. umbellulata* as in common wheat, were sequenced, and their deduced amino acid sequences compared for the 58 *Ae. umbellulata* accessions. In total, 14 polymorphic sites were observed at the *Pina-U1* locus and 28 at the *Pinb-U1* locus; 11 of the sites for *Pina-U1* and 21 sites for *Pinb-U1* were nonsynonymous substitutions. Recently, four *Pina-U1* alleles (*Pina-U1-I* to *Pina-U1-IV*) and three *Pinb-U1* alleles (*Pinb-U1-I, Pinb-U1-II,* and *Pinb-U1-III*) were reported in *Ae. umbellulata* (Cuesta et al., 2013). Here, five additional putative PINA-U1 and ten PINB-U1 amino acid sequences were found in *Ae. umbellulata* (Table 1, Fig. 1).

Pina-U1 sequences include nine subfamilies of alleles, four previously known (*Pina-U1a* to *Pina-U1d*) based on their putative amino acid sequences, which were reported as *Pina-U1-I* to *Pina-U1-IV* by Cuesta et al. (2013). The *Pina-U1-II* (*Pina-U1b*) and *Pina-U1-III* (*Pina-U1c*) alleles were absent in the 58 *Ae. umbellulata* accessions, and five alleles, *Pina-U1e* to *Pina-U1i*, were newly identified (Table 1). All nine *Pina-U1* alleles are different from the *Pina-D1a* functional allele, and harbor at least three nonsynonymous substitutions compared with the *Pina-D1a* sequence (Fig. 1A). Ten subfamilies of *Pinb-U1* alleles, named *Pinb-U1d* to *Pinb-U1m*, were newly identified in the 58 *Ae. umbellulata* accessions examined (Table 1), whereas the three known alleles, *Pinb-U1a, Pinb-U1b,* and *Pinb-U1c* (Cuesta et al. 2013), were absent. Frameshift mutations occurred in the four *Pinb-U1* alleles (Fig. 1B). All 13 *Pinb-U1* types were different from the *Pinb-D1a* functional allele sequence, and also had five nonsynonymous substitutions compared with the *Pinb-D1a* sequence. One of the five substitutions was found in the conserved domain of PINB, a lysine to arginine substitution at the 73th amino acid residue.

The values for nucleotide diversity (π) were 0.0046 for *Pina* and 0.0086 for *Pinb*, which were higher than values that we previously reported (π = 0.0036) based on three single-copy genomic sequences, which included a gene controlling flowering time, *Ppd-1*, an ascorbate peroxidase gene, *APX*, and a carotenoid biosynthetic cyclase gene, *e-LCY* (Okada et al., 2017).

The nucleotide diversity was higher in *Pinb* than in *Pina*. Values for Tajima's *D* statistic (Tajima, 1989) and Fu and Li's *D** statistic (Fu and Li, 1993) were used to evaluate evolutionary neutrality in the *Pina* and *Pinb* genes. The Fu and Li *D** test statistic was significantly negative for *Pinb*, but the *D* and *D** statistics were not significant for *Pina* (Table 2). The negative statistic for *Pinb*, however, was significant due to the *Pinb-U1m* sequence of KU-12190.

3.2. Variation in grain-related traits in Ae. umbellulata *and the synthetic hexaploids*

Six traits (AS, PL, GL, GW, LWR, and CS) of at least 50 hulled grains were evaluated for each of the six *Ae. umbellulata* accessions using *SmartGrain* software. KU-12204 showed the largest values for all of these traits except CS (Supplemental Table 1). The *SmartGrain* data indicated that KU-12204 and KU-12186 respectively produced the largest and smallest grains of the six accessions. These traits were also measured in the allohexaploid synthetics and Ldn. Ldn had higher values for the four grain size-related traits, AS, PL, GL, and GW, than the six synthetic lines (Supplemental Table 1). Grains of the Ldn/KU-12204 synthetic line were the largest of the AABBUU synthetics. Except for LWR, each of the AABBUU synthetics exhibited values in-between Ldn and the parental *Ae. umbellulata* accession. The grain shape of the AABBUU synthetics was similar to that of the synthetic wheat lines with AABBDD genome.

Grain weight and diameter evaluated by SKCS varied widely, with KU-12204 showing the largest size and weight of grains among the six *Ae. umbellulata* accessions (Table 3). Large variation in grain hardness was observed in the six accessions, although the grain size in *Ae. umbellulata* was too small for a precise evaluation. High grain weight and diameter were observed in the six AABBUU synthetic lines, which were similar to those of Ldn rather than the parental *Ae. umbellulata* accessions (Table 3). The SKCS hardness indexes showed more than 70 in Ldn, *Ae. umbellulata* and most of the AABBUU synthetics, and were much higher in the AABBUU synthetics than in the AABBDD synthetic wheat lines. The kernel texture differed among the synthetics, with the Ldn/KU-12204 synthetic line showing the

lowest hardness index. In hard red spring wheat, the hardness value of SKCS is negatively correlated with kernel weight (Martin et al., 2001). However, no wrinkled grain was observed in the synthetics examined in the present study, and the negative correlation was not observed among the synthetics. Thus, the grain shape and weight were not related to the hardness in the AABBUU synthetics.

PC analysis based on the nine traits was performed for the six AABBUU synthetic hexaploids, their parental accessions, and six synthetic wheat lines with the AABBDD genome. The first and second values for the nine traits, PC1 and PC2, were mainly related to grain size and shape, respectively (Supplemental Table 2). The SKCS grain hardness largely contributed to PC2 and PC3. The cumulative proportion of PC1, PC2, and PC3 was 96.7% of the total variation in the grain-related traits. Scatter plots with the two PCs clearly divided Ldn, the *Ae. umbellulata* accessions, and the AABBUU synthetic hexaploids, and partially overlapped between the AABBDD and AABBUU synthetics (Fig. 2). The PC1 values explained the differences well, and the PC1 values of the AABBUU synthetics were in-between those of Ldn and *Ae. umbellulata*.

3.3. SEM of grain transverse sections and starch granules

SEM observation of transverse sections of mature wheat grains has been an effective approach for monitoring adhesion between the starch granules and matrix proteins (Barlow et al., 1973; Chen et al., 2005; Okamoto et al., 2012). The surface of starch granules is cleanly separated from the matrix proteins in soft wheat, whereas the starch granules are tightly bound with the matrix proteins in hard wheat (Chen et al., 2005). With these observations in mind, we compared the starch granules in transverse sections of mature grains of the six *Ae. umbellulata* accessions. In grains of the *Ae. umbellulata* accessions, a single aleurone layer covered the endosperm, and endosperm cells were well ordered under the aleurone layer (Fig. 3). Large and small starch granules filled the inside of the endosperm cells in the mature grains. Four accessions, KU-4026, KU-4087, KU-4103, and KU-12186, showed tight adhesion of the starch granule surface to the matrix proteins. The starch granules in grains of

these accessions were embedded in the matrix proteins without any gaps, and broken starch granules were frequently observed in the grain sections. This observation indicated that these four *Ae. umbellulata* accessions have a hard texture. In KU-4030 and KU-12204, some starch granules appeared to be smoothly rounded and separated from the matrix proteins, and others were tightly attached to the matrix proteins and embedded in them (Fig. 3), meaning that the endosperm texture of these two accessions was also considered to be hard.

Similar to most cultivars of durum wheat, Ldn displayed hard wheat-type starch granules that tightly filled the inside of the grain endosperm (Fig. 4). Broken large starch granules were frequently observed in Ldn. The large starch granules appeared to be rounder in Ldn than in the six Ldn/*Ae. umbellulata* synthetic hexaploid lines. Endosperm cells were more clearly ordered under the single aleurone layer and were covered with thicker cell walls in the six synthetic lines than in Ldn. No structural differences in adhesion of the starch granules and matrix proteins were observed among the six synthetic lines. The starch granules in the mature grains were tightly embedded in the matrix proteins, and broken large granules frequently appeared in the six synthetics, as observed in Ldn. This observation indicated that all six synthetic lines have a hard texture.

Double staining of the transverse sections with FB28 and acid fuchsin revealed that proteins abundantly accumulated within the endosperm of the mature grains in the synthetics and their parental accessions (Fig. 5). Cell walls were much more clearly visualized in the endosperm of the *Ae. umbellulata* accessions than in those of Ldn or the synthetic hexaploids. Especially under the aleurone layer, cell walls were more clearly observed in the synthetic hexaploid lines than in Ldn. This observation indicated that the cell wall might be thicker in the endosperm of the synthetics than in Ldn.

3.4. Expression analysis of Pin *proteins*

The puroindolines of mature grains were compared by 2D gel electrophoresis in the synthetic hexaploids and their parental accessions (Fig. 6). The soft wheat cultivar Norin 61 had spots corresponding to PINA and PINB on the gels, whereas they were absent in Ldn.

Two *Ae. umbellulata* accessions, KU-4026 and KU-4103, generally exhibited only a PINA spot, while the other three accessions, KU-4030, KU-4087, and KU-12204, generally exhibited a PINB spot. No spots for PINA or PINB were observed in KU-12186. The six synthetics showed the same 2D gel electrophoresis profiles for the puroindolines as their parental *Ae. umbellulata* accessions (Fig. 6). In Ldn/KU-12186, as in KU-12186, PINA and PINB were absent.

4. Discussion

Intraspecific variation in grain hardness was observed in *Ae. umbellulata*. SEM of transverse sections of mature grains clearly indicated that the six *Ae. umbellulata* accessions could be regarded as having a hard-type endosperm texture (Fig. 3). The starch granules of mature grains from the *Ae. umbellulata* accessions were tightly embedded in the protein matrix, and broken starch granules were frequently observed. The grain hardness phenotype was supported by the measured SKCS hardness indexes. The grain texture of most of the *Aegilops* species previously examined are soft; only *Ae. sharonensis* Eig is hard (Chen et al., 2005). The hard-type endosperm texture was confirmed in all *Ae. umbellulata* accessions examined, and appears to be a rare characteristic in *Aegilops* species.

Two-dimensional gel electrophoresis showed that PINA, PINB, or both were missing in the mature grains of the *Ae. umbellulata* accessions examined (Fig. 6). In addition, all *Pina-U1* and *Pinb-U1* alleles contained synonymous or nonsense mutations compared to the wild-type alleles of the D-genome homoeologous genes (Fig. 1). All hard cultivars contain alleles in either *Pina* or *Pinb* that differ from the soft cultivars of common wheat, and both PINA and PINB are required for the soft texture if protein accumulation is sufficient in the mature grains (Giroux and Morris, 1998). For example, expression of the wild-type *Pinb-D1* allele (*Pinb-D1a*) can complement the sequence alteration observed in *Pinb-D1b* and can alter the endosperm texture from hard to soft in transgenic plants of common wheat (Beecher et al., 2002). Therefore, similarly to in common wheat, the types of PINA and PINB are considered

to determine the grain character of *Ae. umbellulata*. In addition to the *Pina* and *Pinb* genotypes, accumulation of PINA and PINB varied among the *Ae. umbellulata* accessions examined (Fig. 6). No accumulation of PINB was reported in common wheat cultivars with *Pinb-D1c* (Ikeda et al., 2005), although *Pinb-D1c* transcription was confirmed in developing grains (Gasparis et al., 2011). Thus, the puroindoline accumulation appears to sometimes be inconsistent with the *Pin* transcript level. Since nucleotide variation in the promoter regions of *Pina* and *Pinb* was found in the diploid genomes of wheat relatives (Lillemo et al., 2002; Bhave and Morris, 2008b), elucidation of the relationship between *Pina* and *Pinb* promoter sequences, amount of protein accumulated, and grain hardness is needed.

Based on the putative amino acid sequences, seven PINA and ten PINB types were found in *Ae. umbellulata* (Table 1). Prior to the present study, four *Pina* and three *Pinb* alleles were reported (Cuesta et al., 2013). Novel alleles were also found for both *Pin* genes, implying that *Aegilops* species have abundant allelic diversity at the *Pin* loci and that screening of additional genetic resources may identify even more alleles. In fact, many *Pina* and *Pinb* alleles have been identified from a limited number of accessions of five diploid *Aegilops* species (Cuesta et al., 2013). Population genetic analysis of these two sequences revealed distinct evolutionary features for *Pina* and *Pinb* (Table 2); the *Pina* nucleotide variation pattern was evolutionarily neutral, whereas the *Pinb* variation was significantly negative. This negative statistic is suggestive of the *Ae. umbellulata Pinb* locus being under purifying selection that removes deleterious mutations, although no significance was observed when excluding the *Pinb-U1m* sequence of KU-12190. In addition, higher intraspecific diversity and a larger number of alleles were observed in *Pinb* than in *Pina*. Distinct evolutionary patterns between *Pina* and *Pinb* have been reported in species in the tribe Triticeae including rye and barley, and positive selection was postulated at the *Pina* locus but not at *Pinb* (Massa and Morris, 2006). Therefore, this evolutionary feature of *Pinb* seemed to be specific to *Ae. umbellulata*.

The SKCS hardness indexes were greater than 60 in all six AABBUU synthetic hexaploids (Table 3). The SKCS values of hard cultivars are generally greater than 70 in

common wheat (Lillemo and Morris, 2000), and therefore the synthetics with the AABBUU genome could be regarded as hard wheat. SKCS indexes for grain hardness showed less than 50 in synthetic wheat hexaploids with the AABBDD genome (Table 3), indicating that the grains exhibit a soft texture and that the softer alleles of *Pin* are expressed in the hexaploid genetic background (Gedye et al., 2004). Our previous SEM observation of the endosperm of AABBDD synthetic hexaploids, in which the A and B genomes are derived from Ldn, also indicated that they have soft-textured grains (Okamoto et al., 2012). In addition, the cell wall of the grain endosperm appears to be thick in *Ae. umbellulata*, a character that was transmitted to grains of the AABBUU synthetics (Fig. 5). For the high SKCS hardness indexes of the AABBUU synthetics (Table 3), transmission of the cell wall thickness from the parental *Ae. umbellulata* accessions might be caused as well as hard-textured grain characters. The grains from KU-12186 lacked any spots in gels corresponding to PINA or PINB in the diploid and AABBUU hexaploid background, and the highest index of grain hardness was observed in the Ldn/KU-12186 synthetic line. Therefore, allelic variation in *Pina-U1* and *Pinb-U1* would contribute to expansion of grain hardness diversity in hexaploid wheat through the AABBUU synthetics. Unlike the D genome of *Ae. tauschii*, the *Ae. umbellulata* genome does not appear to have the potential to markedly decrease the grain hardness in hexaploid genetic backgrounds that carry the AABB genome.

5. Conclusions

Various alleles of *Pina* and *Pinb* were found in the *Ae. umbellulata* accessions showing a hard-textured grain character. Moreover, AABBUU synthetic hexaploids, derived from interspecific crosses between durum wheat and the *Ae. umbellulata* accessions, showed hard grain texture due to transmission of the hard texture from the U-genome donors. The allelic divergence in the *Pina-Pinb* region of *Ae. tauschii* has been reflected in large variation in the SKCS hardness index of the AABBDD synthetic hexaploids, hopefully contributing to expansion of the *Pina* and *Pinb* diversity in soft-textured common wheat (Gedye et al., 2004;

Bhave and Morris, 2008a). In contrast, the various *Pina* and *Pinb* genotypes of *Ae. umbellulata* are expected to broaden the grain hardness diversity in hard-textured common wheat.

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Appendix A. Supplementary data

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Table 1. Intraspecific distribution of the *Pin* alleles in 58 *Ae. umbellulata* accessions

Pin types	Accession numbers of *Ae. umbellulata*
Pina alleles	
Pina-U1a (*Pina-U1-I*)	KU-2752, KU-4028, KU-4030*, KU-4041
Pina-U1d (*Pina-U1-IV*)	KU-2760, KU-2932, KU-4006, KU-4007, KU-4010, KU-4017, KU-4024, KU-4025, KU-4026*, KU-4035, KU-4039, KU-4043, KU-4046, KU-4074, KU-4079, KU-4081, KU-4087*, KU-4088, KU-4103*, KU-4109, KU-5949, KU-12198, KU-12204*, KU-12701, KU-8-5
Pina-U1e (*Pina-U1-V*)	KU-2770, KU-4042, KU-4052, KU-4068, KU-4070, KU-4075, KU-4080, KU-4086, KU-5901, KU-5910, KU-5911, KU-5913, KU-5924, KU-5928, KU-5931, KU-5934, KU-5954, KU-12200, KU-12202, KU-12207a, KU-11482a, KU-8-7
Pina-U1f (*Pina-U1-VI*)	KU-4001
Pina-U1g (*Pina-U1-VII*)	KU-5947, KU-5948
Pina-U1h (*Pina-U1-VIII*)	KU-12180, KU-12186*, KU-12189
Pina-U1i (*Pina-U1-IX*)	KU-12190
Pinb alleles	
Pinb-U1d (*Pinb-U1-IV*)	KU-2752, KU-2760, KU-4006, KU-4007, KU-4010, KU-4028, KU-4030*, KU-4039, KU-4041, KU-4043, KU-4074, KU-4079, KU-4103*, KU-4109, KU-5934, KU-5947, KU-12198
Pinb-U1e (*Pinb-U1-V*)	KU-2770, KU-2932, KU-4024, KU-4025, KU-4042, KU-4046, KU-4052, KU-4068, KU-4070, KU-4075, KU-4080, KU-4081, KU-4087*, KU-4088, KU-5901, KU-5910, KU-5911, KU-5913, KU-5924, KU-5928, KU-5931, KU-5949, KU-5954, KU-12200, KU-12202, KU-12204*, KU-12207a, KU-11482a, KU-8-7
Pinb-U1f (*Pinb-U1-VI*)	KU-4001, KU-4086, KU-5948, KU-12180
Pinb-U1g (*Pinb-U1-VII*)	KU-4035
Pinb-U1h (*Pinb-U1-VIII*)	KU-12186*, KU-12189
Pinb-U1i (*Pinb-U1-IX*)	KU-12701
Pinb-U1j (*Pinb-U1-X*)	KU-4017
Pinb-U1k (*Pinb-U1-XI*)	KU-4026*
Pinb-U1l (*Pinb-U1-XII*)	KU-8-5
Pinb-U1m (*Pinb-U1-XIII*)	KU-12190

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

*The accessions are parents of the Ldn/*Ae. umbellulata* synthetic hexaploid lines.

Table 2. Summary of nucleotide variation at *Pin* loci in the *Ae. umbellulata* population

Gene		Entire region	Synonymous	Nonsynonymous
Pina-U1	# of sites	446	103.945	340.055
	# of variable sites	14	4	11
	π	0.00458	0.0507	0.00445
	θ	0.00727	0.00831	0.00699
	Tajima's *D*	-1.11 NS	-0.86 NS	-1.03 NS
	Fu and Li's *D**	-1.77 NS	-1.26 NS	-1.54 NS
Pinb-U1	# of sites	452	93.67	311.33
	# of variable sites	25	7	17
	π	0.00855	0.11045	0.16074
	θ	0.01305	0.21603	0.21603
	Tajima's *D*	-1.10 NS	-1.26 NS	-0.78 NS
	Fu and Li's *D**	-3.75**	-2.77*	-3.17*

NS: not significant

*: significant at the 5% level

**: significant at the 1% level

Table 3. Grain characters measured by SKCS

Sample	Genome	Hardness	Weight (mg)	Diameter (mm)	Moisture (%)
Langdon	AABB	93.7 ± 12.8	46.4 ± 8.9	2.8 ± 0.4	12.1 ± 0.4
KU-4030	UU	79.0 ± 19.6	23.5 ± 8.1	1.6 ± 0.4	9.1 ± 0.6
KU-4026	UU	97.0 ± 17.9	24.3 ± 9.4	1.8 ± 0.4	8.7 ± 0.8
KU-4087	UU	98.4 ± 14.3	22.7 ± 7.0	1.8 ± 0.3	9.4 ± 0.4
KU-4103	UU	71.5 ± 19.9	20.7 ± 6.4	1.6 ± 0.3	8.7 ± 0.6
KU-12186	UU	99.1 ± 19.1	21.3 ± 7.6	1.7 ± 0.3	7.2 ± 0.9
KU-12204	UU	69.2 ± 13.5	26.4 ± 7.8	1.9 ± 0.3	8.9 ± 0.6
Ldn/KU-4030	AABBUU	75.8 ± 15.6	39.7 ± 8.8	2.4 ± 0.4	7.7 ± 1.0
Ldn/KU-4026	AABBUU	76.7 ± 18.2	44.0 ± 15.5	2.7 ± 0.6	6.7 ± 0.8
Ldn/KU-4087	AABBUU	77.6 ± 13.2	38.0 ± 10.2	2.4 ± 0.4	7.7 ± 0.8
Ldn/KU-4103	AABBUU	76.4 ± 17.4	42.1 ± 12.1	2.5 ± 0.5	7.2 ± 0.7
Ldn/KU-12186	AABBUU	79.6 ± 15.0	43.8 ± 9.6	2.6 ± 0.4	7.2 ± 1.0
Ldn/KU-12204	AABBUU	60.7 ± 13.4	45.4 ± 10.8	2.7 ± 0.4	7.4 ± 0.9
Ldn/KU-2076	AABBDD	35.2 ± 13.3	48.5 ± 9.3	2.8 ± 0.3	12.3 ± 0.3
Ldn/KU-2103	AABBDD	49.4 ± 14.1	38.3 ± 9.0	2.4 ± 0.3	13.0 ± 0.3
Ldn/KU-2109	AABBDD	23.0 ± 11.2	39.9 ± 6.6	2.5 ± 0.4	12.4 ± 0.3
Ldn/IG47202	AABBDD	29.2 ± 13.7	37.0 ± 8.7	2.2 ± 0.3	13.7 ± 0.6
Ldn/KU-2105	AABBDD	33.3 ± 12.5	56.7 ± 9.2	3.0 ± 0.3	12.9 ± 0.7

Data are represented as mean ± standard deviation.

Figure legends

Figure 1. Allelic diversity of the *Pina* and *Pinb* genes in *Ae. umbellulata*. Primary structures of puroindolines encoded by *Pina* (A) and *Pinb* (B) are shown. The structures of puroindoline encoded by *Pina-D1a* and *Pinb-D1a* of *Ae. tauschii* are designated as rectangles at the top of the figure. The structures of puroindolines encoded by *Pina-U1* and *Pinb-U1* alleles are shown as horizontal lines. The vertical bars with one-letter abbreviations of amino acids correspond to the sites with amino acid substitutions between puroindolines encoded by *Pina-D1a* and *Pina-U1* alleles or *Pinb-D1a* and *Pinb-U1* alleles. The gray box in the rectangles indicates the conserved domain.

Figure 2. Graph of the first two axes from the PC analysis based on nine grain-related traits. The first component (*x*), mainly related to grain size, accounts for 67.4%, and the second (*y*), related to grain shape, for 20.7% of the total variation. Diagonal crosses, vertical crosses, triangles, and the circle denote *Ae. umbellulata*, synthetic hexaploids with AABBUU genome, synthetic wheat lines with AABBDD genome, and Ldn, respectively.

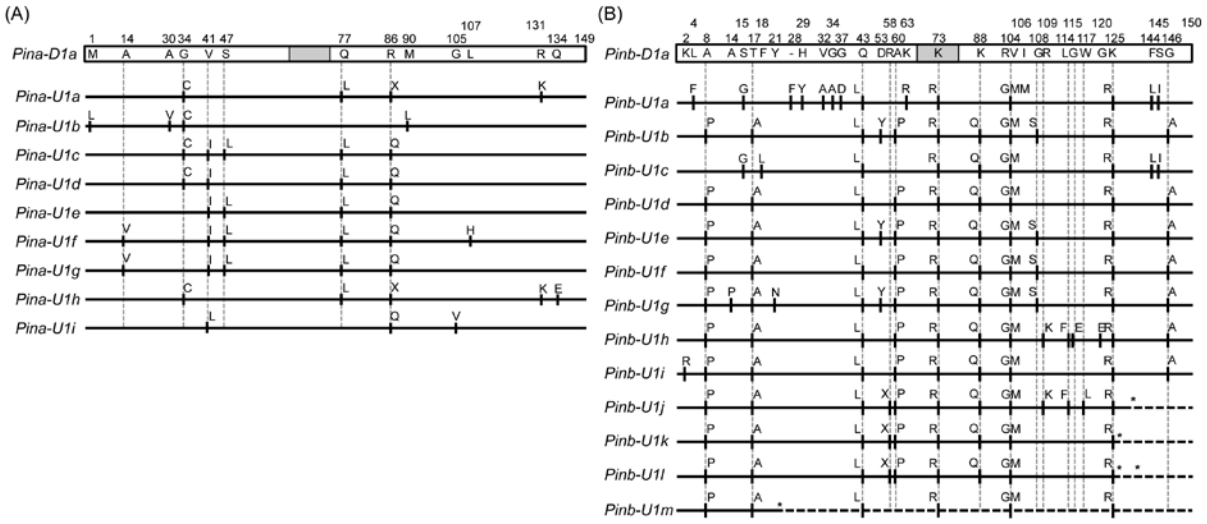
Figure 3. Scanning electron micrographs of transverse sections of *Ae. umbellulata* grains. Photos of the outside (left) and expanded inside (right) regions in the mature grain are represented for each accession. Arrows indicate cell wall. Arrowheads represent attached matrix proteins in sections of KU-12204 and KU-4030. SC; seed coat. AL; aleurone layer, LS; large starch granule, BG; broken large starch granule.

Figure 4. Scanning electron micrographs of transverse sections of mature grains in synthetic hexaploids with the AABBUU genome and parental tetraploid wheat. Photos of the outside (left) and expanded inside (right) regions in the mature grain are represented for each accession. Arrows indicate cell wall. SC; seed coat. AL; aleurone layer, LS; large starch granule, BG; broken large starch granule.

Figure 5. Fluorescence microscope images of transverse sections of mature grains in synthetic AABBUU hexaploids and their parents. The sections were stained with FB28 for cell walls (blue) and acid fuchsin for protein (red).

Figure 6. Two-dimensional gel electrophoresis of Triton X-114-soluble fractions extracted from mature grains of synthetic AABBUU hexaploids and their parents. Grains of a common wheat cultivar, Norin 61, were used as a positive control for PINA (a) and PINB (b) proteins.

Fig. 1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Pina-U1a
Pina-U1b
Pina-U1c
Pina-U1d
Pina-U1e
Pina-U1f
Pina-U1g
Pina-U1h
Pina-U1i
(B)
Pinb-D1a
Pinb-U1a
Pinb-U1b
Pinb-U1c
Pinb-U1d
Pinb-U1e
Pinb-U1f
Pinb-U1g
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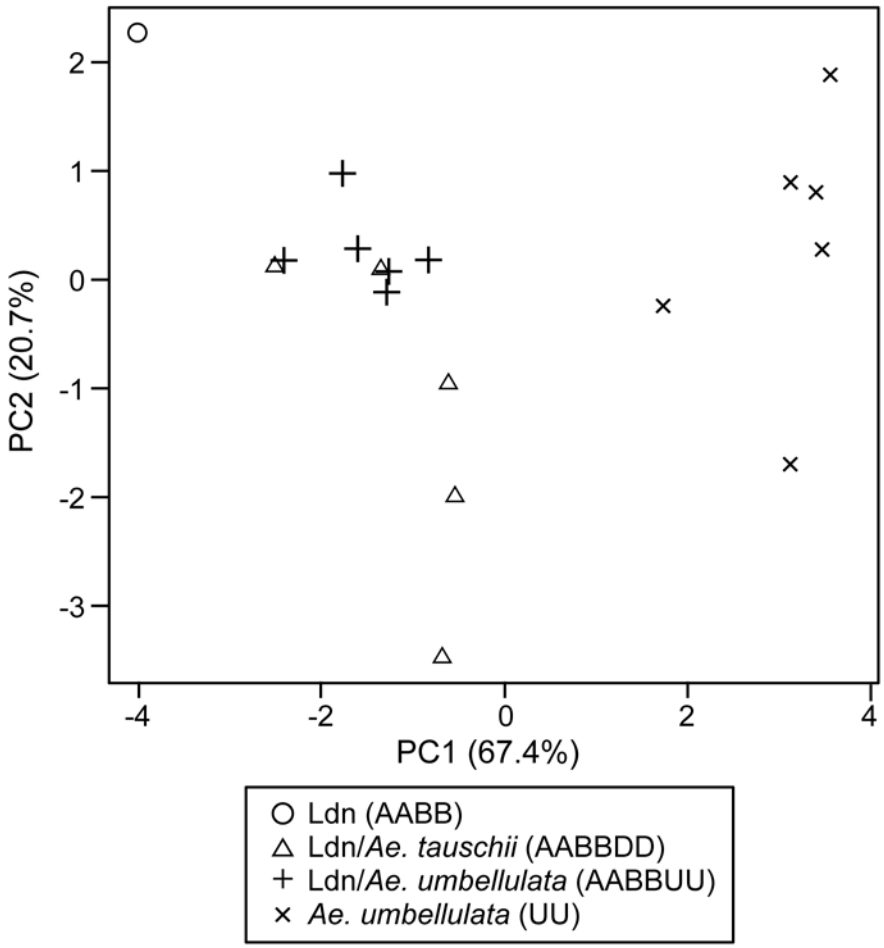
Pinb-U1m

Fig. 2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PC1 (67.4%)
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-2
0
2
4
Ldn (AABB)
Ldn/Ae. tauschii (AABBDD)
Ldn/Ae. umbellulata (AABBUU)
Ae. umbellulata (UU)

Fig. 3

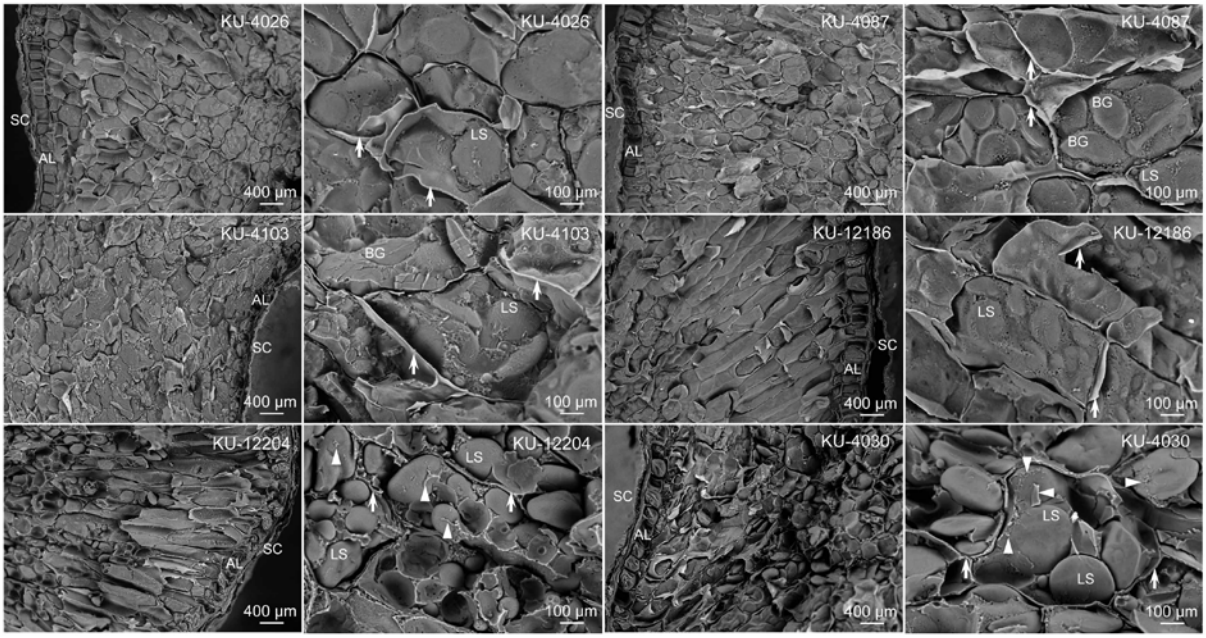
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Fig. 4

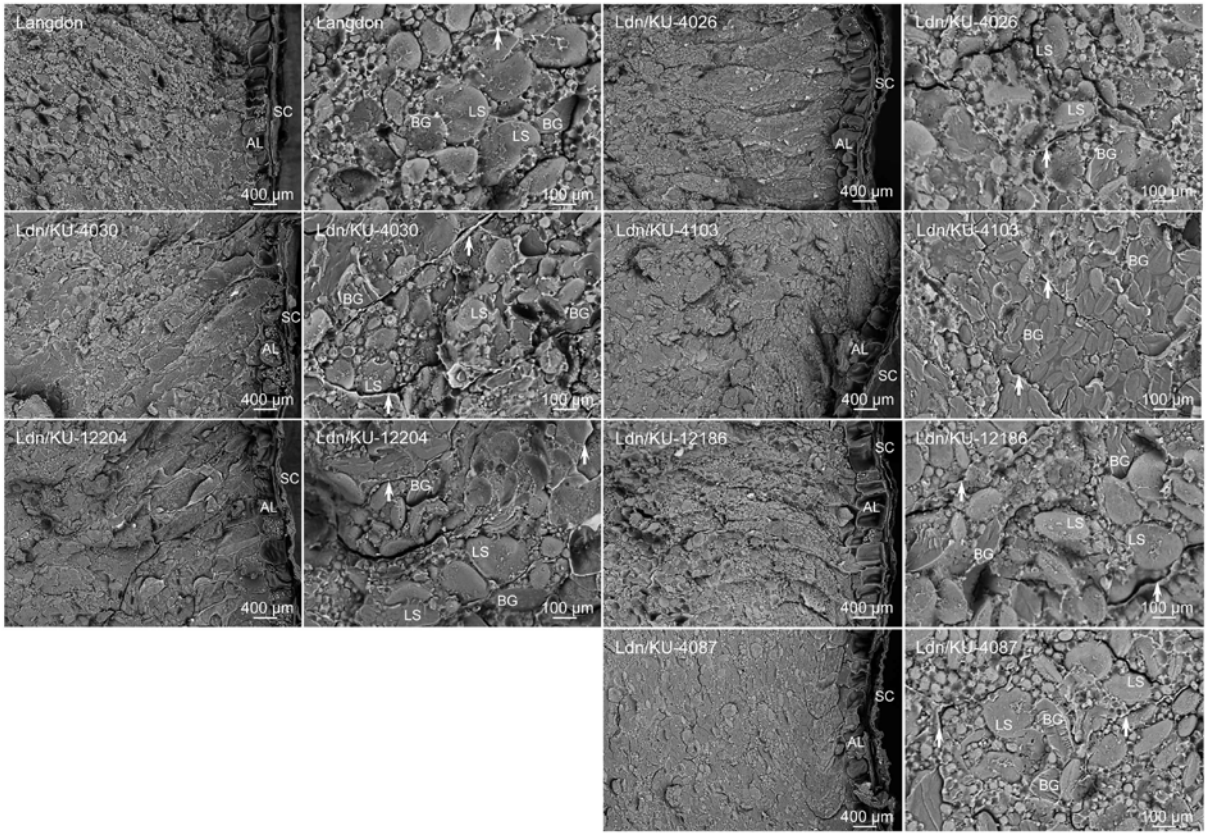
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Fig. 5

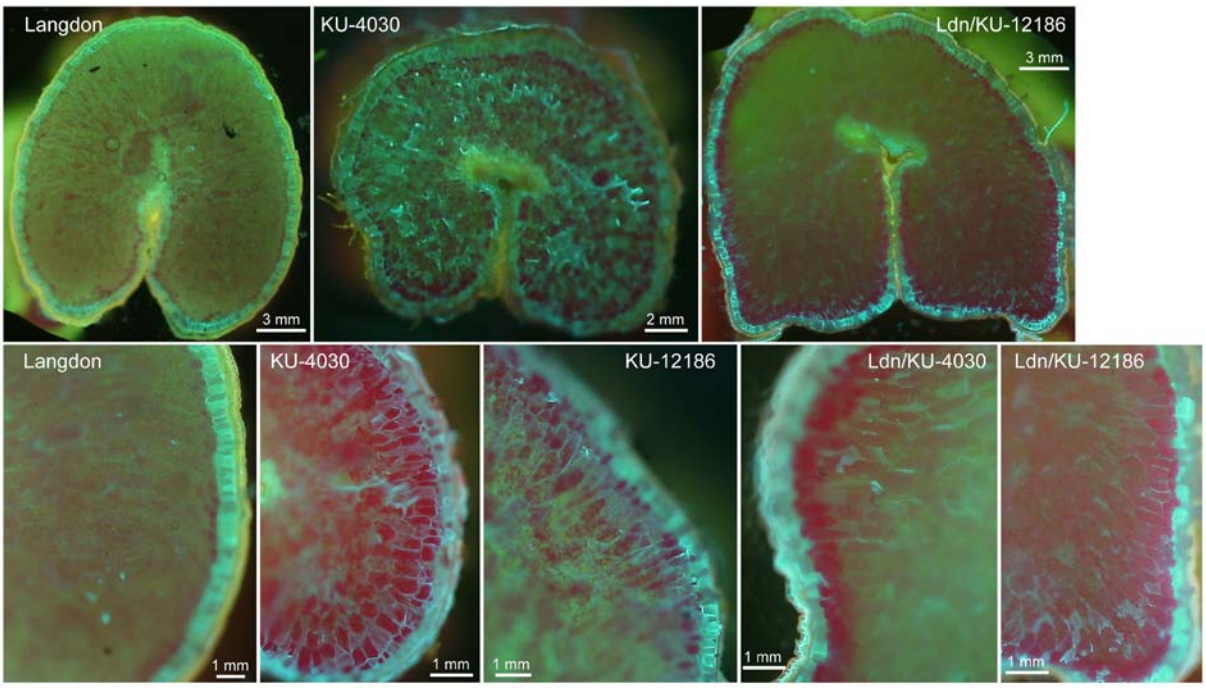
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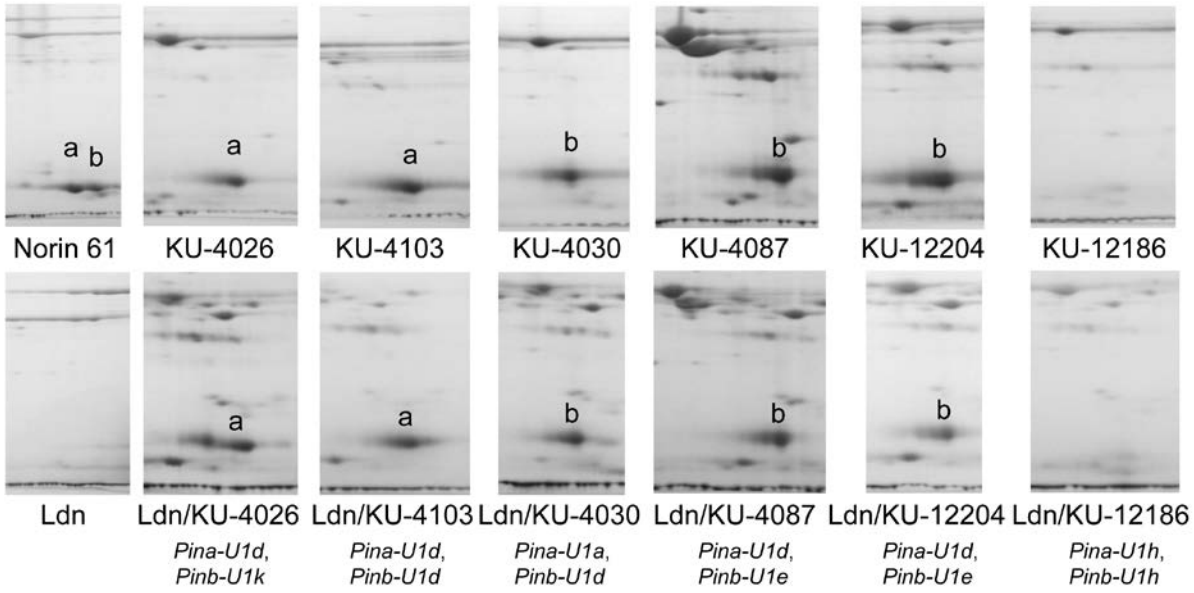
Fig. 6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Norin 61
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KU-4087
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Pina-U1d,
Pinb-U1k
Ldn/KU-4103
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Pinb-U1d
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Pinb-U1d
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Pinb-U1e
Ldn/KU-12204
Pina-U1d,
Pinb-U1e
Ldn/KU-12186
Pina-U1h,
Pinb-U1h

Supplemental Table 1. Grain-related traits measured using *SmartGrain* software

Sample	Area size (AS) (mm^2)	Perimeter length (PL) (mm)	Grain length (GL) (mm)	Grain width (GW) (mm)	Length- width ratio (LWR)	Circularity (CS)
Langdon	29.8 ± 4.8	26.6 ± 3.6	9.7 ± 1.2	4.2 ± 0.4	2.3 ± 0.2	0.5 ± 0.1
KU-4030	11.1 ± 2.4	14.4 ± 1.7	6.0 ± 0.7	2.5 ± 0.3	2.4 ± 0.2	0.7 ± 0.0
KU-4026	11.3 ± 2.3	14.4 ± 1.5	5.8 ± 0.6	2.6 ± 0.3	2.3 ± 1.0	0.7 ± 0.1
KU-4087	11.6 ± 2.4	15.0 ± 1.7	6.1 ± 0.7	2.5 ± 0.3	2.5 ± 0.2	0.6 ± 0.0
KU-4103	9.9 ± 1.6	14.6 ± 1.5	6.1 ± 0.7	2.1 ± 0.2	2.9 ± 0.3	0.6 ± 0.1
KU-12186	9.8 ± 1.7	14.3 ± 1.4	5.7 ± 0.6	2.3 ± 0.2	2.5 ± 0.2	0.6 ± 0.1
KU-12204	14.6 ± 2.7	17.1 ± 1.8	7.1 ± 0.8	2.6 ± 0.2	2.7 ± 0.2	0.6 ± 0.0
Ldn/KU-4030	22.3 ± 3.4	21.5 ± 1.7	9.0 ± 0.7	3.2 ± 0.3	2.8 ± 0.3	0.6 ± 0.0
Ldn/KU-4026	23.8 ± 5.7	21.8 ± 3.1	9.0 ± 1.3	3.4 ± 0.5	2.6 ± 0.3	0.6 ± 0.1
Ldn/KU-4087	20.2 ± 3.7	20.7 ± 2.5	8.5 ± 1.1	3.2 ± 0.4	2.7 ± 0.2	0.6 ± 0.0
Ldn/KU-4103	20.8 ± 3.6	21.1 ± 1.8	8.7 ± 0.9	3.1 ± 0.3	2.8 ± 0.2	0.6 ± 0.0
Ldn/KU-12186	21.0 ± 3.5	21.5 ± 1.9	8.6 ± 0.9	3.3 ± 0.3	2.7 ± 0.3	0.6 ± 0.1
Ldn/KU-12204	24.4 ± 3.4	22.7 ± 1.7	9.3 ± 0.7	3.4 ± 0.3	2.7 ± 0.2	0.6 ± 0.0
Ldn/KU-2076	18.9 ± 2.4	19.6 ± 1.2	8.0 ± 0.5	3.2 ± 0.3	2.5 ± 0.2	0.6 ± 0.0
Ldn/KU-2103	17.9 ± 2.4	19.7 ± 1.7	8.1 ± 0.7	2.9 ± 0.3	2.8 ± 0.2	0.6 ± 0.0
Ldn/KU-2109	16.5 ± 2.0	18.9 ± 1.1	7.9 ± 0.5	2.8 ± 0.3	2.9 ± 0.3	0.6 ± 0.1
Ldn/IG47202	16.5 ± 2.1	19.7 ± 1.0	8.3 ± 0.4	2.6 ± 0.3	3.2 ± 0.4	0.5 ± 0.0
Ldn/KU-2105	21.0 ± 2.3	20.9 ± 1.4	8.6 ± 0.6	3.4 ± 0.3	2.5 ± 0.3	0.6 ± 0.0

Data are represented as mean ± standard deviation.

Supplemental Table 2. Eigenvectors for the first, second and third PCs for the nine grain-related traits

Trait	PC1	PC2	PC3
Area size (AS)	-0.39	0.17	-0.16
Perimeter length (PL)	-0.40	0.08	-0.21
Grain length (GL)	-0.40	-0.02	-0.13
Grain width (GW)	-0.37	0.30	-0.02
Length-width ratio (LWR)	-0.07	-0.68	-0.25
Circularity (CS)	0.27	0.37	0.49
Grain hardness	0.16	0.52	-0.63
Grain weight	-0.38	0.01	0.34
Grain diameter	-0.38	0.06	0.31
Contribution rate (%)	67.4	20.7	8.6
Total contribution rate (%)	67.4	88.1	96.7