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**Genetic mapping of a novel recessive allele for non-glaucousness in wild diploid wheat
Aegilops tauschii: implications for the evolution of common wheat**

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Abstract

Cuticular wax on the aerial surface of plants has a protective function against many environmental stresses. The bluish-whitish appearance of wheat leaves and stems is called glaucousness. Most modern cultivars of polyploid wheat species exhibit the glaucous phenotype, while in a wild wheat progenitor, *Ae. tauschii*, both glaucous and non-glaucous accessions exist. *Iw2*, a wax inhibitor locus on the short arm of chromosome 2D, is the main contributor to this phenotypic variation in *Ae. tauschii*, and the glaucous/non-glaucous phenotype of *Ae. tauschii* is usually inherited by synthetic hexaploid wheat. However, a few synthetic lines show the glaucous phenotype although the parental *Ae. tauschii* accessions are non-glaucous. Molecular marker genotypes indicate that the exceptional non-glaucous *Ae. tauschii* accessions share the same genotype in the *Iw2* chromosomal region as glaucous accessions, suggesting that these accessions have a different causal locus for their phenotype. This locus was assigned to the long arm of chromosome 3D using an F₂ mapping population and designated *W4*, a novel glaucous locus in *Ae. tauschii*. The dominant *W4* allele confers glaucousness, consistent with phenotypic observation of *Ae. tauschii* accessions and the derived synthetic lines. These results implied that glaucous accessions of *Ae. tauschii* with the *W2W2iw2iw2W4W4* genotype could have been the D-genome donor of common wheat.

Keywords: allopolyploid speciation, cuticular wax, intraspecific variation, synthetic hexaploid, wheat

Introduction

Cuticular wax deposition on the surface of terrestrial plants functions as a barrier against biotic and abiotic stresses, such as drought conditions, pathogenic attack, and UV radiation (Shepherd and Griffiths 2006; Guo et al. 2016). Glauousness is a visible trait: a bluish-whitish appearance of leaf and stem, which are covered with wax crystals. In common wheat (*Triticum aestivum* L.), the glaucous phenotype is mainly controlled by two homoeologous loci for wax production, *W1* and *W2*, and two homoeologous loci for wax inhibition, *Iw1* and *Iw2*. *W1* and *Iw1* have been assigned to the short arm of chromosome 2B (2BS), and *W2* and *Iw2* to 2DS (Tsunewaki 1966; Tsunewaki and Ebana 1999; Wu et al. 2013). Wheat plants with the glaucous phenotype possess either or both of the *W* dominant alleles. The *Iw* loci are epistatic to the *W* loci, and either of these wax inhibitors is sufficient for non-glauousness (Tsunewaki and Ebana 1999; Zhang et al. 2013). The *W* loci control accumulation of the cuticular wax compounds, and the *w* recessive alleles resulting in the non-glauous phenotype lead to the absence of β -diketone and its derivatives on the wheat plant surface (Adamski et al. 2013; Zhang et al. 2013). Recent progress has shown that the *W1* locus constitutes a β -diketone biosynthetic gene cluster including *DMP* (*Diketone Metabolism-Polyketide Synthase*), *DMC* (*Diketone Metabolism-P450 CYP709J4*), and *DMH* (*Diketone Metabolism-Hydrolase/Carboxylesterase*) (Hen-Avivi et al. 2016), and that the *Iw1* dominant allele generates a long noncoding RNA possessing an inverted repeat sequence with high homology to *DMH* at the *W1* locus (Huang et al. 2017). The miRNA derived from the *Iw1* transcript, miRW1, represses *DMHs* in both *W1* and *W2* (Huang et al. 2017), which is consistent with the epistasis of the *Iw* loci to the *W* loci.

Besides these four main wax loci, genetic factors in cuticle biosynthesis and glaucousness

have been reported in common and tetraploid wheat. A wax production locus, *W3*, and a glume glaucousness inhibition locus, *Iw3*, were identified on 2BS in common wheat and on 1BS in wild emmer wheat, respectively (Dubcovsky et al. 1997; Wang et al. 2014; Zhang et al. 2015). In addition, a glaucous locus, *Ws*, was assigned to 1AS in durum wheat (Gadaleta et al. 2009). A major quantitative trait locus (QTL) for flag leaf glaucousness was found on chromosome 3A and contributes to increasing glaucousness in common wheat (Bennett et al. 2012). QTLs for flag leaf wax content were also detected on chromosomes 1B, 3D and 5A (Mason et al. 2010; Mondal et al. 2015). Additional QTLs with minor effects on the degree of glaucousness have been reported on many chromosomes of common wheat (Börner et al. 2002; Kulwal et al. 2003). Moreover, four transcription factor genes, *TaMYB31* and *TaMYB74*, orthologs of *Arabidopsis* *R2R3-MYB*, and *TaWXPL1D* and *TaWXPL2B* (*WXP-like*), orthologous to *Medicago truncatula* *WAX PRODUCTION* (*WXP*), play important roles in regulation of cuticle biosynthesis in common wheat (Bi et al. 2016, 2017). A fatty acyl-coenzyme A reductase, *FAR5*, is involved in biosynthesis of the leaf blade cuticular wax (Wang et al. 2015). Thus, many genes in addition to the major phenotypic marker loci function in regulation of the cuticular wax content on the wheat plant surface.

Most cultivated varieties of tetraploid wheat show the glaucous phenotype, although non-glaucous accessions are often found in wild tetraploid wheat (Tsunewaki and Ebana 1999; Simmonds et al. 2008). These observations suggest that the glaucous phenotype could have been artificially selected during tetraploid wheat domestication. Almost all varieties of common wheat are glaucous, carry the dominant alleles of *W1* and *W2* and lack the *Iw1* and *Iw2* genes, suggesting that the D-genome donor of common wheat, *Aegilops tauschii* Coss., had the recessive *iw2* allele (Tsunewaki 1966; Tsunewaki and Ebana 1999). Out of 210

accessions of the *Ae. tauschii* core collection, only 20, distributed from the Transcaucasus to the southerneastern coastal region of the Caspian Sea, show the glaucous phenotype (Nishijima et al. 2014). The *Iw2* locus mainly determines the *Ae. tauschii* intraspecific variation in glaucousness, and the glaucous accessions of *Ae. tauschii* have the *W2W2iw2iw2* genotype (Watanabe et al. 2005; Nishijima et al. 2014). Most of the non-glaucous accessions appear to possess the *W2W2Iw2Iw2* genotype, whereas two of the 210 *Ae. tauschii* accessions, KU-2104 and KU-2105, exceptionally show the non-glaucous phenotype despite their putative *iw2iw2* genotype (Nishijima et al. 2014). Therefore, the two non-glaucous accessions might carry a mutated allele of another glaucousness-determining locus outside of the *Iw2* locus. Of the 82 synthetic hexaploid lines, which were derived from crosses between the tetraploid wheat cultivar Langdon (Ldn), which has the glaucous *W1W1iw1iw1* genotype, and each of 82 *Ae. tauschii* accessions (Kajimura et al. 2011; Adamski et al. 2013), 80 show the same glaucous or non-glaucous phenotype as their parental *Ae. tauschii* accessions (Nishijima et al. 2014). However, two synthetic hexaploids, Ldn/KU-2104 and Ldn/KU-2105, exhibit the glaucous phenotype, suggesting that the non-glaucous phenotype of these two *Ae. tauschii* accessions might be caused by a defect in a cuticular wax biosynthesis-related gene, possibly a recessive mutation of *W2*. Here, our goal was genetic analysis of the non-glaucous phenotype in KU-2105. The aims of this study were (1) to elucidate the chromosomal position of the causal gene for the non-glaucous phenotype in the two exceptional *Ae. tauschii* accessions and (2) to discuss the possibility of the two non-glaucous accessions being the D-genome donor of common wheat.

Materials and methods

Plant materials and phenotype evaluation

In total, 23 accessions of *Aegilops tauschii* Coss. were used for genotyping with the *Iw2*-linked molecular markers. These accessions contained the three non-glaucous accessions KU-2154, KU-2104 and KU-2105 and 20 glaucous accessions (Table S1).

An F₂ mapping population with a population size of 200 was generated from a cross between two *Ae. tauschii* accessions, KU-2105 (a non-glaucous accession) and KU-2126 (a glaucous accession). Seeds of the F₂ population were sown in November 2015, and the F₂ plants were grown during the 2015-2016 season in an experimental field of Kobe University. The glaucous phenotype of each individual was visibly judged from the whitish surface of sheaths, peduncles, and spikes after the flowering stage.

Genotyping of the *Ae. tauschii* accessions with the *Iw2*-linked markers

Three *Iw2*-linked markers, *S51038-8*, *S10812-1*, and *S10812-13*, which had been developed in our previous study (Nishijima et al. 2014), were used for genotyping the 23 *Ae. tauschii* accessions. *S10812-1* and *S10812-13* were derived from the *Ae. tauschii* scaffold 10812 to which the *Iw2* locus was subsequently assigned (Jia et al. 2013; Huang et al. 2017). Table 1 shows the primer sequences, PCR annealing temperatures, and relevant restriction enzymes. PCR conditions for genotyping were as in our previous report (Nishijima et al. 2014), and the PCR products and their digests were resolved in 2% agarose or 13% non-denaturing polyacrylamide gels, stained with ethidium bromide, and visualized under UV light.

Linkage map construction

For single sequence repeat (SSR) genotyping of the parental accessions and F₂ individuals, total DNA was extracted from leaves of each plant, and SSR marker information was obtained from the GrainGenes website (<https://wheat.pw.usda.gov/GG2/index.shtml>). PCR conditions and electrophoresis of the PCR products were performed as described above. The MAPMAKER/EXP version 3.0 package was used for linkage map construction and genetic mapping of the glaucous locus with a threshold for log-of-odds (LOD) score of 3.0 (Lander et al. 1987).

Results and discussion

Between the two parental accessions of *Ae. tauschii*, a difference in glaucousness was clearly observed on the surface of sheaths, peduncles, and spikes (Fig. 1A). To assign the genetic locus determining the glaucous/non-glaucous trait in wheat, an F₂ mapping population with 200 individuals was generated from a cross between glaucous (KU-2126) and non-glaucous (KU-2105) accessions. Of the 200 F₂ individuals, 145 showed the glaucous phenotype and 55 were non-glaucous. Segregation significantly fit a 3:1 ratio ($\chi^2 = 0.67$, $P = 0.41$), consistent with Mendelian segregation of alleles of a single gene. This indicates that a single locus is related to the trait, and that the allele representing the glaucous phenotype was dominant. The recessiveness of the KU-2105-type allele supports our previous observation that a synthetic hexaploid line from an interspecific cross between Ldn (*W1W1iw1iw1*) and KU-2105 was glaucous (Nishijima et al. 2014), suggesting that KU-2105 carries the *iw2iw2* genotype and that the causal locus for the non-glaucous phenotype of KU-2105 should be distinct from the *Iw2* locus.

To confirm whether KU-2105 has the recessive allele at the *Iw2* locus, one non-glaucous accession (KU-2154), 20 glaucous accessions, KU-2104, and KU-2105 were genotyped with three *Iw2*-linked markers (Nishijima et al. 2014). KU-2104 and KU-2105 shared the same genotype with all the glaucous accessions at the *S51038-8* and *S10812-13* loci, while KU-2154 had a different genotype (Fig. 1B). At the *S10812-1* locus, the 20 glaucous accessions showed two alleles; 15 accessions revealed the same allele and the remaining five accessions had another allele. The two non-glaucous accessions, KU-2104 and KU-2105, exhibited the same genotype as the five glaucous accessions at this locus, whereas KU-2154, a non-glaucous accession with the *Iw2Iw2* genotype, possessed a different allele. These genotyping results indicated that KU-2104 and KU-2105 could possess the *iw2iw2* genotype. The D-genome donor of common wheat could have been *Ae. tauschii* accessions carrying the *iw2* recessive allele (Tsunewaki 1966; Tsunewaki and Ebana 1999; Nishijima et al. 2014). Thus, the existence of non-glaucous *Ae. tauschii* accessions with the *iw2iw2* genotype suggests that the *Ae. tauschii* accessions that brought the D genome to common wheat were not necessarily glaucous.

To examine the linkage of the causal locus of the non-glaucous phenotype of KU-2105 and W2, linkage maps were constructed in the KU-2105/KU-2126 population. In total, 61 markers constituted seven linkage groups, and the total map length was 837.5 cM with an average 13.7 cM interval between markers (Figs. 1B and S1). However, the non-glaucous locus of KU-2105 was not assigned to chromosome 2D, indicating that the causal locus was neither W2 nor *Iw2* on 2DS. The causal locus was assigned to the long arm of chromosome 3D (Fig. 2). To our knowledge, the 3DL gene is a novel locus controlling glaucousness on the plant surface in *Ae. tauschii*, and is hereby defined as W4. No gene related to the glaucousness

variation has been reported on 3DL in common wheat. Therefore, the non-glaucous allele of *W4* could not have been brought to common wheat varieties from *Ae. tauschii*. A QTL for flag leaf glaucousness was previously found on chromosome 3A as a major contributor to the glaucousness variation under southern Australian conditions in common wheat (Bennett et al. 2012), while the homoeologous relationship between the 3A QTL and *W4* is still unknown. Recent advances in studies of wheat wax production and the β -diketone biosynthetic pathway (Adamski et al. 2013; Zhang et al. 2013; Hen-Avivi et al. 2016) and in the DeNovoMAGIC assemblies of wheat genome sequences by NRGene (<https://wheat-urgi.versailles.inra.fr>) will help further identification of candidate genes for the *W4* locus.

Two non-glaucous cultivars carrying the recessive *w2* allele have been observed in common wheat (Tsunewaki 1966). The common wheat cultivar Chinese Spring, with the *W1W1w2w2* genotype, exhibits a weak glaucous phenotype, and another cultivar, Salmon, is non-glaucous and possesses the *w1w1w2w2* genotype (Tsunewaki 1966). However, no loss-of-function mutations of the *W2* locus have been found in *Ae. tauschii*. Allelic differences at the *Iw2* locus can explain most of the glaucous/non-glaucous variation in *Ae. tauschii* (Nishijima et al. 2014), and the exceptional non-glaucous accessions of *Ae. tauschii* carry the *w4* recessive allele on 3DL as mentioned above. These observations suggest that the loss-of-function *w2* mutation occurred on 2DS after the speciation of common wheat. In contrast to *w2*, the recessive *w1* allele appears in wild emmer wheat accessions showing the non-glaucous phenotype (Tsunewaki 1966; Hen-Avivi et al. 2016). A non-glaucous accession of wild emmer wheat, TTD140, lacks the cluster region of β -diketone metabolic genes at the *W1* locus (Hen-Avivi et al. 2016). Functional conservation of the *W2* locus among *Ae. tauschii* accessions should be confirmed at the nucleotide sequence level in future study.

In conclusion, the present study showed that *W4* on 3DL, a novel locus for wax production on the wheat plant surface, partly contributes to the glaucous/non-glaucous variation in the natural population of *Ae. tauschii*. The two exceptional non-glaucous accessions, KU-2104 and KU-2105, were assumed to possess the *W2W2iw2iw2w4w4* genotype. The lack of *Ae. tauschii* accessions with either the *w2w2iw2iw2* or *w2w2Iw2Iw2* genotype obtained from habitats covering the entire species range suggests that the recessive *w2* allele observed in common wheat was not introduced through the allohexaploid speciation event. Therefore, glaucous accessions of *Ae. tauschii* with the *W2W2iw2iw2W4W4* genotype could have been the D-genome donor of common wheat. Glaucous accessions have been collected from Armenia to the southwestern coastal part of the Caspian Sea (Table S1). As mentioned in our previous report (Nishijima et al. 2014), *Ae. tauschii* subspecies *strangulata* (Eig) Tzvel, postulated to be the D-genome donor of common wheat (Jaaska 1981; Dudnikov 2017), was not included in the glaucous accessions (Table S1). Recent population structure studies showed that *Ae. tauschii* accessions could have diverged into three major lineages, TauL1, TauL2 and TauL3, and that two sublineages could be recognized in both TauL1 and TauL2 (Matsuoka et al. 2015). Out of the 20 accessions with *W2W2iw2iw2W4W4*, 19 (the exception being IG127015) belonged to TauL2; 14 grouped to TauL2a and 5 to TauL2b (Table S1). This observation is consistent with the assumption that the *Ae. tauschii* accessions associated with the birth of common wheat belong to TauL2 (Wang et al. 2013; Matsuoka et al. 2015). The subspecies *strangulata* accessions, defined by markedly moniliform spikes, are distributed only in a TauL2b and TauL2 admixture, and only one QTL region determining the distinct spikelet and grain morphology of the two *Ae. tauschii* subspecies, *tauschii* and *strangulata*, was mainly associated with the spikelet morphological divergence in TauL2

(Nishijima et al. 2017). Therefore, *Ae. tauschii* accessions that had the W2W2iw2iw2W4W4 genotype and were genetically close to subspecies *strangulata* may have been a direct progenitor of common wheat.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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shed light on the origins of wheat D-genome genetic diversity and pinpoint the geographic origin of hexaploid wheat. *New Phytol* 198:925–937

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Figure legends

Figure 1. An exceptional non-glaucous accession of *Ae. tauschii*. (A) Phenotypic difference between the parental accessions, KU-2105 (non-glaucous) and KU-2126 (glaucous). (B) PCR analyses of three *Iw2*-linked markers in 23 *Ae. tauschii* accessions; from left to right, KU-2154, KU-2126, the other 19 glaucous accessions, KU-2104, and KU-2105. An amplified PCR fragment of *S51038-8* and a digested (lower) PCR fragment of *S10812-13* were obtained only in KU-2154. At the *S51038-8* locus, a single band was detected only in KU-2154. At the *S10812-13* locus, the size of the PCR fragment in KU-2154 was smaller than in the others. At the *S10812-1* locus, the 23 accessions exhibited three alleles.

Figure 2. Linkage map of chromosome 3D in the KU-2105/KU-2126 F₂ population. The putative position of the centromere is indicated by the arrowhead.

Table 1. *Iw2*-linked markers used in this study

Marker name	Primer sequence (5'- to -3')	Marker type	Restriction enzyme	Annealing temp. (°C)
<i>S51038-8</i>	TGGCAGTGAAACCTCCCAA AAACATCACCACATTTCTCTAGTTG	Dominant	—	54
<i>S10812-1</i>	TACAACCTTGGGCCAGTCAC CGTCGTCTTCCCTACAAATCG	SSR	—	58
<i>S1-812-13</i>	CTCTTCAAGCCCCGATCCG ACGGAGAAGTGTTATCCAGTCC	CAPS	<i>HhaI</i>	58

Fig. 1

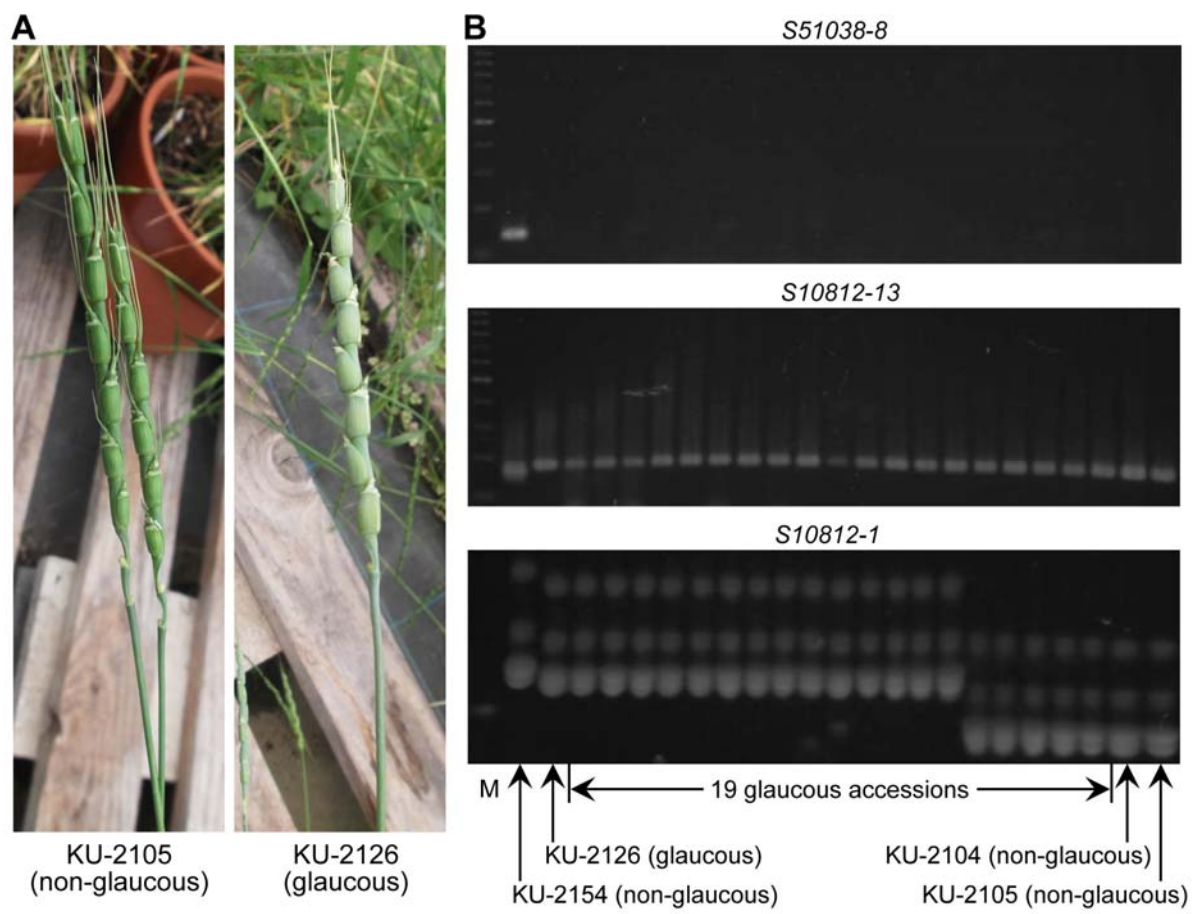
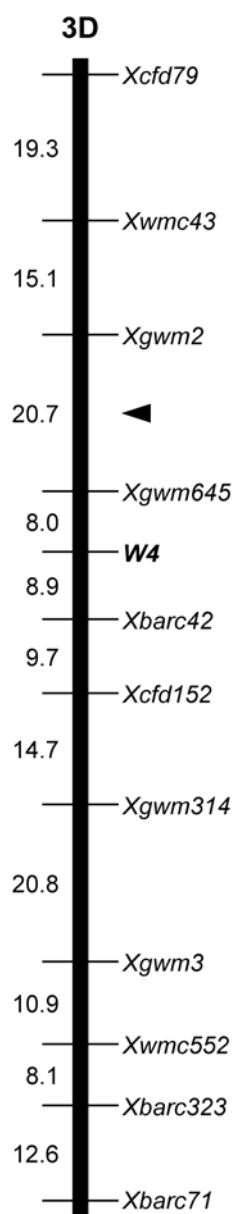


Fig. 2



Supplementary Fig. 1

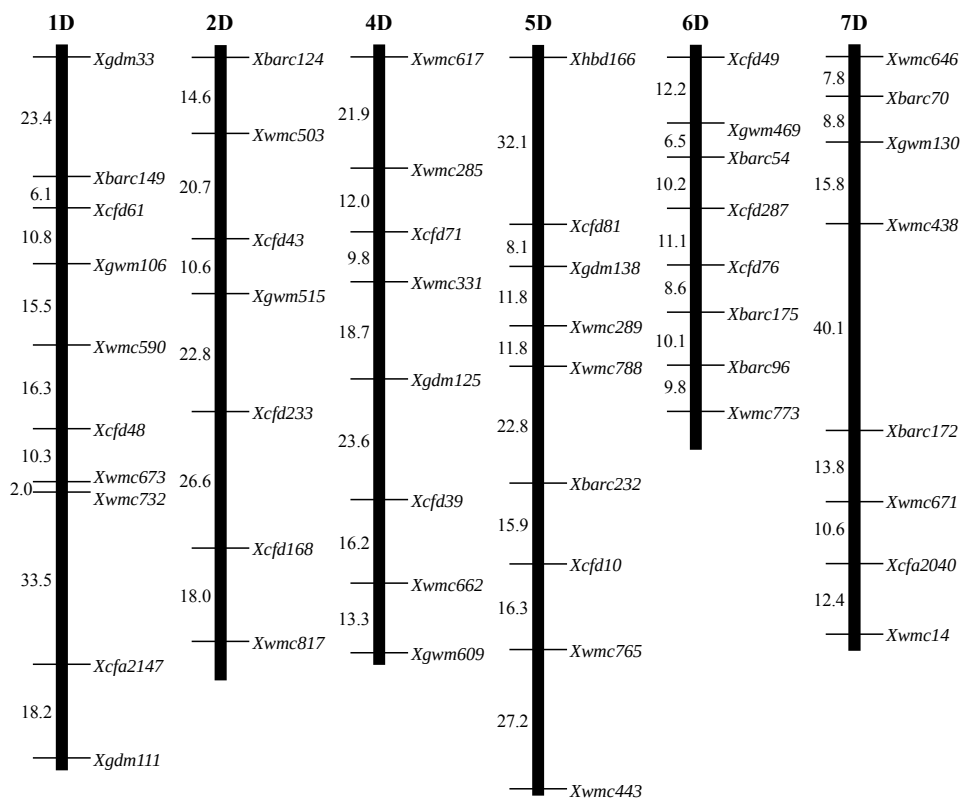


Figure S1. Linkage maps of six of the seven chromosomes, with the exception of chromosome 3D, in the KU-2015/KU-2126 mapping population of *Ae. tauschii*.

Supplementary Table 1

Table S1. Passport data of 20 *Ae. tauschii* accessions that have the W2W2iw2iw2W4W4 genotype and exhibit the glaucous phenotype

Accession	Country	Lower taxon	Locality	Latitude ^a	Longitude ^a	Sublineage ^b
KU-2811	Armenia	var. typica	Cytoanka Park, Erevan	40.245	44.621	TauL2a
KU-20-8	Iran	var. typica	25 km WSW of Firuzkuh	35.874	52.654	TauL2a
KU-2106	Iran	var. typica	14 km NW of Pahlavi	37.674	49.4	TauL2b
KU-2118	Iran	var. typica	Khoy	38.622	45.103	TauL2a
KU-2124	Iran	var. typica	Techalousse (near Chalus)	36.717	51.46	TauL2a
KU-2126	Iran	var. typica	Techalousse (near Chalus)	36.717	51.46	TauL2a
KU-2156	Iran	var. typica	7.3 km SW from Avej to Hamadan	35.633	49.396	TauL2a
KU-2158	Iran	var. meyeri	Ramsar	36.954	50.745	TauL2b
KU-2159	Iran	var. typica	Ramsar	36.954	50.745	TauL2b
KU-2160	Iran	var. meyeri	Ramsar	36.954	50.745	TauL2b
KU-2155	Iran	var. typica	7.3 km SW from Avej to Hamadan	35.633	49.396	TauL2a
PI 486267	Turkey	-	6km south of Semdinli	37.2	44.617	TauL2a
KU-2069	Iran	var. typica	2 km N of Karaj (Suburbs of Tehran)	35.848	51.037	TauL2b
KU-2111	Iran	var. typica	Ardabil	38.347	48.421	TauL2a
IG 127015	Armenia	-	Megri distr. Zichk	39.078	46.306	TauL1b
AE 1037	Georgia	-	20 km NW Citeli-Ckaro, Rayon	41.714	44.354	TauL2a
KU-2827	Georgia	var. typica	Suburbs of Tibilisi (Botanical Garden)	41.759	44.852	TauL2a
KU-20-7	Iran	var. typica	2 km N of Karaj (Suburbs of Tehran)	35.848	51.037	TauL2a
KU-2086	Iran	var. typica	11 km WSW of Firuzkuh	35.916	52.77	TauL2a
KU-2112	Iran	var. typica	19 km SW of Ardabil (Ardabil - Surab)	38.261	48.289	TauL2a

^aThe latitude and longitude values were estimated in our previous reports (Matsuoka et al. 2008; Takumi et al. 2009).

^bMatsuoka et al. (2015)