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Effect of the U genome on grain hardness in nascent synthetic hexaploids derived from interspecific hybrids between durum wheat and *Aegilops umbellulata*

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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

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

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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 −25 °C 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).