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# Monogalactosyldiacylglycerol deficiency in tobacco inhibits the cytochrome $b_6f$-mediated intersystem electron transport process and affects the photostability of the photosystem II apparatus

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ABSTRACT

Monogalactosyldiacylglycerol (MGDG) is the most abundant lipid component of the thylakoid membrane. Although MGDG is believed to be important in sustaining the structure and function of the photosynthetic membrane, its exact role in photosynthesis in vivo requires further investigation. In this study, the transgenic tobacco plant M18, which has an MGDG deficiency of approximately 53%, and which contains many fewer thylakoid membranes and exhibits retarded growth and a chlorotic phenotype, was used to investigate the role of MGDG. Chlorophyll fluorescence analysis of the M18 line revealed that PSII activity was inhibited when the plants were exposed to light. The inactive linear electron transport found in M18 plants was mainly attributed to a block in the intersystem electron transport process that was revealed by P700 redox kinetics and PSI light response analysis. Immunoblotting and Blue Native SDS-PAGE analysis suggested that a reduction in the accumulation of cytochrome $b_6f$ in M18 plants is a direct structural effect of MGDG deficiency, and this is likely to be responsible for the inefficiency observed in intersystem electron transport. Although drastic impairments of PSII subunits were detected in M18 plants grown under normal conditions, further investigations of low-light-grown M18 plants indicated that the impairments are not direct structural effects. Instead, they are likely to result from the cumulative photodamage that occurs due to impaired photostability under long-term exposure to relatively high light levels. The study suggests that MGDG plays important roles in maintaining both the linear electron transport process and the photostability of the PSII apparatus.



## 1. Introduction

In oxygen-evolving photosynthetic organisms, the thylakoid membrane is the specific site where the light reaction takes place. Through the close cooperation of four important cofactor-protein complexes embedded in the lipid matrix, namely photosystem II (PSII), photosystem I (PSI), cytochrome $b_6f$ ($Cytb_6f$) and ATP synthase (ATPase), thylakoid membranes efficiently convert solar energy to active chemical energy in the form of ATP and NADPH and supply these to the subsequent $CO_2$ assimilation process that occurs in the chloroplast stroma (in algae and higher plants) or the cytosol (in cyanobacteria) and through which stable chemical energy is finally stored in the form of carbohydrate. It is generally accepted that the thylakoid membrane, which is mainly composed of glycolipids instead of the phospholipids found in other biological membranes, provides an exclusive matrix in which the photosynthetic apparatus is able to sustain its structural and functional integrity. In higher plants, the thylakoid membrane is characterized by high levels of two galactolipids, namely monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG). These account for approximately 50% and 30% of the total thylakoid lipids, respectively, while two other lipids, sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG), each account for only 5–12% [1–3]. Besides their high abundance in the thylakoid membrane, galactolipids are rich in polyunsaturated fatty acyl chains, which give them unusual biophysical features [4] and enable them to maintain the appropriate fluidity and flexibility of the membrane [2]. In the case of MGDG, a small galactose head group and two acyl chains rich in double bonds, which occupy a larger space, give it a conical molecular shape and a tendency to form the so-called hexagonal $H_{II}$ structure in vitro. In the case of DGDG, however, an additional galactose molecule attached to the head group gives it a cylindrical molecular shape, thus making it well suited to the formation of a bilayer [4]. In fact, MGDG is the only non-bilayer-forming lipid in the thylakoid membrane and is thus considered to be crucial for the organization of a highly stacked thylakoid membrane structure [5]. In addition, crystallographic studies have shown that MGDG is also the most

*Abbreviations:* PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; UDP, uridine diphosphate; $Q_A$, primary quinone acceptors of photosystem II; PTGS, post-transcriptional gene silencing

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abundant integral lipid in the PSII complex [6,7] (the so-called "functional lipids" [2]), and this may be an essential factor in several processes, such as PSII monomer–monomer interactions and the PSII repair process (for recent reviews, see [8,9]).

In seed plants, the biosynthesis of MGDG exclusively occurs in plastids, and its final step is catalyzed by MGDG synthase (EC 2.4.1.46, UDP-galactose: 1, 2-diacylglycerol 3-β-D-galactosyltransferase), which transfers galactose from UDP-galactose to diacylglycerol (DAG) [10,11]. Although three functional MGDG synthases [MGD1 (A-type), MGD2 and MGD3 (B-type)] have been identified in the model plant *Arabidopsis thaliana*, MGD1, encoded by the *AtMGD1* gene, is considered to be predominantly responsible for the biosynthesis of the bulk of the MGDG synthesized during thylakoid membrane biogenesis in green tissues [12,13]. The significance of MGDG in maintaining the structural and functional integrity of the thylakoid membrane in vivo has been substantiated by studies of two Arabidopsis MGD1 mutants. In the *mgd1-1* mutant [14], which has an MGDG deficiency of approximately 42%, the chloroplasts were severely underdeveloped and contained fewer internal thylakoid membranes, especially the grana lamellae. In addition, the *mgd1-1* mutant was found to contain about 50% less chlorophyll per plant than was found in wild type plants, and this deficiency was responsible for its chlorotic phenotype. Analysis of *mgd1-2* [15], a null mutant in which MGDG and DGDG are almost absent, revealed that the development of photosynthetic membranes almost completely fails in these plants and no chlorophyll or PSII proteins accumulate, leading to the loss of both photosynthetic activity and photoautotrophic ability. Nevertheless, compared with DGDG, whose roles in photosynthesis have been extensively investigated using the *dgd1* mutant [16] and the *dgd1 dgd2* double mutant [17] (for reviews, see [3,18]), our understanding of the exact roles of MGDG in photosynthetic activities is limited at present. It is impossible to address this issue by studying the *mgd1-2* mutant due to the disorganization of the thylakoid membranes that is a characteristic of this mutant line. Aronsson et al. [19] investigated the photosynthetic properties of *mgd1-1* and suggested that a 42% deficiency of MGDG in Arabidopsis has no impact on photosynthetic efficiency or the accumulation of photosynthetic proteins in low-light-grown plants, though the mutant was more susceptible to light stress. Although obtaining viable mutants with greater MGDG deficiencies would be of significant benefit to further investigations, isolating such mutants would be problematic because it is difficult to control the extent of the MGDG knockdown that results from genetic manipulation. Moreover, severe lipid mutants, especially of dwarfish Arabidopsis plants, always have survival difficulties.

Thanks to the broad evolutionary conservation and distribution of the A-type MGDG synthase in plant species and its predominant role in MGDG biosynthesis in green tissues, it is possible to expand our understanding of the role of MGDG in photosynthesis by studying other plants. We previously identified several transgenic tobacco lines with MGDG deficiencies that resulted from post-transcriptional gene silencing (PTGS) of *NtMGD1* [20]. In this study, a well-established transgenic plant line, designated as M18, with about 53% MGDG deficiency was utilized to investigate the role of MGDG in sustaining photosynthetic structure and function in vivo. Like Arabidopsis, tobacco has been shown to be a typical "16:3 plant" [21], which means that both the prokaryotic pathway (characterized by a 16-C fatty acid at the sn-2 position in the MGDG molecule) and the eukaryotic pathway (characterized by an 18-C fatty acid at the sn-2 position in the MGDG molecule) are involved in MGDG biosynthesis [22]. This means that, to some extent, it is reasonable to discuss our results in parallel with those obtained previously in Arabidopsis. The MGDG deficiency of greater than 50% that is found in the M18 line is expected to provide us with significant new insight into MGDG function.

Investigation of the *mgd1-1* mutant showed that low-light-grown mutant plants suffered from increased PSII photoinhibition compared with wild type plants when subjected to a short-term high light treatment [19]. As photoinhibition always involves the loss of active PSII centers, which need to be recovered by degradation and synthesis of the D1 protein [23], the increase in PSII photoinhibition seen in the mutant plants implies that MGDG may play an important role in maintaining the photostability of the PSII apparatus. In this respect, it would be advisable to take the photoinhibition event into account when investigating photosynthesis in MGDG-deficient plants, especially when different light intensities are used for growth. In the present study, photoinhibition was a major focus and was considered to be an important factor in distinguishing between the direct and indirect effects of MGDG deficiency in M18 tobacco.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Wild type (WT) common tobacco (*Nicotiana tabacum* L. cv. Wisconsin-38) was used as the control plant in this study. WT and $T_0$ generation M18 plants were maintained by micropropagation on 0.8% solidified agar containing half-strength Murashige and Skoog medium (pH 5.8) supplemented with 3% sucrose. Seedlings in flasks were grown in a growth chamber at 28 °C (daytime) and 26 °C (nighttime) with a light intensity of approximately 60 μmol $m^{-2}$ $s^{-1}$ under a photoperiod of 14 h light/10 h dark. For examination of tobacco plants grown under normal conditions, 3- to 4-week-old seedlings were transferred to soil and grown for another 4 weeks in a greenhouse at 25–28 °C under controlled light conditions with a maximum daily light intensity of approximately 500 μmol $m^{-2}$ $s^{-1}$ at noon in a natural photoperiod. To examine the tobacco plants grown under low light conditions, 3- to 4-week-old seedlings were transferred to soil and grown for another 2 weeks with a light intensity of 100 μmol $m^{-2}$ $s^{-1}$ and with all other conditions maintained as described for seedlings grown in the growth chamber. Unless otherwise stated, all measurements were carried out using the fully expanded leaves (fourth or fifth leaves from the top) of the tobacco plants.

### 2.2. RNA extraction, isolation of NtMGD2 cDNA and RT-PCR analysis

Total RNA was extracted from the tobacco leaves using a Transzol reagent kit (TransGen Biotech Co., Ltd., Beijing, China) in accordance with the manufacturer's instructions. After treatment with DNase I to eliminate any DNA contamination, 3–5 μg of total RNA from each sample was used as the template for first strand cDNA synthesis by the M-MLV reverse transcriptase (Promega, USA). To isolate an *NtMGD2* cDNA fragment, several B-type *MGD* cDNA and EST sequences identified in a database (*A. thaliana MGD2*, NM_122048; *A. thaliana MGD3*, NM_126865; *Vigna unguiculata*, EF466098; *Glycine max*, AW666268) were aligned and the following degenerate PCR primers were designed: sense, 5′-ATGCAR(A/G)CAY(C/T)ATW(A/T)CCATTGTG-3'; antisense, 5'-TCACAR(A/G)GCTCCCATCCAT-3'. A partial *NtMGD2* sequence was obtained by PCR amplification and 3' rapid amplification of cDNA ends (3'-RACE) [24] was then carried out. For RT-PCR analysis of *NtMGD1* and *NtMGD2* transcript levels, specific primers were designed as follows: *NtMGD1*-sense, 5'-ACTCAAGAACCCACTAACCC-3'; *NtMGD1*-antisense, 5'-CTGTCCAGCAATGTAATCAT-3'; *NtMGD2*-sense, 5'-CATTGTGGGTTCTTAAATGG-3'; *NtMGD2*-antisense, 5'-GGAATGTTTGCAAGTGGACC-3'. The expression level of a tobacco *ACTIN* was used as the internal control and the primer sequences used were as follows: *ACTIN*-sense, 5'-CCCTCCCACATGCTATTCT-3'; *ACTIN*-antisense, 5'-AGAGCCTCCAATCCAGACA-3'.

### 2.3. Lipid extraction and fatty acid analysis

Lipid extraction was carried out in general accordance with an established protocol [25] but with minor modifications. For total fatty acid analysis, a moiety of the lipid extract was directly