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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