Comparative High-Resolution Mapping of the Wax Inhibitors $Iw_1$ and $Iw_2$ in Hexaploid Wheat

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Abstract

The wax (glaucousness) on wheat leaves and stems is mainly controlled by two sets of genes: glaucousness loci ($W_1$ and $W_2$) and non-glaucousness loci ($Iw_1$ and $Iw_2$). The non-glaucousness ($Iw$) loci act as inhibitors of the glaucousness loci ($W$). High-resolution comparative genetic linkage maps of the wax inhibitors $Iw_1$ originating from Triticum dicoccoides, and $Iw_2$ from Aegilops tauschii were developed by comparative genomics analyses of Brachypodium, sorghum and rice genomic sequences corresponding to the syntenic regions of the $Iw$ loci in wheat. Eleven $Iw_1$ and eight $Iw_2$ linked EST markers were developed and mapped to linkage maps on the distal regions of chromosomes 2BS and 2DS, respectively. The $Iw_1$ locus was located within a 0.96 cM interval flanked by the BE498358 and CA499581 EST markers that are collinear with 122 kb, 202 kb, and 466 kb genomic regions in the Brachypodium 55 chromosome, the sorghum 6S chromosome and the rice 4S chromosome, respectively. The $Iw_2$ locus was located in a 4.1 to 5.4-cM interval in chromosome 2DS that is flanked by the CJ886319 and CJ519831 EST markers, and this region is collinear with a 2.3 cM region spanning the $Iw_1$ locus on chromosome 2BS. Both $Iw_1$ and $Iw_2$ co-segregated with the BF474014 and CJ876545 EST markers, indicating they are most likely orthologs on 2BS and 2DS. These high-resolution maps can serve as a framework for chromosome landing, physical mapping and map-based cloning of the wax inhibitors in wheat.

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Introduction

The outermost wax layer protects plants from many types of biotic and abiotic stresses, such as drought, phytophagous insects, pathogens, solar radiation, and freezing temperatures [1,2]. One of the most important roles of the cuticle is to limit transpiration to reduce water loss and this provides a key mechanism for plant survival in water-limited environments, such as deserts, high mountains, saline-alkali lands, and coastal ecosystems [3,4]. Worldwide, bread wheat (Triticum aestivum L.) is one of the most important food sources for human beings. The wheat leaf, stem and, in some cases, spike surfaces are coated with cuticular waxes that confer a glaucousness characteristic [3,6]. Physiological studies in wheat by Johnson et al. [7] and Richards et al. [8] showed that glaucousness reduces transpiration and increased water use efficiency. More recently Zhang et al. demonstrated that glaucousness reduced cuticle permeability in the terms of non-stomatal water loss and chlorophyll efflux [9]. Bread wheat cultivars with non-glaucousness traits exhibit significant yield increases with reduced solar radiation losses that enable continued photosynthesis during the grain filling period [10], and the trait may also provide resistance to aphids [11].

Glaucousness and non-glaucousness are parallel variations in wheat and its relatives. Classical genetic studies have shown that both the glaucousness and the non-glaucousness stem and leaf phenotypes are controlled by two sets of loci; the wax production genes $W_1$ and $W_2$ and the wax inhibitor genes $Iw_1$ and $Iw_2$, respectively. The $Iw_1$ and $Iw_2$ non-glaucousness loci function as inhibitors of the $W_1$ and $W_2$ glaucousness loci, and could also inhibit other wax production genes in the wax pathway [3,12,13]. Genetic analyses have indicated that the $W_1$ wax production gene and the $Iw_1$ wax inhibition gene are located on chromosome 2BS with a genetic distance of 2 cM [12]. However, $W_2$ and $Iw_2$ are separated on chromosome 2DS where the $Iw_2$ locus is close to the centromere [12–15]. Two loci, $Iw_3$ and $W_5$, were also reported conditioning wax on spikes in wheat. Non-glaucousness locus $Iw_3$ was mapped on chromosome 1BS [16] and the $W_5$ gene on the short arm of chromosome 1AS is responsible for glaucous spikes [17]. In addition to these genes, a major QTL ($QW.aww-3A$) that accounts for up to 32 percent of the flag leaf glaucousness variation has been detected in a doubled-haploid (DH) population [18].

Molecular mapping and cloning of genes controlling epicuticular wax in wheat is of great interests for understanding interactions between non-glaucousness genes ($Iw$) and glaucousness genes ($W$), as well as their effects on yield, and biotic and...
abiotic stresses. The *Iw1* locus originating in wild emmer is closely linked to the *Xdo456 RFLP* marker at the end of chromosome arm 2BS [19]. Liu et al. found that the *Iw1* locus is 18.77 cM away from the powdery mildew resistance gene *MIM1701* on chromosome 2BS [20]. Simmonds et al. also reported that the *Iw1* (*Vir*) gene conditioning a non-glaucousness phenotype maps to chromosome 2BS [10]. In a tetraploid wheat background, Yoshiya et al., have found that *W1* is linked to *Iw1* [21], but the relationship between *Iw1* and *Iw1* was not confirmed [21], and in an *A. tauschii* F2 segregating population, the non-glaucous locus *Iw2* was located on chromosome 2DS [22]. In another report, the dominant non-glaucous locus *Iw3672* (*Iw2*) derived from a synthetic hexaploid wheat also mapped on 2DS by simple sequence repeat (SSR) and expressed sequence tag (EST) markers [23]. During development of a wheat genetic linkage map with a doubled haploid (DH) population derived from the TA4152–60 [23]. The newly developed F1 population consisting of 1161 recombinant inbred lines (RILs) was used in crosses with Xuezao, a glaucousness common wheat line, and ND495, a glaucousness common wheat line and ND495, a glaucousness common wheat line, as well as glaucous and non-glaucous bulks, were genotyped on each F2 plant, F3 family, RILs, and DH lines in field trials with adult plants. Chromosomal assignment and bin mapping of markers linked to the wax inhibition genes *Iw1* and *Iw2* were carried out with Chinese Spring (CS) and homoeologous group 2 nullisomic-tetrasomics [33], ditelosomics [34] and deletion lines [35].

**PCR and product analysis**

Total genomic DNA was isolated from leaves by use of a cetyl trimethylammonium bromide (CTAB) protocol [36]. Non-glaucous and glaucous bulks, assembled with equal amounts of DNA from 10 homoygous non-glaucous and 10 homoygous glaucous F2 plants, were used for bulked segregant analysis (BSA) [37]. SSR and EST markers located on the short arms of homoeologous group 2 chromosomes were chosen for polymorphism screening [38,39]. http://wheat.pw.usda.gov/cgi-bin/graingenes/browse.cgi?class=marker. Primers for EST markers were designed from EST sequences derived from the public NCBI EST database. The primer-designed criteria included a Tm of 50–65°C with no greater than a 3°C difference between primer pairs. Primer sequences and information about the bin-mapped EST markers are available at the GrainGenes database (http://wheat.pw.usda.gov). Polymorphic markers between the parental wheat lines, as well as glaucous and non-glaucous bulks, were genotyped on each F2 plant, RILs, and DH lines.

Polymere chain reaction (PCR) was performed in 10 μl reactions containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTPs, 25 ng of each primer, 50 ng of genomic DNA, and 0.75 U of *Pfu* DNA polymerase. Amplification of DNA was conducted at 94°C for 5 min, followed by 40 cycles at 94°C for 45 s, 50–60°C (depending on specific primers) for 45 s, and 72°C for 90 s, and reactions were terminated after a final extension at 72°C for 10 min. PCR products were mixed with 2 μl of loading buffer (98% formamide, 10 mM EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol), separated on 8% non-denaturing polyacrylamide gels (39 acrylamide : 1 bisacrylamide), and visualized following silver staining.

**Comparative genomics analysis and EST marker development**

Polymorphic bin-mapped EST markers flanking the non-glaucousness loci *Iw1* and *Iw2* were used in BLAST searches of the *Brachypodium*, sorghum, and rice genome sequences to find orthologous genomic regions. Orthologous gene pairs in the corresponding genomic regions between the three species were located and used to search homologous wheat ESTs (http:// blast.ncbi.nih.gov/Blast.cgi) that were used to design PCR primers using *Primer5.0* (http://www.genome.wi.mit.edu/ftp/pub/ software/primer5.0). Polymorphic EST markers between non-glaucous and glaucous parental lines, as well as the bulk segregants, were used for genotyping three mapping populations to construct high-resolution genetic linkage maps.

**Data analysis and genetic linkage map construction**

Chi-squared (χ²) tests for goodness-of-fit were performed to estimate deviations of observed data from theoretically expected segregation ratios. Linkages between molecular markers and the wax inhibition loci were analyzed using Mapmaker 3.0 with a LOD score threshold of 3.0 [40]. The genetic linkage map was drawn with the software Mapdraw V2.1 [41].
Results

Genetic analyses of wax inhibitors Iw1 and Iw2 in hexaploid wheat

At the adult plant stage, leaves and stems of the common wheat line, WE74, and the TA4152–60 and W7984 synthetic hexaploid wheat lines were non-glaucous, whereas the common wheat lines, Xuezao, ND495, and Opata M85, were glaucous. F1 plants from the Xuezao/WE74, ND495/TA4152–60, and W7984/Opata M85 crosses were non-glaucous suggesting that the wax inhibitor genes in WE74, TA4152–60 and W7984 are dominant. The F2 M85 crosses were non-glaucous, suggesting that the wax inhibitor gene. Since non-glaucousness originates from wild emmer in WE74 and synthetic wheat lines in TA4152–60 and W7984, the non-glaucousness loci should be designated Iw1.

Identification of SSR markers linked to Iw1

Because the Iw1 and Iw2 non-glaucousness genes are located on chromosomes 2BS and 2DS, respectively [10,12,19–24], SSR markers assigned to 2BS and 2DS were used preferentially for BSA. Linkage of the Xbarc297 and Xc4925 polymorphic SSR markers to Iw1 on chromosome 2BS was confirmed after genotyping the F2 segregating populations of the parental lines Xuezao and WE74, as well as the non-glaucous and glaucous DNA pools of Xuezao/WE74 (Fig. 1b). Two SSR markers Xgwm614 and Xgwm210, previously linked to Iw1 on chromosome 2BS [10], were not polymorphic between Xuezao and WE74, or the non-glaucous and glaucous DNA pools, and therefore could not be used for Iw1 mapping.

Physical bin map of Iw1 and the linked SSR and EST markers

A set of Chinese Spring homoeologous group 2 nullisomic-tetrasomics, ditelosomics, and deletion lines [33–35] were employed for physical bin mapping of Iw1. The Xc4925 and Xbarc297 SSR markers were located on chromosome 2BS bin 0.84–1.00 (Fig. 1a), indicating that the non-glaucousness locus Iw1 was mapped on the distal part of 2BS.

Comparative analysis of the Iw1 genomic region

The BE498358 EST sequence was used as a query to perform a Blast search against the Brachypodium, sorghum, and rice genome sequences. This search revealed that Bradi5g01130, Sb06g01410, and Os04g0136700 are orthologs of BE498358 located on the 5S, 6S, and 4S chromosomes of Brachypodium, sorghum, and rice respectively. Putative genes flanking Bradi5g01130, Sb06g01410, and Os04g0136700 were annotated and compared to identify orthologous gene pairs between the three species. The results indicate that a 462 kb genomic region in the Brachypodium chromosome 5S from Bradi5g01020 to Bradi5g01430 is syntenic to a 3.9 Mb region from Sb06g001110 to Sb06g002790 on sorghum 6S and a 5.6 Mb region from Os04g0118900 to Os04g0101500 on rice 4S (Table 2; Fig. 1). Brachypodium genes in the syntenic genomic region were then used to find homologous wheat ESTs to design primers and for polymorphism screening of the parental lines Xuezao and WE74, and the non-glaucous and glaucous DNA pools. Ten polymorphic EST markers were developed and used to genotype F2 individuals and to construct a high-resolution genetic linkage map of Iw1 (Table 3; Fig. 1b).

Table 1. Genetic analysis of wax inhibitors Iw1 and Iw2 in hexaploid wheat.

| Mapping population | Non-glaucousness | Glaucousness | Total | \( \chi^2 \) | \( \chi^2_{0.05} \) |
|--------------------|-----------------|--------------|-------|------------|----------------|
| WE74 (Iw1)         | 10              | 0            | 10    | 0.00       | 3.84           |
| Xuezao             | 10              | 0            | 10    | 0.00       | 3.84           |
| Xuezao/WE74        | 10              | 0            | 10    | 0.00       | 3.84           |
| Xuezao/WE74 F2     | 3730            | 1219         | 4949  | 0.36       | 3.84           |
| Xuezao/WE74 F3     | 1269(A)+2461(H) | 1219(B)      | 4949  | 1.16       | 5.99           |
| W7984 (Iw2)        | 27              | 0            | 27    | 0.00       | 3.84           |
| Opata M85          | 20              | 0            | 20    | 0.00       | 3.84           |
| W7984/Opata M85 F1 | 26              | 0            | 26    | 0.00       | 3.84           |
| W7984/Opata M85 RILs | 549            | 612          | 1161  | 3.41       | 3.84           |

A, H, B represent homozygous non-glaucousness, heterozygous and homozygous glaucousness, respectively.

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Bradi5g01220 in Brachypodium, a 202 kb genomic region (Sb06g001290 to Sb06g001410) with 13 predicted genes in sorghum, and a 466 kb genomic region in rice (Os04g0132300 to Os04g0137100) that has 12 predicted genes (Table 2; Fig. 1).

Comparative genetic mapping of Iw1 and Iw2

EST markers linked to Iw1 were used to genotype the 1161 ITMI RILs and 120 DH lines of ND495/TA4152–60 to develop a high-resolution genetic linkage map of Iw2. Eight EST markers linked to Iw1 were polymorphic between ND495 and TA4152–60, as well as W7984 and Opata M85, and were used to construct a linkage map of Iw2 on wheat chromosome 2DS (Fig. 1). The Iw2 locus co-segregated with EST markers BF474014 and CJ876545, and is flanked by the ESTs CJ886319 and CJ519831 within a 4.1 cM sequence in the ITMI RIL population (Fig. 1f) and a 5.4 cM interval in the ND495/TA4152–60 DH population (Fig. 1g) on wheat chromosome 2DS which is collinear with a 2.3 cM genomic region spanning the Iw1 locus on 2BS (Fig. 1).

Discussion

The aerial surfaces of most plants are coated by epicuticular waxes whose chemical and physical properties have important roles in interactions between plants and the environment. In wheat and its relatives, almost all species have parallel variations of glaucousness and non-glaucousness except for Einkorn (A genome), which is non-glaucousness [6,12,14]. Genetic and cytological studies indicate that glaucousness is mainly controlled by two dominant genes, W1 and W2, that are located on the distal of 2BS and proximal of 2DS, respectively; and are thought to be homologous [12,13]. However, the glaucousness phenotype (controlled by W1 and W2) is inhibited by the non-glaucousness Iw1 and Iw2 loci located on 2BS and 2DS, respectively [12,14,15]. These results indicate that the glaucousness locus (W) itself, and interactions between the non-glaucousness (Iw) and glaucousness (W) loci are responsible for wax phenotypes in different wheat tissues.

Cloning of wheat genes responsible for glaucousness and non-glaucousness will provide useful information about molecular
interactions between the W and Iw loci, and the mechanisms whereby the waxy phenotypes are regulated. Our development of a high-resolution genetic linkage map is a first step towards fine mapping and map-based cloning of the glaucousness and non-glaucousness loci. However, additional refinements to the linkage maps are necessary before we can clone the respective genes and understand their relationships.

Comparative genomics analyses have been applied widely to develop high-resolution genetic linkage maps of interesting genes in wheat [25,42,43]. Macro-colinearity has been observed between wheat homoeologous group 2 chromosomes and Brachypodium chromosome 5, rice chromosome 4, and sorghum chromosome 6 [25,44–46]. Several studies have also revealed high levels of micro-colinearity in particular genomic regions between wheat, Ae. tauscii, Brachypodium, and rice [20,47–51] even through their synteny is often interrupted by inversions, deletions, duplications, and rearrangements [44,46,47,49].

In this study, we have found that a 3.2 cM genomics region spanning the Iw1 locus in wheat chromosome 2BS was highly syntenic to a 462 kb genomic region on Brachypodium chromosome 5S, a 3.9 Mb region on sorghum 6S, and a 5.6 Mb region on rice chromosome 4S (Fig. 1). However, gene duplications, insertions,

Table 2. Colinearity between Brachypodium, sorghum and rice in the syntenic genomic region of wheat wax inhibitors Iw1 and Iw2.

| Wheat EST | Brachypodium | Sorghum | Rice | Annotation |
|-----------|--------------|---------|------|------------|
| CJ519831  | Bradi5g01430 | Sb06g001110 | Oo04g0118900 | Hypothetical protein |
| CJ949174  | Bradi5g01280 | Sb06g001240 | Oo04g0129500 | Sec42-like transport protein |
| Bradi5g01270 | Sb06g001250 | Oo04g0129600 | Sec23/Sec24 zinc finger domain containing protein |
| Bradi5g01260 | Tyrosine specific protein phosphatase-like |
| Bradi5g01250 | Tyrosine specific protein phosphatase-like |
| Bradi5g01240 | Hypothetical protein transferase family protein |
| CJ777783  | Bradi5g01230 | Sb06g001270 | Oo04g0131900 | UDP-glucose:sterol glucosyltransferase |
| CA499581  | Bradi5g01220 | Sb06g001290 | Oo04g0132300 | AAR2 family protein |
| Bradi5g01210 | Sb06g001310 | Oo04g0132500 | LRR receptor-like serine/threonine-protein |
| Bradi5g01200 | N-acetylanalolamime amidohydrolase |
| Bradi5g01190 | Unknown protein/serine-type peptidase |
| BF474014  | Bradi5g01180 | Sb06g001350 | Oo04g0136700 | CBS domain containing protein |
| CJ876545  | Bradi5g01160 | Oo04g0136900 | Conserved hypothetical protein |
| Bradi5g01150 | LIM, zinc-binding; Ubiquitin interacting motif; DA1-large seed size |
| CD927782  | Oo04g0137100 | Pectate lyase 15-like |
| BE498358  | Bradi5g01130 | Sb06g001410 | Oo04g0137400 | Hypothetical protein |
| Bradi5g01110 | NB-ARC domain |
| Bradi5g01100 | NB-ARC domain |
| Bradi5g01090 | NB-ARC domain |
| Bradi5g01080 | NB-ARC domain |
| Bradi5g01070 | NB-ARC domain |
| Bradi5g01060 | Sb06g001460 | Oo04g0147200 | Hypothetical protein |
| BQ841470  | Bradi5g01050 | Sb06g001460 | Oo04g0150300 | Hypothetical protein |
| Bradi5g01040 | Hypothetical protein |
| Bradi5g01030 | Sb06g001490 | Oo04g0154800 | Hypothetical protein |
| Cj886319  | Bradi5g01020 | Sb06g002790 | Oo04g0185500 | Zinc finger family protein |
| CD882067  | Bradi5g01010 | Oo04g0185100 | CDT1-like protein a, chloroplastic-like |

Table 3. EST markers of the wax inhibitors mapped in the Iw1 and Iw2 genomic regions.

| Wheat EST | Forward sequence (5'–3') | Reverse sequence (5'–3') | Annotation |
|-----------|--------------------------|--------------------------|------------|
| CJ519831  | ATACCAAGCCTATCTAGACACTG | AAGGCATACTCAACAGAAATCA | |
| CJ949174  | TGCTTGGGAATCTGTTAGATGC | CTAACAATCTTGAGACCTTT | |
| CJ777783  | GCACGCCAAAGCTGCGACA | CAGCTGCTTACCTGCGAGGA | |
| CA499581  | GCACTGCTCTGCTCCTC | GCACCTGCTTATGCTCCCT | |
| BF474014  | CCAGTACCTCGAGTCCCAGA | CGAAAGGGGCTTGACCTT | |
| CJ876545  | CCCCAACAAGGGGCGTCGATCT | | |
| CD927782  | TACCCGCACTCCACCCACACA | CACGTGACCACGAGAGACATC | |
| BE498358  | CAACATTCTACCCCCACACA | CAGCCTGACCTGAGACG | |
| BQ841470  | TGGTTACCTCTGATAGAGA | GGAATCCTCATTGCAGAGA | |
| CJ886319  | CACACCTGCTTCTTCTTCT | TGGGACAAACTCTAGGAGA | |
| CD882067  | CACGCAAGACGCTATCATCAT | TGGGACAACCATCTGACGACA | |
| CA695634  | GAAACATTCCTAGTATGAGA | GGAATCCTCATTGCAGAGA | |

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In this study, we have found that a 3.2 cM genomics region spanning the Iw1 locus in wheat chromosome 2BS was highly syntenic to a 462 kb genomic region on Brachypodium chromosome 5S, a 3.9 Mb region on sorghum 6S, and a 5.6 Mb region on rice chromosome 4S (Fig. 1). However, gene duplications, insertions,
and deletions were also observed in the syntenic genomic regions between wheat, *Brachypodium*, rice and sorghum. The *Iw1* co-segregating EST marker CJ076545 (orthologous to Bradi5g01160) is not found in the syntenic genomics regions of rice and sorghum, indicating that the *Brachypodium* gene order can serve as a better model than rice and sorghum for developing closely linked markers in wheat. However, another *Iw1* co-segregating EST marker, CD927782 (orthologous to Oe4g0136900), was not located in the syntenic genomics region of *Brachypodium* and sorghum, implying that the rice and sorghum genes can provide alternative information for marker development of some wheat genes.

The bread wheat genome consists of three subgenomes (A, B, and D) that diverged from a common ancestor about 2.5–4.5 MYA [52,53]. The three subgenomes are still very closely related after hundreds of thousands of years of independent evolution and genetic linkage maps and comparative analyses over the past 20 years have revealed substantial conservation of orthologs among the A, B, and D subgenomes [38,39]. Conventional genetic analyses have also suggested that the *W1* and *W2* glaucousness loci are duplicated genes and that the *Iw1* and *Iw2* non-glaucousness loci are also duplicated [12,13]. Additional molecular mapping experiments have revealed that both *Iw1* and *Iw2* are located on the distal part of chromosomes 2BS and 2DS, suggesting that they also may be orthologs. Low polymorphisms are observed on chromosome 2DS compared to chromosome 2BS, and of 11 EST derived markers mapping in the *Iw1* genomic region, only 7 are located on the *Iw2* genetic linkage map (Fig. 1). An F2 mapping population containing 4949 plants was used to narrow *Iw1* to a 0.96 cM genomic region flanked by the CA499581 and BE498358 markers in wheat. However, another *Iw1* co-segregating EST marker, CD927782 (orthologous to Oe4g0136900), was not located in the syntenic genomics region of *Brachypodium* and sorghum, implying that the rice and sorghum genes can provide alternative information for marker development of some wheat genes.

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**Author Contributions**

Conceived and designed the experiments: ZL QS HP. Performed the experiments: HW JQ JH XZ. Analyzed the data: HW JQ JH ZW JX. Contributed reagents/materials/analysis tools: HW JQ JH XZ SO YL DZ ZW. Analyzed the data: HW JQ JH ZW JX. Wrote the paper: HW JH ZL.

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