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Characterization and gene mapping of a chlorophyll-deficient mutant *clm1* of *Triticum monococcum* L.

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Abstract

Diploid wheat *Triticum monococcum* L. is a model plant for wheat functional genomics. Chlorophyll-deficient mutant (*clm1*) was identified during manual screening of the ethylmethane sulphonate (EMS)-treated M_2 progenies of *T. monococcum* accession pau14087 in the field. The *clm1* mutant, due to significantly decreased chlorophyll content compared with the wild-type (WT), exhibited pale yellow leaves which slowly recovered to green before flowering. The *clm1* mutant showed early flowering, reduced number of tillers, trichome length and density, and different shape as compared with the WT. At the same time, *clm1* mutant culm had more chlorophyll-containing parenchymatous tissues compared to WT, presumably to absorb more sunlight for photosynthesis. Genetic analysis indicated that the *clm1* mutation was monogenic recessive. The *clm1* mutant was mapped between Xgwm473 and Xwmc96 SSR markers, with genetic distances of 2.1 and 2.6 cM, respectively, on the $7A^mL$ chromosome.

Additional key words: bulk segregant analysis, diploid wheat, ethylmethane sulfonate, SSR marker.

Introduction

Chlorophyll (Chl) plays significant role in absorption of sunlight by photosynthetic reaction centers (Liu *et al.* 2007) and approximately 100 terawatts of energy is captured by plants (Nealson and Conrad 1999). Mutations in the genes of Chl biosynthesis or related pathway result in Chl-deficient mutants or leaf-color mutants. These mutants are ideal materials for fundamental research in photosynthesis, photomorphogenesis, hormone physiology, resistance mechanism, and identification of gene functions (Parks and Quail 1991, Mochizuki *et al.* 2001, Stern *et al.* 2004, Beale 2005). The chemical mutagen, ethylmethane sulfonate (EMS), has been successfully applied in wheat and rice (Wang *et al.* 2009, Ansari *et al.* 2012, Tian *et al.* 2012).

The diploid wheat (*T. monococcum*, A^mA^m) is an ideal material for induced mutations which can be easily characterized and transferred to polyploid wheat. Certain characteristics of *T. monococcum* make it an attractive diploid model for gene discovery in wheat (Wicker *et al.* 2001) and application of several functional genomics approaches. Firstly, the diploid *T. monococcum* has a small genome size (5 700 Mb) compared with hexaploid wheat (17 300 Mb). Secondly, the existence of a very high level of polymorphism for DNA based markers, conservation of colinearity and synteny with other cereal crops, and high resistance against various wheat diseases are the other attractive features. Finally, a large bacterial artificial chromosome (BAC) library is available in *T. monococcum*.

Several Chl-deficient mutants have been identified

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Abbreviations: BSA - bulk segregant analysis; Chl - chlorophyll; *clm1* - chlorophyll-deficient mutant; EMS - ethylmethane sulfonate; M_2 - second generation after mutagenesis; PCR - polymerase chain reaction; RILs - recombinant inbred lines; SEM - scanning electron microscopy; T.S. - transverse section; WT - wild-type.
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in wheat (Freeman *et al.* 1982, Falbel and Staehelin 1994, Guo *et al.* 1996), rice (Kaul and Bhan 1977, Wu *et al.* 2007, Chen *et al.* 2009), barley (Dunford and Walden 1991), soybean (Zhang *et al.* 2011), *Arabidopsis* (Long *et al.* 1993, Hsieh and Goodman 2005, Kim *et al.* 2006), and tobacco (Chang *et al.* 2001). In the hexaploid wheat, *T. aestivum*, some Chl mutants like Driscoll's *chlorina* (Pettigrew *et al.* 1969) and *chlorina-1* (Sears and Sears 1968) have been mapped on the A genome, and *Chlorina-214* (Washington and Sears 1970) on the B genome. These wheat mutants exhibit reduced Chl *b* content, a significant reduction in light harvesting complex, and formation of grana-deficient thylakoid membranes (Falbel *et al.* 1996). *CD3*, a non-lethal Chl-deficient mutant, was also identified in hexaploid wheat (Freeman *et al.* 1982). Some orthologous *chlorina* mutants similar to *CD3* have been mapped to chromosome 7 of the A, B, and D parental diploid wheat genomes in both hexaploid (*T. aestivum*) and tetraploid (*T. turgidum*) wheat (Falbel and Staehelin 1994).

The rapid development of new simple sequence repeat (SSR) molecular markers has been increasingly exploited to develop high-density genetic maps in diploid wheat, *T. monococcum* (Dubcovsky *et al.* 1996, Singh *et al.* 2007). The first sequence coverage of the wheat genome (*T. aestivum,* Chinese Spring line 42) has been released and is available *via EMBL*/GenBank and *CerealsDB* for genomic analysis and application (http://www.cerealsdb.uk.net).

This article deals with the characterization and mapping of a novel, EMS induced, Chl deficient mutant (*clm1*) in diploid wheat, *T. monococcum*. The results will not only promote map-based cloning of *clm1* gene but also could have far reaching implications and applications in the investigation of photosynthesis and chloroplast development pathway in wheat and other cereals.

Materials and methods

The *clm1* mutant used in the present study was isolated from diploid wheat, *T. monococcum* accession pau14087, at the Punjab Agricultural University, Ludhiana, after seed treatment with 0.25 % EMS. This mutant was identified during manual screening of the M_2 EMS-treated population in the field. The seeds of *clm1*, the WT parent, and an accession pau5088 of *T. boeoticum* (the wild and tall progenitor of *T. monococcum*) were planted at the Indian Institute of Technology (IIT), Roorkee, in November 2005. A recombinant inbred lines (RILs) population of *T. boeoticum* pau5088 × *T. monococcum* pau14087 was also planted in the field (Singh *et al.* 2007) and some RILs were chosen at random to develop a negative bulk without the mutation. The *clm1* mutant was crossed with both the WT parent, *T. monococcum*, and its wild progenitor, *T. boeoticum*, for developing F_2 populations for inheritance and mapping studies, respectively. The F_2 populations were planted at IIT, Roorkee, in 2007 and 2008 in 2 m rows with row-to-row distance of 30 cm and plant to plant distance of 10 cm following the standard package of agronomic practices for wheat cultivation.

The total Chl *a* and *b* content of *clm1* mutant and *T. monococcum* were estimated according to Arnon (1949) and Koski (1950). Fresh leaf tissue was extracted with 85 % (v/v) acetone and absorbance was recorded on a spectrophotometer (*Lambda 25 UV/VIS, Perkin Elmer*, Shelton, USA) at 663 and 644 nm.

The samples for scanning electron microscopy (SEM) were prepared according to Mou *et al.* (2000). The tissue samples were immersed in 2.5 % (v/v) glutaraldehyde at room temperature for 2 h and dehydrated in 50 % (v/v) ethanol for 5 min, in 70 % for 30 min (twice), 90 % for 30 min (twice), and 100 % for 30 min (twice). Finally, in mixture of ethanol and amyl acetate 3:1 for 30 min, 2:2 for 30 min, 1:3 for 30 min, and in pure amyl acetate for 30 min. The samples were kept for critical point drying for 40 min and mounted onto metal stubs with double-sided carbon tape. For sputter coating, a thin layer of gold was applied over the samples using an automated sputter coater. These samples were then analyzed using scanning electron microscope (*Leo 435*, Cambridge, USA) and the surface images were taken at 150-fold magnification.

For histological examination, the second internode of stems were excised, fixed in formalin + acetic acid + ethanol (FAA), and dehydrated in a graded ethanol series and finally in xylene. For sectioning, the tissues were embedded in paraffin wax (*Sdfine*, Mumbai, India) at 60 °C, and sectioned to 10 μm thickness on a rotary microtome. The tissues were stained with toluidine blue O (0.06 %, m/v) as suggested by Johansen (1940). Transverse sections of the stems were observed under a light microscope (*Axiostar plus 1169-151, Carl Zeiss*, Oberkochen, Germany) at different magnifications.

The genomic DNA from parents and the F_2 population was extracted following the cetyltrimethyl ammonium bromide (CTAB) method as described by Saghai-Maroof *et al.* (1984). A number of high density molecular maps have been developed in wheat (Somers *et al.* 2004) including a RIL population between *T. monococcum* acc. pau14087, used for *clm1* mutant isolation, and *T. boeoticum* acc. pau5088, used for making populations, with the *clm1* mutant (Singh *et al.* 2007). The primers for anchored SSR markers at about 10 cM from each of the diploid wheat chromosomes and polymerase chain reaction (PCR) protocols were carried out in a thermo cycler (*Applied Biosystems*, Singapore) according to methods described by Singh *et al.* (2007). The PCR products were separated on 8 % (m/v) polyacrylamide gels according to the length of the amplified fragments and stained with ethidium bromide.

To study the inheritance of *clm1* mutant, it was crossed with its WT parent *T. monococcum* and

T. boeoticum for molecular mapping. The F_1 plants were advanced to F_2 generations. The phenotypic data on leaf color (chlorotic *vs*. green) was recorded on each of the plant of the F_2 populations. χ^2-test was applied to test the goodness of fit to the segregation ratio.

For bulk segregant analysis (BSA), positive bulk of 12 *clm1* mutant plants was made from homozygous mutant F_2 *chlorina* plants from the respective F_2 mapping population of *T. monococcum* × *T. boeoticum*. The negative bulk was prepared from 15 RILs of *T. monococcum* pau5088 × *T. boeoticum* pau14087 cross without any mutant in the parents. An equal amount of DNA from each plant of both bulks was pooled along with parents. These two sets of bulks along with the parents were used to identify putative SSR markers linked to the *clm1* mutant. Genotyping of the debulked F_2 plants was done with the putatively linked marker (Xgwm473) and two other closely linked markers (Xwmc96 and Xcfd68) as per the diploid wheat map (Singh *et al.* 2007). These three SSR markers were used to genotype individual plants of the F_2 mapping population. A linkage map was constructed with *MAPMAKER/EXP v. 3.0* (Lander *et al.* 1987, Lincoln *et al*. 1993) according to the linkage data of the *clm1* loci and polymorphic SSR markers in the F_2 mapping population.

Results

The Chl-deficient mutants (*clm1*) were erect with shorter stems and roots, small flag leaf, lower tiller numbers, less spikelets per spike, and slower growth rate as compared to WT parent *T. monococcum* (Fig. 1*A,B*). The change in color was observed in the *clm1* mutant in comparison to WT at 14 d after sowing. The *clm1* mutant exhibited yellow-green leaves (14 to 56 d after sowing) which gradually changed from pale yellow to yellowish green (approximately 70 d after sowing) and became pale green just before flowering (approximately 84 d after sowing). The *clm1* mutant was more sensitive to a herbicide *Topik* as compared to WT.

Chlorophyll content was measured in the *clm1* and WT at 14-d intervals up to 84 d (Table 1).

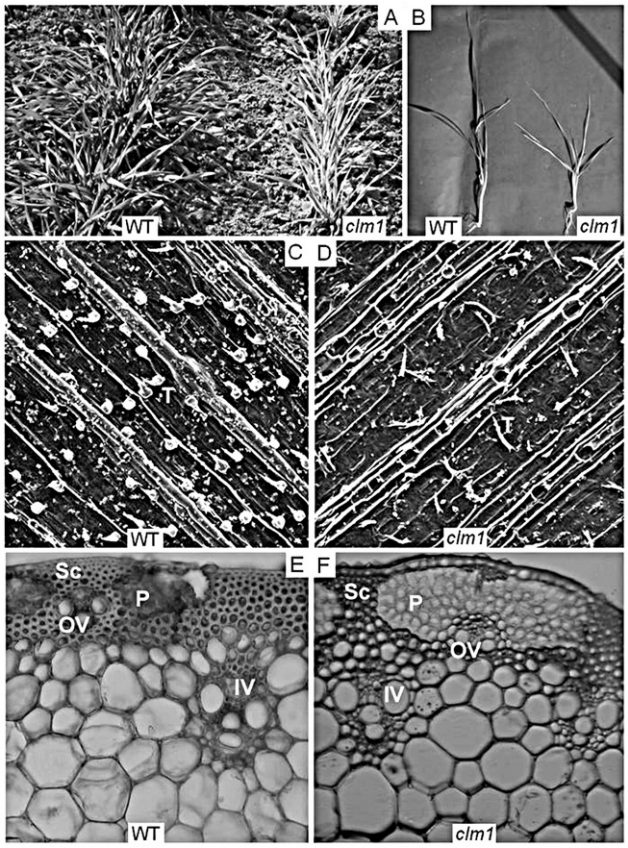

Fig. 1. Morphological and histological comparison of *T. monococcum* wild type (WT) and its *clm1* mutant: *A* - field view; *B* - 25-d-old seedling; *C* and *D* - scanning electron microscopy of trichomes on upper leaf surface of WT and *clm1* mutant (45-d-old seedlings (150×);. *E* and *F* - transverse section of second internode (toluidine blue staining) of WT and *clm1* mutant (Sc - sclerenchyma, P - parenchyma; OV - outer or peripheral vascular bundles; IV - inner vascular bundle; T - trichome).

Table 1. Chlorophyll content [mg g^{-1}(f.m.)] in the leaves during ontogeny of *T. monococcum* WT and *clm1* mutant (mean ± SE, $n = 3$).

Ontogeny	WT			*clm1*		
	Chl *a*	Chl *b*	Chl *a*+*b*	Chl *a*	Chl *b*	Chl *a*+*b*
Day 14	1.10 ± 0.02	0.26 ± 0.016	1.36 ± 0.02	0.21 ± 0.01	0.07 ± 0.009	0.28 ± 0.01
Day 28	1.82 ± 0.02	0.38 ± 0.02	2.20 ± 0.03	0.31 ± 0.01	0.11 ± 0.01	0.42 ± 0.02
Day 56	1.98 ± 0.03	0.41 ± 0.02	2.39 ± 0.02	0.64 ± 0.02	0.21 ± 0.01	0.85 ± 0.02
Day 84	2.16 ± 0.03	0.47 ± 0.02	2.63 ± 0.03	1.08 ± 0.02	0.41 ± 0.01	1.49 ± 0.02

The *clm1* mutant showed reduced content of total Chl (43.3% to 84.5 % reduction) and an increased Chl *a*/*b* ratio. In comparison to the WT, the content of Chl *a* and Chl *b* remained significantly reduced throughout the whole *clm1* development with the lowest values at 14 d seedling stage. Increased Chl *a*/*b* ratio occurred probably because of the relatively lower synthesis of Chl *b* than Chl *a*. However, *clm1* mutant accumulated significant quantities of Chl in the upper leaves to become slightly green at flowering stage.

The morphology and distribution of trichomes in WT and *clm1* mutant varied in length, density, and type. In *clm1* mutant, the number of trichomes was low whereas in the WT they were packed densely. In the leaves of WT, three types of trichomes were found. One type had a round globular base without a pointed tip which occurred near the stomata and over the veins, the second type had a globular base with a short pointed tip and the third type had a globular base with a long pointed tip. All three types were observed over the upper leaf surface although the third type was rare. In the *clm1* mutant, only one type of elongated trichomes with a pointed tip and without a globular base were observed. All the trichomes in the WT and *clm1* were unbranched. A higher density of glandular and globular trichomes was observed over the leaf veins of the WT, whereas no trichomes were observed over the leaf veins in *clm1*. It indicated the pleiotrophic effect of *clm1* locus which not only affects the Chl content but also plays a significant role in controlling the shape and types of trichomes (Fig. 1*C,D*).

To determine whether the less Chl accumulation was associated with a change in the histology of *clm1* mutant, transverse sections of the culm of the WT and mutant were stained with toluidine blue. The transverse section revealed that the diameter of the second internode in the WT was larger than that in the *clm1* mutant. Cell density (number of cells per unit area) of the internode was similar in both the *clm1* mutant and WT. The amount of sclerenchymatous tissues in the *clm1* mutant was significantly reduced and only small strips of sclerenchyma were observed attached to the epidermis on the outer side and to vascular bundles towards the inner side in the *clm1* mutant. However, in WT, sclerenchyma formed a thick and dense layer towards the periphery and around the vascular bundles. Large chloroplast-containing parenchyma, which was similar to mesophyll cells of the leaves, was present in the *clm1* mutant probably to absorb more sunlight and to increase the photosynthetic rate. The increased size of the parenchyma was negatively correlated with the reduced amount of Chl in the chloroplast presumably in an effort to compensate for the reduced photosynthetic rate in *clm1*. In WT, few Chl-containing parenchyma tissues were present. In *clm1*,

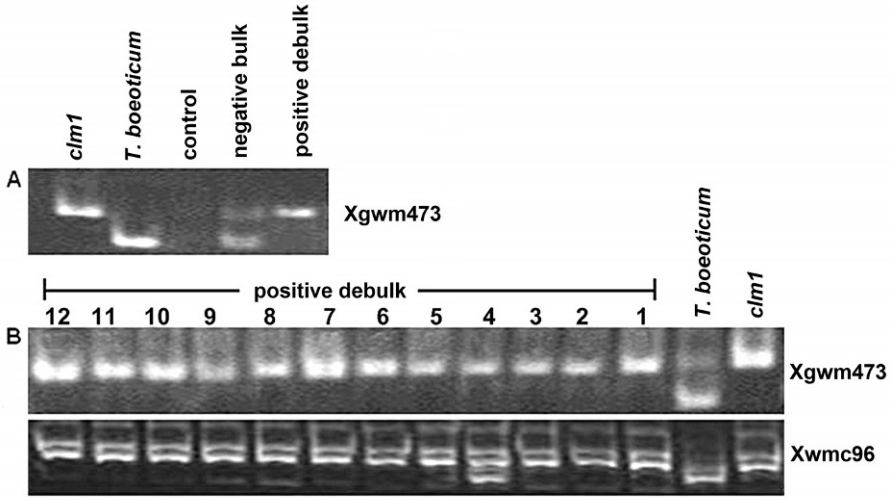

Fig. 2. *A* - Identification of putative SSR marker Xgwm473 linked with *clm1* mutant through bulk segregant analysis. *B* - PCR amplification of debulks of positive bulks (1 - 12) of F_2 plants using putatively linked marker Xgwm473 identified in BSA and its closely linked marker Xwmc96 (Singh *et al*. 2007).

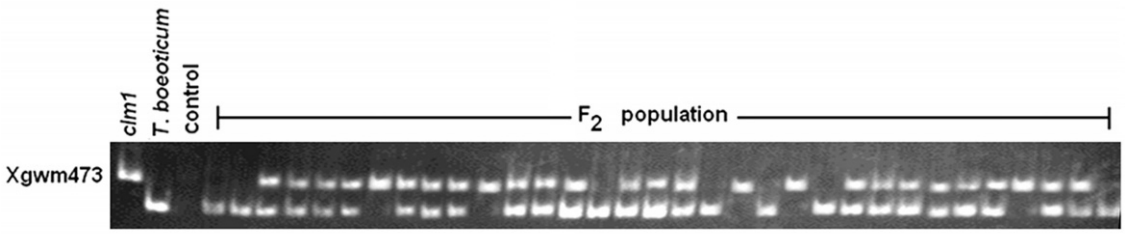

Fig. 3. The segregation of SSR marker *Xgwm473* in representative 35 F_2 plants of *clm1* × *T. boeoticum* cross.

peripheral vascular bundles were surrounded by parenchyma from the outer side, whereas in WT, peripheral vascular bundles were completely surrounded by sclerenchyma. (Fig. 1*E*,*F*).

The F_1 obtained from the cross of *T. monococcum* × *clm1* mutant was like the WT parent indicating that the *clm1* mutant was recessive. The segregation for Chl-deficiency was found in the F_2 population. Out of 160 F_2 plants in total, 117 plants were WT and 43 were found Chl-deficient (chlorina) which gave a good fit to 3:1 ratio indicating that the *clm1* mutant was monogenic recessive.

Anchored SSR markers (133) polymorphic between *T. monococcum* and *T. boeoticum* (Singh *et al.* 2007) were used for BSA. The marker Xgwm473 and Xwmc96 (not shown in BSA) located on chromosome $7A^mL$ showed polymorphism between the positive and negative bulk for *clm1* (Fig. 2*A*). Debulking of positive bulk plants also showed close linkage with markers xgwm473 and Xwmc96 in the *clm1* mutant. Genotyping of the debulked F_2 plants with the putatively linked markers from BSA confirmed their close linkage with *clm1* mutant (Fig. 2*B*). To map the *clm1* mutant, F_2 mapping population developed by crossing the *clm1* mutant with *T. boeoticum* acc. pau 5088, a non-chlorina and tall wild progenitor of *T. monococcum*, was used. A total of 160 individual F_2 plants were used for genotyping of *clm1* F_2 populations (Fig. 3). Co-segregation analysis of individual markers, Xgwm473, Xwmc96, and Xcfd68 located on chromosome $7A^mL$ and *clm1* using the marker and mutant genotypes of F_2 plants, was carried out with the help of recombination frequency among markers at each locus. Based on the data on recombination frequency, the marker Xgwm473, Xwmc96, and Xcfd68 were mapped at distances of 2.1, 2.6, and 4.6 cM, respectively, from the *clm1* gene on chromosome $7A^mL$ (Fig. 4).

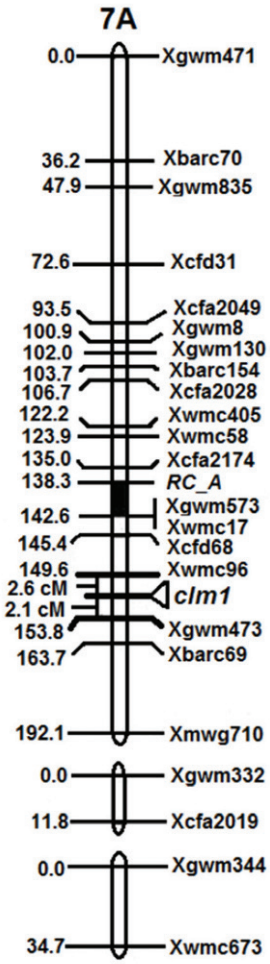

Fig. 4. Mapping of *clm1* mutant on chromosome 7AL of *T. monococcum* (Singh *et al.* 2007).

Discussion

The EMS-induced Chl-deficient mutant *clm1* was used for phenotypic and molecular characterization in the present study. The *clm1* mutant was found to be controlled by a single recessive gene. Several Chl-deficient mutants have been identified in tetraploid and hexaploid wheat and mapped on A, B, and D genomes (Sears and Sears 1968, Pettigrew *et al.* 1969, Washington and Sears 1970, Freeman *et al.* 1987, Falbel and Staehelin 1994, Guo *et al.* 1996). However, no Chl-deficient mutant has been reported in diploid wheat. The *clm1* mutant exhibits a yellow green leaf phenotype and reduced content of Chl *a* and *b* at the seedling stage. The Chl content reached maximum of 67 % of the WT, *T. monococcum*, just before flowering and the upper leaves of the *clm1* mutant ultimately turned green. This suggests that the *clm1* mutant exhibited delayed greening during photomorpho-genesis because of a slower rate of Chl accumulation. The *clm1* mutation affected Chl

synthesis most drastically in the early developmental stages and recovered in the later stage during flowering suggesting that it may be a development stage specific or a conditional mutant. These results were consistent with those of Wu *et al.* (2007), Du *et al.* (2009) and Zhang *et al*. (2011) .

Three types of trichomes were observed in the WT. However, elongated trichomes with pointed tip and without a globular base clearly different from the trichomes present in the *T. monococcum* were present in the *clm1* mutant. This indicated that *clm1* mutation not only affected the Chl content but also had a pleitropic effect on leaf trichome morphology, type, and distribution. The transverse section of second internode revealed an increase in the quantity of Chl-containing parenchyma in *clm1* mutant. Several Chl-deficient mutants have been reported in tetraploid and hexaploid wheats. All these mutants exhibited reduced Chl *b* content, a significant reduction in light harvesting CP complex, and formation of grana-deficient thylakoid membranes (Falbel *et al.* 1996). Some of these mutants have been mapped to chromosome 7 A, B, or D genomes in both hexaploid (*T. aestivum*) and tetraploid (*T. turgidum*) wheat (Falbel and Staehelin 1994).

In the F_2 population between the *clm1* mutant and its WT, *T. monococcum*, WT phenotype and the mutant showed a segregation ratio of 3:1 (green : yellow plants) indicating that the *clm1* mutant was controlled by a single recessive nuclear gene. Such mutants probably could not have been recovered in polyploid wheat because of their orthologous loci on other genomes unless multiple mutants were induced at all the loci or certain loci were silenced during evolution. The *clm1* mutant has been mapped to chromosome $7A^m$ of diploid wheat, *T. monococcum*, which is similar to the findings previously reported for Chl-deficient mutants, such as *CD3* in hexaploid wheat on the homoeologous chromosome 7 (Falbel and Staehelin 1996). Similar mutants were obtained in rice (Huang *et al.* 2008), barley (Liu *et al.* 2008), and soybean (Zhang *et al.* 2011). The photosynthetic metabolic pathway is very complex, and many genes have remained undiscovered. Several QTLs and genes related to Chl metabolic pathway have been mapped in rice (Chen *et al.* 2007, 2009, Shen *et al.* 2007, Huang *et al.* 2008, Jiang *et al.* 2008, Wang *et al.* 2009, Li *et al.* 2010, Sang *et al.* 2010), and soybean (Zou *et al.* 2003, Zhang *et al.* 2011). Map-based cloning was done for yellow green leaf 1 (*ygl1*), a Chl-deficient recessive mutant reported in rice with yellow green leaves, decreased Chl content, and delayed chloroplast development in young plants (Wu *et al.* 2007). The *clm1* mutant also had reduced Chl content and inheritance similar to *ygl1*.

Chl deficiency is one of the most important agronomic traits that affect not only grain production but also the usefulness of cereal straws as animal forage. As an important locus regulating wheat Chl synthesis, knowledge of *clm1* (and its orthologs in other cereals) could make a significant contribution to the future improvement of wheat for enhanced Chl content and photosynthetic rate.

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