Breeding Science 63: 169–175 (2013)
doi:10.1270/jsbbs.63.169

Characterization and mapping of novel chlorophyll deficient mutant genes in durum wheat

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The yellow-green leaf mutant has a non-lethal chlorophyll-deficient mutation that can be exploited in photosynthesis and plant development research. A novel yellow-green mutant derived from *Triticum durum* var. Cappelli displays a yellow-green leaf color from the seedling stage to the mature stage. Examination of the mutant chloroplasts with transmission electron microscopy revealed that the shape of chloroplast changed, grana stacks in the stroma were highly variable in size and disorganized. The pigment content, including chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotene, was decreased in the mutant. In contrast, the chl*a*/chl*b* ratio of the mutants was increased in comparison with the normal green leaves. We also found a reduction in the photosynthetic rate, fluorescence kinetic parameters and yield-related agronomic traits of the mutant. A genetic analysis revealed that two nuclear recessive genes controlled the expression of this trait. The genes were designated *ygld1* and *ygld2*. Two molecular markers co-segregated with these genes. *ygld 1* co-segregated with the SSR marker wmc110 on chromosome 5AL and *ygld 2* co-segregated with the SSR marker wmc28 on chromosome 5BL. These results will contribute to the gene cloning and the understanding of the mechanisms underlying chlorophyll metabolism and chloroplast development in wheat.

Key Words: durum wheat, yellow-green leaf mutant, genetic mapping, agronomic traits.

Introduction

Chlorophyll (Chl) is a vital biomolecule that sustains the life processes of all plants. Chl plays a critical role in photosynthesis by absorbing light and transferring light energy to the reaction centers of the photosynthetic system. Thus, Chl is essential for plant development and agricultural production (Eckhardt *et al.* 2004, Flood *et al.* 2011). The phenotypes of leaf color mutations are varied and are affected by different genetic and environment factors. Among the numerous leaf color mutants, the yellow-green leaf color (chlorina) mutant is a special phenotype. The physiology and genetic mechanisms of this mutant are distinct from those of the other leaf color mutants. Yellow-green leaf color mutants have been identified in many higher plants, such as *Arabidopsis thaliana* (Jarvis *et al.* 2000, Liu *et al.* 2003), rice (Jung *et al.* 2003, Moon *et al.* 2008), barley (Bellemare *et al.* 1982, Preiss and Thornber 1995), maize (Asakura *et al.* 2008, Mei *et al.* 1998), sunflower (Mashkina and Gus'kov 2002) and wheat (Falbel *et al.* 1996, Giardi *et al.* 1995, Hui *et al.* 2012). The effects of these mutations on chloroplast development, photosynthesis, chlorophyll *b* accumulation and the levels of light-harvesting chlorophyll *a*/*b* proteins (Kosuge *et al.* 2011) have been examined. However, the rate of natural mutation is an estimated 10^{-5}–10^{-8} in higher plants and is thus notably low. Artificial methods have been applied to obtain these precious mutant resources. Radiation, for example, is an effective way to induce various mutations in higher plants (Morita *et al.* 2009).

To date, several yellow-green leaf mutant genes have been mapped and cloned. *ygl1* is a yellow-green leaf mutant gene in rice. The *YGL1* gene encodes an enzyme required for Chl *a* biosynthesis. A point mutation (Pro-198 to Ser) in the *YGL1* gene reduces Chl synthase activity (Wu *et al.* 2007). *chl1* and *chl9*, which have been isolated from chromosome 3 of two rice chlorina mutants, encode the ChlD and ChlI subunits of Mg-chelatase, respectively. These two genes play an important role in chloroplast development by modulating MgProto (Zhang *et al.* 2006). In wheat, the homoeologous chlorina loci have been mapped onto the homoeologous group 7 chromosomes. These loci include the *cn-A1* locus on chromosome 7A, the *cn-B1* locus on the 7B and the *cn-D1* on chromosome 7D. These mutations reduce the expression of the light-harvesting Chl *a*/*b* complex II (Klindworth *et al.* 1995, Watanabe and Koval 2003).

In this study, we characterized the yellow-green leaf mutant *ygld* in durum wheat and mapped the mutated genes of the $F_{2:3}$ populations with SSR markers. The mutations affecting the agronomic traits were also investigated.

Communicated by H. Tsujimoto
Received July 30, 2012. Accepted December 6, 2012.
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Materials and Methods

Plant materials

The *ygld* (yellow-green leaf durum) mutant was introduced from Italy, derived from *Triticum durum* var. Cappelli treated by gamma radiation (Tomarchio *et al.* 1983). The mutant displays yellow-green leaves throughout development. The $F_{2:3}$ segregation populations were used for genetic analysis and mapping. These populations were made by crossing the *T. durum* cultivar Langdon with the normal green-leaf plant and the ygld mutant.

Pigment content and fluorescence kinetic parameters

The content of Chl (chlorophyll) and Cars (carotenoid) was measured using a DU 800 UV/Vis Spectrophotometer (Beckman Coulter) according to the method detailed by Lichtenthaler (1987). The fluorescence kinetic parameters were measured using the Hansatech Fluorecence Monitoring System-FMS-2. Each experiment was repeated three times.

Transmission electron microscopy analysis

The wild-type and *ygld* mutant leaf samples were collected from 1-week- and 4-week-old plants. All plants were grown under a controlled environment with the same light intensity, temperature and living conditions. First, the leaf sections which were cut to about 5 mm in length from fresh leaves, were quickly fixed in a solution of 2% glutaraldehyde. Next, the sections were fixed in a solution of 1% OsO_4 and the samples were stained with uranyl acetate and dehydrated in ethanol. The thin sections were embedded in Spurr's medium. Finally, the samples were sliced to 50 nm in thickness, and stained again then examined using a JEOL 100 CX electron microscope.

Agronomic trait analysis

The agronomic traits of the $F_{2:3}$ populations were examined. Both populations were grown in Beijing (39.54°N). The F_2 population was planted in 2009 and the F_3 population was planted in the fall of 2010. A total of 7 agronomic traits were investigated. These traits included the plant height (cm) (PH), number of spikes per plant (NSP), number of spikelets per spike (NSS), spike length (cm) (SL), number of grains per spike (NGS), grain yield per plant (GYP) and 1000-grain weight (TGW).

Microsatellite analysis

Total DNA was extracted from the sample leaves using the CTAB method (Murray and Thompson 1980). Each PCR reaction was conducted in a total volume of 15 μl containing 1.2 μl of template DNA (approximately 50 ng), 0.09 μl of Taq DNA polymerase (5 U/μl, Fermentas), 1.5 μl of 10 × PCR buffer [200 mM $(NH_4)_2SO_4$, 750 mM Tris-HCl (pH 8.8, 25°C)], 1.2 μl of 25 mM $MgCl_2$, 0.12 μl of dNTP (25 mM), 1.2 μl of primers (2 μM) and 9.69 μl of H_2O. The amplification experiments were subjected to 94°C for 5 min. This step was followed by 35 cycles of 94°C for 45 s, 48–63°C (depending on the microsatellite primer annealing temperature) for 45 s and 72°C for 45 s. The extension step was performed at 72°C for 10 min. Each PCR product was mixed with 3 μl of loading buffer (98% formamide, 0.3% of each bromophenol blue and xylene cyanol and 10 mM of EDTA), denatured at 95°C for 5 min and immediately cooled on ice. Electrophoresis was carried out in a 5% denatured polyacrylamide gel with 1 × TBE (90 mM Tris-borate, 2 mM EDTA) for 90 min. A silver-staining experiment was later performed according to the method of Tixier *et al.* (1997).

Linkage analysis

The F_2 generation derived from crossing the yellow-green leaf mutant *ygld* and *T. durum* cultivar Langdon was used for mapping. The phenotypes of the F_2 individuals were confirmed by investigating the F_3 families in the field at different growth stages. A total of 794 F_2 individuals were used for genetic mapping. To determine the map position of the *ygld* genes on the wheat chromosomes, 546 pairs of SSR markers on the whole durum wheat chromosomes were selected from Somers *et al.* (2004), Paux *et al.* (2008) and Xue *et al.* (2008). The polymorphic markers between parents were first picked out. Then BSA (bulked segregant analysis) method was used to screen for the polymorphic markers between the green and yellow-green leaf DNA bulks. Each bulk was composed of 6 individuals from the F_2 generation. To exclude the possibility of other reasons which could cause the yellow-green leaf phenotype, all the DNA samples of F_2 were phenotypically validated using the F_3 families. The MAPMAKER/EXP ver. 3.0 program, which is based on the maximum likelihood method, was used for linkage analysis (Lander *et al.* 1987). The map distances between markers and genes were derived from the Kosambi function (Kosambi 1944).

Results

Phenotypic characterization of the ygld mutant

Leaf-color mutations are diverse and can occur at different growth stages. The plants with the mutations conferring severe Chl deficiency died early. Some plants with other mutations eventually regained the normal green leaf color (Chen *et al.* 2009, Dong *et al.* 2007, Pereira *et al.* 1997). Compared with the wild-type, the *ygld* mutant exhibited yellow-green leaves from germination through maturity. Even though the mutant was less vigorous and was delayed in its development, the yellow-green leaf color mutation in the *ygld* mutant was not lethal (Fig. 1).

The TEM analysis showed that there were no differences in the size or number of the chloroplasts between the mutant and wild type plants. However, the shape and structure of the chloroplasts were different between groups. The wild-type plants had ellipsoidal chloroplasts. In the *ygld* mutant, the shape of chloroplasts changed from ellipsoidal to circular. The mutant grana stacks were highly variable in size and

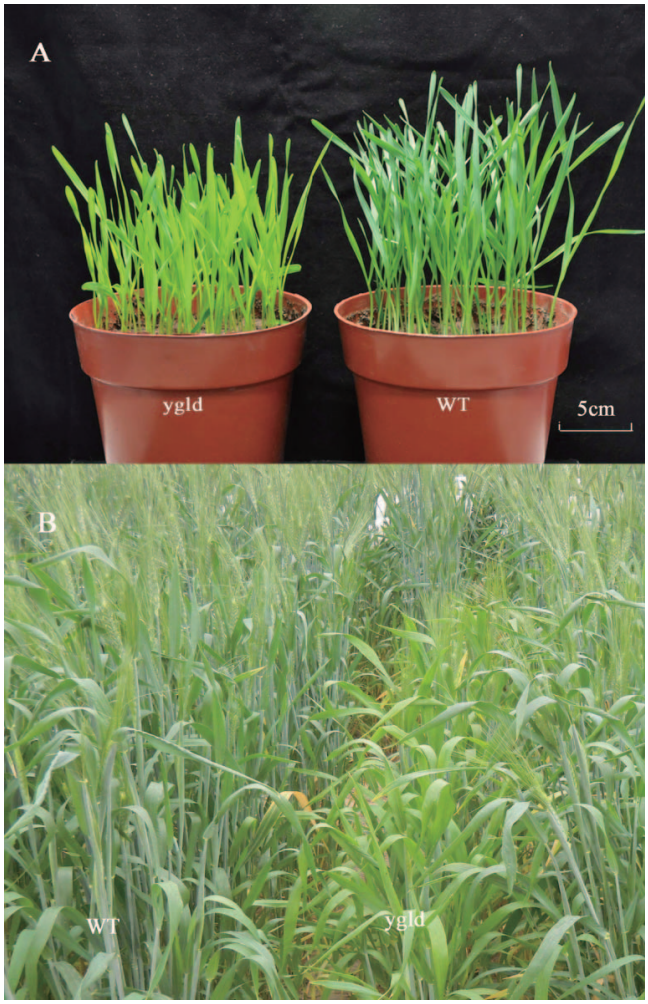

Fig. 1. The phenotypes of the *ygld* mutant and the wild type. A: The phenotypes of the *ygld* mutant and the wild type at the seedling stage. A mutant with yellow-green leaves is shown on the left. A wild-type plant with normal green leaves is shown on the right. Both are cultivated in the same enviroment for 10 days. B: Phenotypes of the *ygld* mutant and wild type at the heading stage. A mutant with yellow-green leaves and reduced height is shown in the middle, which is inhibited in growth and about a week later than the normal plants. The wild-type lines are shown on the left.

appeared disorganized in the stroma region. In the wild type, the chloroplasts had uniform diameters and had approximately parallel grana stacks and lamella (Fig. 2).

Pigment content and fluorescence kinetic parameter analysis

The quantity of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoid (Car) as well as the ratio of chlorophyll *a*/chlorophyll *b* (Chl *a*/*b*) were measured. These measurements were compared between groups at different times during development (Table 1). The content of the pigments changed with developmental stage, but the levels of Chl *a*, Chl *b* and Car in the mutant were lower throughout development compared with the wild type. Chl accumulation in the mutant was slower than that in wild type and did not reach the wild-type level. Additionally, increasing the Chl *a*/Chl *b* ratio meant that Chl *b* was more reduced than Chl *a* in the mutant. Therefore, the *ygld* mutant is deficient especially in Chl *b*.

The fluorescence kinetic parameters (Table 2) in Fo and Fm of the yellow-green leaf mutant were decreased significantly compared with the wild type. This reduction corresponded to the reduction in chl. The ϕPSII, NPQ and ETR were decreased by 16.7%, 16.6% and 25.5%, respectively. These results indicated that photosynthesis efficiency was decreased in the mutant. The Fv/Fm values of the wild type and the mutant were not significantly different. This result implied that the primary light energy conversion of PSII in the mutant was retained at wild-type levels (Wang *et al.* 2009).

The effects of the mutations on yield traits

The yield-related traits of the F_2 and parts of the F_3 populations were investigated in the 2 crop seasons. To comprehensively understand the effects of the *ygld* phenotype on the yield-related traits, a survey of 7 agronomic traits was carried out (Table 3). We measured the plant height (cm) (PH), number of spikes per plant (NSP), number of spikelets per spike (NSS), spike length (cm) (SL), number of grains of spike (NGS), grain yield per plant (GYP) and 1000-grain weight (TGW). With the exception of SL, all of the agronomic traits in the mutant were significantly reduced compared with wild-type plants. The Chl deficiency throughout development inhibited photosynthesis and consequently affected the accumulation of biomass and the development of the plant.

Genetic analysis of the ygld phenotype

The F_2 population, which was derived from crossing the *ygld* mutant with Langdon, was used to investigate the Mendelian segregation ratio. The F_2 population contained 794 individuals in total: 51 of them exhibited yellow-green leaves, whereas the other individuals showed normal green leaves. A χ^2 analysis was applied to test for the deviation of the *ygld* mutant in the F_2 population from the expected segregation ratio of 15:1 ($\chi^2_{(15:1)} = 0.0056$, $p > 0.9$). The segregation ratio indicated that the *ygld* phenotype was controlled by two recessive nuclear genes. Neither gene alone could give rise to the leaf color abnormality. The genes were designated as *ygld1*(yellow-green leaf durum 1) and *ygld2* (yellow-green leaf durum 2).

Genetic mapping of the two recessive genes

To map the *ygld1*and *ygld2* genes, 546 pairs of markers were screened for polymorphisms. Among these markers, 132 pairs showed polymorphisms between the two parents. Six and 3 SSR markers were linked with the *ygld1* and *ygld2* genes, respectively (Table 4 and Fig. 3). The gene *ygld1* was located on chromosome 5AL and was flanked by the SSR markers cfa2185 and cfa126 at genetic distances of 16.0 cM and 8.5 cM, respectively. Moreover, the SSR

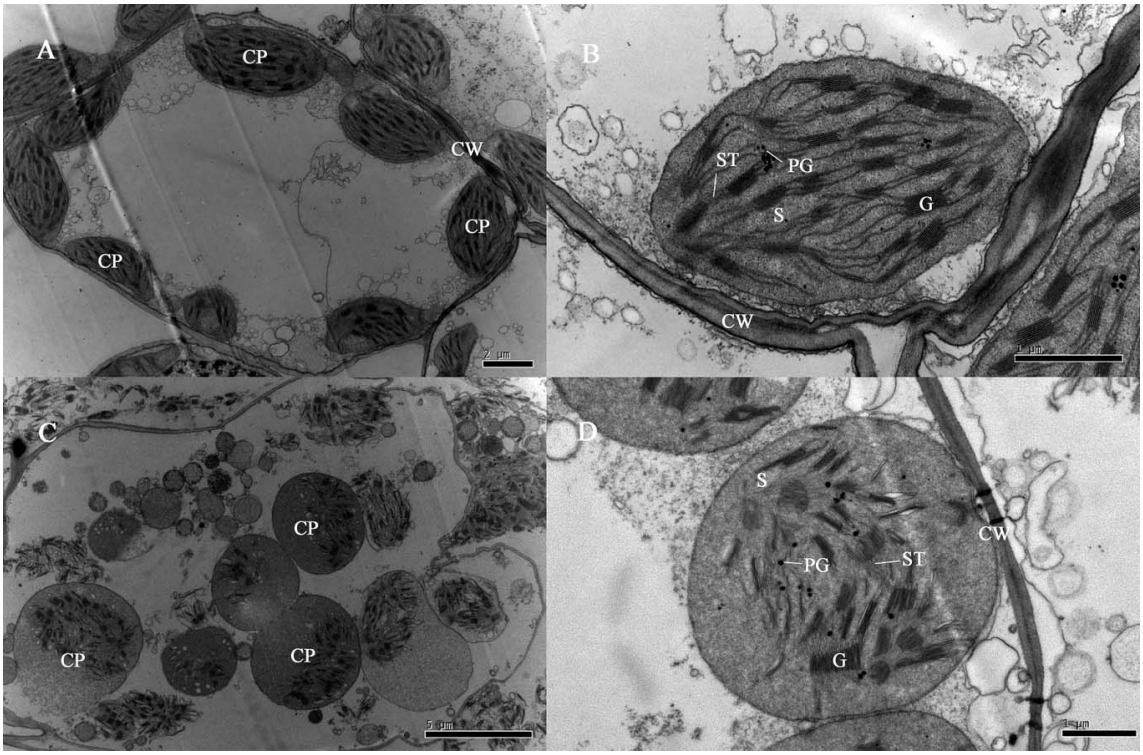

Fig. 2. Transmission electron microscopic analysis of chloroplasts in the *ygld* mutant and the wild type. A and B: Chloroplasts in the wild type (wt) plants with normal green leaves. C and D: Chloroplasts in the *ygld* mutant with yellow-green leaves. CP, chloroplast; PG, plastoglobule; G, grana; S, stroma; CW, cell wall; ST, stroma thylakoid. Scale bars on the lower right corner are 2 μm, 1 μm, 5 μm and 1 μm.

Table 1. The pigment content in the leaves of the wild-type and the *ygld* mutant in mg g^{-1} fresh weight

Growth stage	Genotype	Chl *a*	Chl *b*	Car	Chl *a*/*b* Ratio
2 weeks	*ygld* mutant	0.99 ± 0.02**	0.23 ± 0.01**	0.21 ± 0.02**	4.38
	Wild type	3.08 ± 0.01	1.1 ± 0.02	0.44 ± 0.10	2.81
4 weeks	*ygld* mutant	2.06 ± 0.16**	0.42 ± 0.10**	0.38 ± 0.01**	4.93
	Wild type	4.15 ± 0.22	1.39 ± 0.05	0.8 ± 0.11	2.98
10 weeks	*ygld* mutant	2.28 ± 0.24**	0.64 ± 0.18**	0.46 ± 0.01**	3.96
	Wild type	4.56 ± 0.36	1.67 ± 0.25	0.86 ± 0.06	2.73

Chl and Car were measured in acetone extracts from second leaf of different growth stages from top. Values shown are the mean SD (±SD) from three independent determinations. ** significant at $P < 0.01$ by T-test.

Table 2. Comparison between the fluorescence kinetic parameters of the *ygld* mutant and the wild type

	Fo	Fm	Fv/Fm	ϕPSII	qP	NPQ	ETR
Wild type	76.5 ± 7.2	350.2 ± 30.6	0.78 ± 0.003	0.42 ± 0.007	0.70 ± 0.014	1.69 ± 0.406	2.71 ± 0.073
Mutant	45.5 ± 4.0**	227.5 ± 20.1**	0.80 ± 0.002	0.35** ± 0.005	0.66 ± 0.006	1.41 ± 0.087	2.02** ± 0.131

Fo: the minimal fluorescence; Fv: the variable fluorescence; Fm: the maximal fluorescence (Fm = Fo + Fv); Fv/Fm: the primary light energy conversion of PSII; ϕPSII: quantum yield of photosystem II electron transport; qP: photochemical quenching coefficient; NPQ: non-photochemical quenching; ETR: apparent photo-synthetic electron transport rate. ** significant at $P < 0.01$ by T-test.

marker wmc110 co-segregated with *ygld1*. The *ygld2* gene was mapped onto chromosome 5BL with 3 markers. Barc142 and barc308 were both at a distance of 8.0 cM and the SSR marker wmc28 cosegregated with *ygld2* (Fig. 3).

Discussion

Chloroplasts and Chl are essential for photosynthesis in higher plants. This fundamental process sustains the life on earth (Chen *et al.* 2005, Inaba and Ito-Inaba 2010, Wang *et al.* 2009). Chl *a* is a component of the light-harvesting complexes (LHCs) and photosynthetic reaction centers. Chl *b* is located in the light-harvesting pigment protein complexes of the PSI and PSII (Masuda *et al.* 2003, Oster *et al.* 2000). Previous studies have indicated that mutants with a higher Chl *a*/Chl *b* ratio have lower LHC levels. As a result of the

Table 3. The relationship between the phenotypes and seven yield-related traits

Phenotype	Trait						
	PH	TGW	SL	NSP	NSS	NGS	GYP
Wild type	132.9 ± 11	34.6 ± 8.1	9.5 ± 4.6	23.4 ± 2.7	46.8 ± 9.1	272.6 ± 171.0	9.5 ± 6.5
Mutant	102.8 ± 14.5**	20.4 ± 5.8**	7.4 ± 1.3	19.5 ± 3.4**	31.1 ± 8.5**	45.7 ± 23.1**	1.0 ± 0.6**

plant height (cm) (PH), number of spikes per plant (NSP), number of spikelets per spike (NSS), spike length (cm) (SL), number of grains of spike (NGS), grain yield per plant (GYP), 1000-grain weight (TGW), ** significant at $P < 0.01$ by T-test.

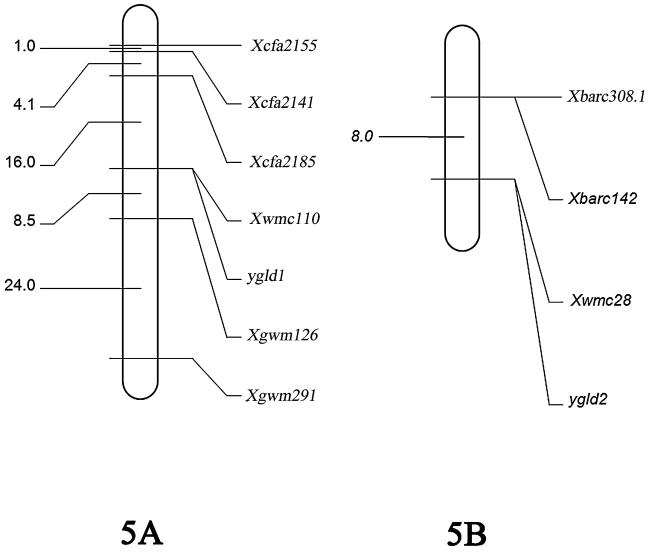

Fig. 3. Genetic map showing the *ygld1* gene on chromosome 5A with the SSR co-segregation marker wmc110 and the *ygld2* gene on chromosome 5B with the SSR co-segregation marker wmc28.

limited chl content, these mutants have abnormal thylakoid membranes (Falbel and Staehelin 1996, Marco *et al.* 1989). The *ygld* mutant with the yellow-green leaf phenotype is a chlorophyll deficient mutant. This mutant has a high chl *a*/chl *b* ratio and has decreased chl *a*, chl *b*, carotenoid levels. These results indicate that the LHC content may be lower in the mutant than in the wild type. Furthermore, the PSII of the *ygld* mutant was not significantly affected according to our measurements of the fluorescence parameters. This result indicated that the PSI in the *ygld* mutant was impaired by the mutation. Thus, the abnormal pigment content and chloroplast structure in the *ygld* mutant may have contributed to the partial repression of chl synthesis. Plants with normal leaf colors have higher chlorophyll content and normal chloroplasts. These characteristics allow the plants to absorb more energy and be more efficient at photosynthesis (Lawlor 2009, Luo and Ren 2006, Wang *et al.* 2003, Wang *et al.* 2010). Therefore, the wild-type plants grow vigorously. The chloroplasts in many leaf color mutants are irreversibly abnormal from the early stages of leaf development (An *et al.* 2011). Therefore, the mutations affect the accumulation of both Chl and biomass throughout plant development. The *ygld* mutant can carry out photosynthesis but grows slowly compared to the wild type. Almost all of the agronomic traits were reduced significantly in *ygld*, but the influence on SL was limit. Further study on the genes of *ygld* would contribute to the improvement in breeding. The study could be useful to understand genetic mechanism of chloroplast biogenesis and exploit in the improvement for wheat photosynthesis. Moreover the differentiation in leaf color could be a significant marker. Leaf color mutant has been used successfully in rice breeding to monitor seed purity in seed production as a phenotypic marker (Chen *et al.* 2007).

Many of these yellow-green leaf mutations have been genetically characterized and used as genetic markers. In rice, 11 yellow-green leaf mutants (chl 1 to10 and ygl1) have been identified. All of these phenotypes are controlled by a single recessive gene (www.gramene.com). Moreover, several yellow-green leaf mutants have been identified from tetraploid wheat (*Triticum turgidum* L.). Further analysis showed that most yellow-green leaf mutants are also controlled by a single recessive gene (Klindworth *et al.* 1995, Luo and Ren 2006, Williams *et al.* 1985). However, Smith identified a mutant controlled by a dominant gene (Smith

Table 4. Molecular markers used for mapping the genes of *ygld1* and *ygld2*

Marker	Tm (°C)	Forward primer (5′→3′)	Reverse primer (5′→3′)
cfa2155	60	TTTGTTACAACCCAGGGGG	TTGTGTGGCGAAAGAAACAG
cfa2141	60	GAATGGAAGGCGGACATAGA	GCCTCCACAACAGCCATAAT
cfa2185	60	TTCTTCAGTTGTTTTGGGGG	TTTGGTCGACAAGCAAATCA
wmc110	61	GCAGATGAGTTGAGTTGGATTG	GTACTTGGAAACTGTGTTTGGG
gwm126	60	CACACGCTCCACCATGAC	GTTGAGTTGATGCGGGAGG
gwm291	60	CATCCCTACGCCACTCTGC	AATGGTATCTATTCCGACCCG
barc308	55	GCGATCTTGCGTGTGCGTAGGA	GCGTGGGATGCAAGTGAACAAT
barc142	52	CCGGTGAGAGGACTAAAA	GGCCTGTCAATTATGAGC
wmc28	51	ATCACGCATGTCTGCTATGTAT	ATTAGACCATGAAGACGTGTAT

1952). Additionally, Varughese and Swaminathan (1968) found a yellow-green leaf trait that was controlled by two recessive genes, but no further analysis was conducted. In the current study, we identified a new yellow-green leaf mutation that was controlled by two complementary recessive genes, specifically, *ygld1* and *ygld2*. These two genes were mapped onto chromosome 5AL and 5BL with the co-segregating markers wmc110 and wmc28, respectively and these two genes might have homoeologous relationship due to their locations. The analysis of the mapping locations and phenotypes showed that *ygld1* and *ygld2* were different from the previously reported genes associated with yellow-green leaf color alterations. Thus, *ygld1* and *ygld2* are novel genes.

Acknowlegements

We are grateful to Juncheng Zhang for his valuable suggestion on genetic mapping, and Yiyuan Li, Lingli Zheng, Lei Pan for their work on phenotype investigaion. This work was supported by National Transgenic Research Project (2008ZX08009-001).

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