Euphytica (2007) 155:71–78
DOI 10.1007/s10681-006-9302-5

# Molecular mapping of a dominant non-glaucousness gene from synthetic hexaploid wheat (*Triticum aestivum* L.)

## Molecular mapping of non-glaucousness gene in wheat

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Received: 12 August 2006 / Accepted: 8 November 2006 / Published online: 10 December 2006


**Abstract** The leaf and stem surfaces of many land plants are covered with a cuticular wax layer that confers a glaucous appearance or white bloom. Synthetic hexaploid wheat Line 3672 was non-glaucous, and common wheat Line 9753 was glaucous. The cuticular wax was characterized using Scanning Electron Microscopy (SEM). A hybrid using 9753 as female parent and 3672 as male parent was made and 108 $F_2$ plants and their $F_3$ progenies were used to map the non-glaucousness gene. Non-glaucousness in Line 3672 was controlled by a single dominant gene, temporarily designated *Iw3672*. Five SSR markers mapped on chromosome 2DS were linked to *Iw3672*. Additionally, two EST-derived markers and a SNP marker were developed and were also linked to *Iw3672*. The order of the eight markers and *Iw3672* was $Xte6_{730}$ /$Xbarc124_{520}$ —*Iw3672* —$Xwe6_{2100/2150}$ —$Xcau96_{287}$ —$Xcfd51_{180/200/230}$ —*Xwe7* —$Xgdm5_{190}$ —$Xgdm35_{246/250}$, with the genetic distance for each interval being 0.9 cM, 1.4 cM, 0.9 cM, 0.9 cM, 1.9 cM, 7.2 cM and 2.5 cM, respectively. We concluded that *Iw3672* is physically mapped on the distal region of wheat chromosome 2DS.

**Keywords** Synthetic hexaploid wheat · Cuticular wax · Non-glaucousness · Molecular marker · EST · SNP

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## Introduction

The leaf and stem surfaces of many land plants are covered with cuticular wax, which confers a glaucous phenotype (Tsunewaki and Ebona 1999). Cuticular wax is composed mainly of saturated, straight very long-chain fatty acids (VLCFAs), alkanes, primary and secondary alcohols, aldehydes, ketones, esters, triterpenes and sterols (Kunst and Samuels 2003). It has been suggested that cuticular wax restricts non-stomatal water loss, protects plants against ultraviolet radiation and reduces water retention on the plant

surface, thus minimizing deposition of dust, pollen and air pollutions (Kunst and Samuels 2003). Moreover, surface wax plays an important role in plant defense against bacterial and fungal pathogens (Jenks et al. 1994), and has been shown to participate in a variety of plant-insect interactions (Eigenbrode and Espelie 1995).

Classical genetic studies in wheat indicated that glaucousness was controlled by single dominant gene (Miczynski 1930; Chavan et al. 1955; Ayad 1953). However, Jensen and Driscoll (1962) demonstrated that non-glaucousness was controlled by a single dominant gene that behaved as an epistatic inhibitor of glaucousness. Cytogenetic analysis showed that glaucousness gene *W1* was located on chromosome 2BS and the dominant inhibitor gene *Iw2* was located on chromosome 2DS. *W1* and *Iw2* have duplicated genes, *W2* and *Iw1*, located on 2DS and 2BS, respectively, demonstrating that the genes for wax production and suppression were non-allelic (Tsunewaki 1962, 1966; Tsunewaki and Ebona 1999). A third glaucousness inhibitor, *Iw3*, was located on chromosome 1BL (Dubcovsky et al. 1997). A gene for spike glaucousness, *WS*, was also mapped distally on chromosome 1BS of wild emmer (Peng et al. 2000). Orthologous loci for glaucousness also occur in barley chromosome 2HS (*gs1*, *gs6* and *gs8*), rye chromosome7RL (*wa1*), and maize (*gl2*) (http://www.grs.nig.ac.jp/wheat/komugi/genes/macgene/GeneSymbol.html). A gene responsible for heavy wax production in durum wheat was located on chromosome 2A (Allan and Vogel 1960), and a gene associated with glaucousness was located on chromosome 2A of Chinese Spring (Tsunewaki 1964).

Molecular markers, including RFLP, RAPD, AFLP and SSR, have been widely used to map genes in crop plants (McIntosh et al. 2003). Microsatellite markers (SSR) have the advantages of being faster, less expensive and requiring smaller DNA samples than RFLP markers and have been used successfully in genetic mapping. Single nucleotide polymorphisms (SNPs) are now becoming important genetic markers for genotyping and mapping because of their abundance and amenability to high-throughout screening. Furthermore, SNP can contribute directly to a phenotype (Thornsberry et al. 2001) or may be associated with a phenotype as a result of linkage disequilibrium (Daly et al. 2001). Despite the extensive use of SNPs to study genetic disorders in humans, there have been fewer extensive surveys of SNPs in plants (Tenaillon et al. 2001). Four methods commonly are used for SNP (or mutation) detection: (i) identification of single strand conformation polymorphisms (SSCP); (ii) heteroduplex analysis; (iii) direct DNA sequencing; and (iv) the recently developed variant detector arrays (VDAs) (Gray et al. 2000). SSCP is the most popular method of SNP detection because of its simplicity, high sensitivity and low cost. For SSCP detection, the DNA fragment spanning the putative SNP is PCR-amplified, denatured and run on a non-denaturing polyacrylamide gel. During the gel run, the single stranded fragments adopt secondary structure according to sequence (Gray et al. 2000). Fragments bearing SNPs are identified by their different migration rates.

Although numerous chromosomal rearrangements occur in non-conserved regions, microsynteny between rice and wheat exists over a large proportion of the expressed genomes and can be used to develop additional molecular markers for fine mapping of wheat (Rota et al. 2004; Conley et al. 2004; Francki et al. 2004). The extensive public EST and genomic sequencing information from rice and wheat have provided resources for functional and comparative genomics analysis across species. The USA. National Science Foundation (NSF) initiative and various private sector organizations have focused on the development, characterization and contig assembly of EST sequences from numerous cDNA libraries of wheat and other related grasses (Francki et al. 2004). Representative ESTs have been mapped to specific regions of wheat chromosomes using deletion lines (Qi et al. 2003). Based on chromosomal location this information can be used to map or identify genes in wheat (Francki and Appels 2002).

Since no molecular markers for wax-related genes were reported in wheat, this study was conducted to investigate the genetic control of non-glaucousness in synthetic wheat Line 3672, and to map the gene(s) using molecular markers.

## Materials and methods

### Plant materials

Synthetic hexaploid wheat Line 3672 (kindly provided by CIMMYT) is non-glaucous, and common wheat Line 9753 is glaucous. The hybrid Line 9753 × Line 3672 was produced and 108 $F_2$ plant and their $F_3$ progenies were used for the study.

Chinese Spring (CS) and certain nullisomic-tetrasomic (N2AT2B, N2AT2D, N2BT2A, N2BT2D, N2DT2A & N2DT2B) and ditelosomic (Dt2AL, Dt2AS, Dt2BL, Dt2DL & Dt2DS) lines were used for chromosome and chromosomal arm assignment of molecular markers.

### Analysis of glaucousness by SEM

For scanning electron microscopy (SEM) analysis, leaf samples were cut into appropriate size and glued to a sample holder. The sample was then transferred to the cold sample stage (+4 °C) inside the SEM and subsequently analyzed with an accelerating voltage of 5 KV. Images were digitally recorded.

### Genomic DNA extraction

Genomic DNA was extracted from healthy leaves by the modified cetyltrimethylammonium bromide (CTAB) method (Saghai-Maroof et al. 1984). DNA pools from waxy and non-waxy leaves were made by bulking DNAs from 10 glaucous and 10 non-glaucous $F_2$ plants, respectively.

### SSR analysis

Two bulked DNA pools and two parents were used for SSR polymorphism analysis. Wheat GDM, GWM, KSM, PK and BARC primers were synthesized according to the published sequences (Röder et al. 1998; http://wheat.pw.usda. gov/). The CAU SSR primers were designed using EST sequences derived from the public EST database in GenBank. The primer-designing criteria included a Tm of 50–65 °C with no greater than a 3 °C difference between primer pairs. Polymorphic primers were then used to amplify DNAs from the 108 $F_2$ individuals.

Each PCR reaction was performed in a total volume of 10 μl containing 10 mM Tris-Hcl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of dNTP, 25 ng of each primer, 50–100 ng genomic DNA and 0.75 U *Taq* DNA polymerase. The amplifications were performed at 94 °C for 3 min, followed by 40 cycles at 94 °C for 1 min, 50–60 °C (depending on the primer pair) for 1 min, and 72 °C for 2 min, with a final extension at 72 °C for 10 min. Three μl PCR products mixed with 2 μl loading buffer were separated on 8% non-denaturing polyacrylamide gels or 5% denaturing polyacrylamide gels. Gels were then silver stained and photographed.

### Primer design for EST and SNP markers

Twenty BAC sequences in the corresponding collinear regions of rice chromosome 4 were selected according to the results of Conley et al. (2004). These BAC sequences were used to search for EST mapped on Bin5-0.47-1.00 of wheat chromosome 2DS in the mapped wheat EST database using BLASTX, TBLASTX and BLASTN. The matched wheat ESTs were used to search for the corresponding rice BAC sequences. Specific primers with predicted amplified products of 200–500 bp were designed by DNAMAN. PCR products were first separated on a 5% denaturing PAGE gel. PCR products of the primers that produced no polymorphism on the denaturing gel were separated on a 10% SSCP gel which was run at 25 °C under constant voltage (480 V) according to the method of Sunnucks et al. (2000). Polymorphic primers were then used to amplify DNAs from the 108 $F_2$ individuals.

### Linkage analysis

MapMaker 3.0 was used to perform linkage analysis and to establish a linkage map with a LOD threshold of 3.0.

## Results

### Analysis of cuticular wax by SEM

Observations in the field indicated that there was no cuticular wax on the leaf and stem surface of

Line 3672 and non-glaucous $F_3$ individuals, but cuticular wax was present on the leaf and stem surfaces of Line 9753 and glaucous $F_3$ individuals from jointing to milk dough stage. The morphology of cuticular wax on $F_3$ plants of 9753 × 3672 at the milk dough stage was examined by SEM. Clear differences in surface wax between glaucous and non-glaucous plants were observed (Fig. 1). The epidermal layers of Line 3672 and non-glaucous $F_3$ individuals were glossy and completely non-glaucous (Fig. 1a, c). However, wax deposition was present over most of the epidermis of the sheaths and leaves of Line 9753 and glaucous $F_3$ individuals. The wax crystals occurred as a high density of fine tubes or rods, oriented perpendicularly to the epidermis and organized either singularly or joined together in groups (Fig. 1b, d).

## Genetic analysis of non-glaucousness in Line 3672

$F_1$ plants of 9753 × 3672 were non-glaucous. $F_2$ individuals and $F_3$ families were grown in the field and frequencies of glaucous and non-glaucous plants were recorded. Among the 108 $F_2$ individuals, 25 were homozygous non-glaucous, 53 heterozygous non-glaucous and 30 glaucous, indicating that the non-glaucousness in Line 3672 was controlled by a single dominant gene ($x^2_{1:2:1} = 0.5$, $P > 0.05$), hereafter designated *Iw3672*.

## Microsatellite mapping of *Iw3672*

Bulked segregant analysis was employed to map *Iw3672*. Among more than 2000 SSR primer pairs tested, five (Table 1), BARC124, CAU96, CFD51, GDM5 and GDM35, amplified polymorphic bands between the glaucous and non-glaucous bulks as well as between Lines 9753 and 3672.

SSR primer pair BARC124 amplified a DNA fragment sized 520 bp from Line 3672 and non-glaucous individuals (Fig. 2a), and SSR primer pair GDM35 amplified two DNA fragments, sized 246 bp and 250 bp, from Line 3672 and non-glaucous individuals (Fig. 2b). EST-SSR primer pair CAU96 amplified a DNA fragment sized 287 bp from Line 3672 and homozygous

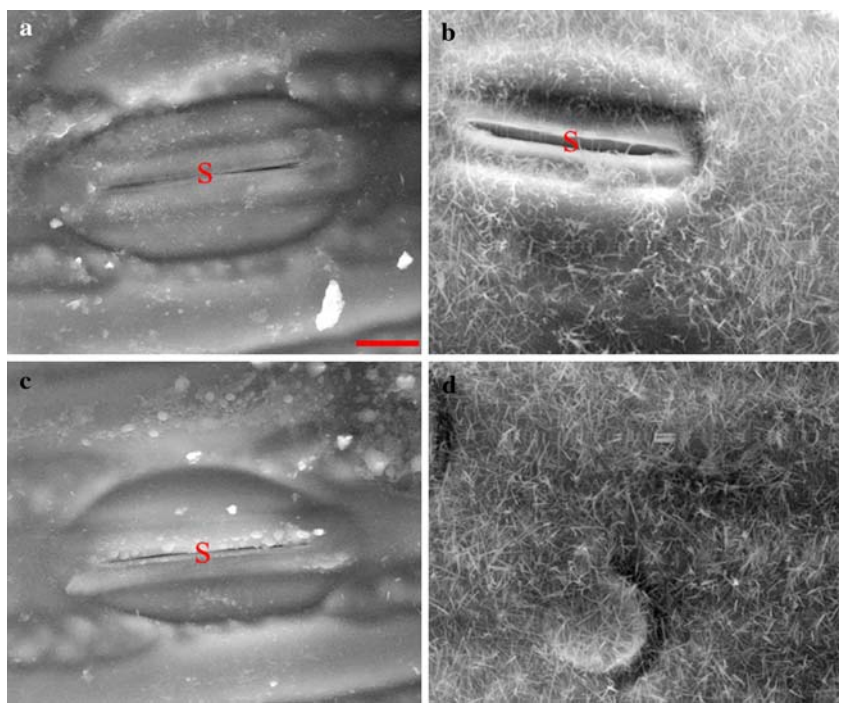


**Fig. 1** Morphology of wheat cuticular wax analyzed by SEM Stomatal pores (s) are indicated. The scale bar represents 10 $\mu$m; (**a**) non-glaucous Line 3672; (**b**) glaucous Line 9753; (**c**) non-glaucous $F_3$ plant; (**d**) glaucous $F_3$ plant

**Table 1** Primer sequences that amplified bands polymorphic between Lines 9753 and 3672

| Primer | Forward sequence (5′–3′) | Reverse sequence (5′–3′) | Corresponding wheat EST | Corresponding rice BAC clone |
|---|---|---|---|---|
| *CAU96* | AGAGTGGAGGAGAAATGAGACC | AGGAATCGAAGATGTAGGCG | – | – |
| CFD51 | GGAGGCTTCTCTATGGGAGG | TGCATCTTATCCTGTGCAGC | – | – |
| BARC124 | TGCACCCCTTCCAAATCT | TGCGAGTCGTGTGGTTGT | – | – |
| GDM35 | CCTGCTCTGCCCTAGATACG | ATGTGAATGTGATGCATGCA | – | – |
| GDM5 | CTAGCCAGAAGGTTACTTTG | CAACATTAACATTAACGCAC | – | – |
| TE6 | ATTGCCATTACTTCCTTTGC | TTAGCGACCTGTGCTCCTT | BE498683 | AL606616 |
| WE6 | CACAAGGGCTCCTCCTGCT | AGCGACCGACGAATCAATC | BE498358 | AL731627 |
| WE7 | ACCCTTTACACTATCTGGTC | GCTGGTTACTTCTGCTTCC | BE498111 | AL731620 |

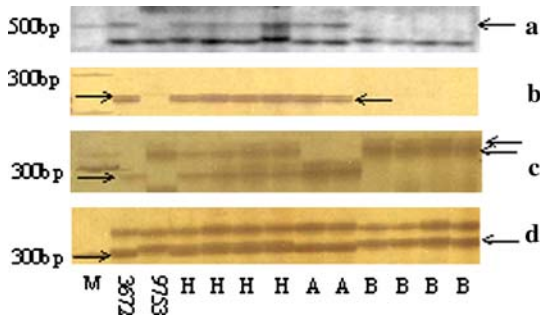


**Fig. 2** DNA fragments amplified by SSR primer pairs: (**a**) BARC124; (**b**) GDM35; (**c**) CAU96; (**d**) GDM5 in $F_2$ population and wheat parents Line 9753 and Line 3672. PCR products were separated on 5% denaturing PAGE and silver stained. A: homozygous non-glaucous plants; H: heterozygous non-glaucous plants; B: glaucous plants. M: Marker, 100 bp DNA ladder

non-glaucous individuals, and two DNA fragments, sized 342 bp and 368 bp, from Line 9753 and glaucous individuals. These three DNA fragments were also present in heterozygous non-glaucous progenies (Fig. 2c). SSR primer pairs GDM5 yielded a DNA fragment sized 190 bp from Line 3672 and homozygous non-glaucous individuals, and a DNA fragment sized 368 bp from Line 9753 and glaucous individuals. Both fragments were present in heterozygous non-glaucous individuals (Fig. 2d). SSR primer pair CFD51 amplified three DNA fragments, sized 180 bp, 200 bp and 230 bp, from Line 3672 and homozygous non-glaucous individuals, and three DNA fragments, sized 140 bp, 150 bp and 170 bp, from line 9753 and glaucous individuals. All six DNA fragments were present in heterozygous non-glaucous individuals.

Among the five SSR markers, $Xcau96_{287}$, $Xcfd51_{180/200/230}$ and $Xgdm5_{190}$ were co-dominant, whereas $Xbarc124_{520}$ and $Xgdm35_{246/250}$ were dominant. The five SSR primer pairs were further tested on all 108 $F_2$ plants (Table 2).

## Chromosome arm assignment and physical mapping of SSR markers

Since *Xcfd51*, *Xgdm5* and *Xgdm35* were already mapped on wheat chromosome 2DS (http://wheat.pw.usda.gov), Chinese Spring (CS), nullisomic-tetrasomic lines (N2AT2D, N2BT2A, N2BT2D, N2DT2A & N2DT2B) and ditelosomic lines (Dt2AL, Dt2AS, Dt2BL, Dt2DL & Dt2DS) were used for chromosome and chromosomal arm assignment of the SSR markers that were not mapped. Marker *Xcau96* was located on wheat chromosomes 2B and 2DS, *Xbarc124* on chromosomes 2A and 2D, and *Xgdm5*, *Xgdm35* and *Xcfd51* on chromosome 2DS (Fig. 3). Hence all five *Iw3672*-linked markers were located on wheat chromosome 2DS. Assuming a similar gene order and location exists between the chromosomes of CS and Lines 3672 and 9753, it was concluded that *Iw3672* was located on chromosome 2DS.

It was already known that *Xcfd51* and *Xbarc124* mapped to the distal region of Bin5-0.47-1.00 of chromosome 2DS (http://wheat.pw.usda.gov/ggpages/SSRclub/GeneticPhysical/groupe2v2.xls). Since *Iw3672* mapped between *Xcfd51* and *Xbarc124*, it was clear that *Iw3672* was physically located on the distal region of chromosome 2DS.

**Table 2** Segregation of *Iw3672* and linked markers in the $F_2$ population derived from Line 9753 × Line 3672

| SSR Locus | *IwIw* | | | *Iwiw* | | | *iwiw* | | |
|---|---|---|---|---|---|---|---|---|---|
| | Marker genotype | | | Marker genotype | | | Marker genotype | | |
| | AA | AB | BB | AA | AB | BB | AA | AB | BB |
| *Xbarc124* | 25 | 0 | 0 | 0 | 52 | 1 | 0 | 0 | 30 |
| *Xcau96* | 22 | 3 | 0 | 1 | 52 | 0 | 0 | 0 | 30 |
| *Xcfd51* | 22 | 3 | 0 | 2 | 51 | 0 | 0 | 0 | 30 |
| *Xgdm35* | 23 | 0 | 2 | 0 | 48 | 5 | 2 | 0 | 28 |
| *Xgdm5* | 18 | 6 | 1 | 4 | 43 | 6 | 0 | 1 | 29 |
| *Xte6* | 25 | 0 | 0 | 0 | 52 | 1 | 0 | 0 | 30 |
| *Xwe6* | 24 | 1 | 0 | 2 | 51 | 0 | 0 | 0 | 30 |
| *Xwe7* | 21 | 4 | 0 | 0 | 52 | 1 | 0 | 0 | 30 |

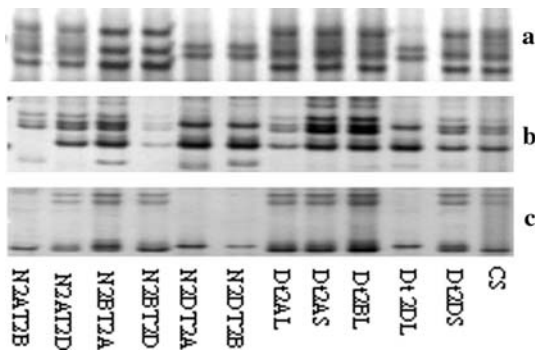


**Fig. 3** Amplification patterns of (**a**) CAU96:, (**b**) BARC124: and (**c**) GDM5, in Chinese Spring nullisomic-tetrasomics and ditelosomics. PCR products were separated on 8% non-denaturing PAGE and silver stained

## Development of EST and SNP markers linked to *Iw3672* gene

Twenty-three wheat ESTs matching well with the 20 rice BAC sequences were selected to identify molecular markers linked to *IW3672*. Three primer pairs (Table 1) amplified polymorphisms between the two parents. Primer pair WE6 amplified two DNA fragments, sized 2100 bp and 2150 bp, from Line 3672 and homozygous non-glaucous individuals, and two DNA fragments, sized 2090 bp and 2140 bp, from Line 9753 and glaucous individuals. All four fragments were present in heterozygous non-glaucous individuals (Fig. 4a). Primer pair TE6 amplified a DNA fragment sized 730 bp specifically from Line 3672 and non-glaucous individuals (Fig. 4b). Primer pair WE7 amplified a DNA fragment from Line 3672 and homozygous non-glaucous individuals, and a DNA fragment from Line 9753 and glaucous individuals in the SSCP gel (Fig. 5). Both fragments were present in heterozygous non-glaucous individuals. The three primer pairs were also further tested on all 108 $F_2$ plants (Table 2).

SSR markers $Xbarc124_{520}$, $Xcau96_{287}$, $Xcfd51_{180/200/230}$, $Xgdm5_{190}$ and $Xgdm35_{246/250}$, ESTs-derived markers $Xte6_{730}$ and $Xwe6_{2100/2150}$, a SNP marker *Xwe7*, and *Iw3672* were assigned to the same linkage group. The gene order was $Xte6_{730}$ /$Xbarc124_{520}$ —*Iw3672*—$Xwe6_{2100/2150}$ —$Xcau96_{287}$ —$Xcfd51_{180/200/230}$ —*Xwe7* —$Xgdm5_{190}$—$Xgdm35_{246/250}$ (Fig. 6).

## Discussion

There have been several studies on the inheritance and cytological localization of genes for glaucousness and non-glaucousness in wheat. Two dominant genes, *W1* and *W2*, for glaucousness were reported and cytologically located on chromosomes 2BS and 2DS, respectively (Tsunewaki 1962, 1966). Two dominant wax inhibitor genes, *Iw1* and *Iw2*, located on 2BS and 2DS were shown to epistatically inhibit the glaucous phenotypes conferred by *W1* and *W2*. Tsunewaki and Ebona (1999) suggested that *Iw2* originated from *T. tauschii* and was located on the distal region of chromosome 2DS. In the present study, we found that the non-glaucous phenotype of synthetic hexaploid wheat Line 3672 was inherited as a single dominant allele named *Iw3672*. Since *Iw3672* mapped in the same region as *Iw2*

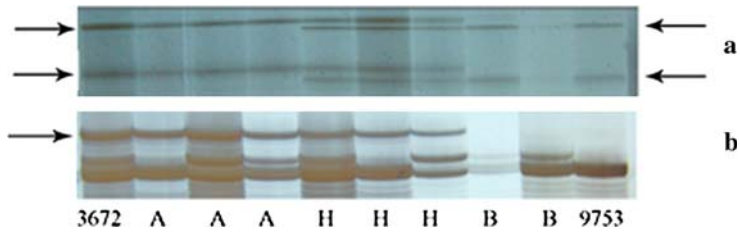


**Fig. 4** DNA fragments amplified by primer pairs (**a**) WE6; and (**b**) TE6 in $F_2$ population and parents Line 9753 and Line 3672. PCR products were separated on 5% denaturing PAGE and silver stained. A: homozygous non-glaucous plants; H: heterozygous non-glaucous plants; B: glaucous plants

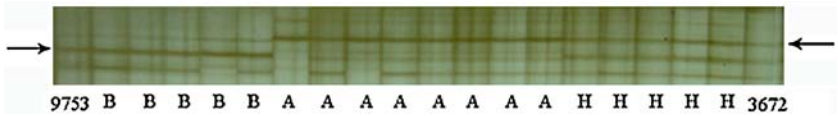


**Fig. 5** DNA fragments amplified by primer pairs: WE7 in the $F_2$ population and parents Line 9753 and Line 3672. PCR products were separated on 10% SSCP gel and silver stained. A: homozygous non-glaucous plants; H: heterozygous non-glaucous plants; B: glaucous plants

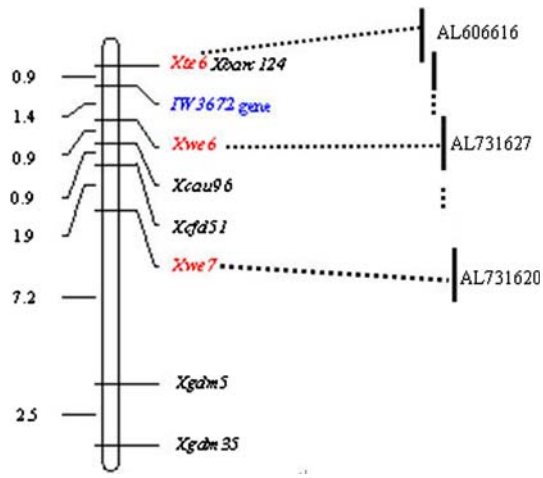


**Fig. 6** Linkage map of *Iw3672* and linked molecular markers, showing micro-collinearity between the gene-based markers and BAC clones of rice chromosome 4

we suggest that *Iw3672* and *Iw2* are the same. PCR markers closely linked to the locus were identified.

**Acknowledgements** This work was financially supported by National Basic Research Program of China (2003CB1143), Fok Ying Tung Education Foundation (94021), and National High-Tech Program.

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