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Research article

The *toc*132*toc*120 heterozygote mutant of *Arabidopsis thaliana* accumulates reduced levels of hexadecatrienoic acid

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ABSTRACT

A null and heterozygous mutant for the *Arabidopsis thaliana TOC*132 and *TOC*120 genes accumulates increased levels of 16:0 and decreased 16:3, suggesting altered homeostasis in fatty acid synthesis. The *FAD*5 gene encodes a plastid desaturase that catalyzes the first step in the synthesis of 16:3 in monogalactosyldiacylglycerol (MGDG). In non-acclimated *toc*132*toc*120+/− mutant plants, the *FAD*5 gene was repressed and this correlated with decreased levels of 16:3. In cold-acclimated mutant however, the *FAD*5 gene was upregulated and there was a small increase in 16:3 levels relative to the non-acclimated mutant plants. The *MGD*1 gene was expressed at control levels and the mutant accumulated levels of MGDG that were similar to the wild type. In the mutant however, MGDG had decreased 16:3 levels, suggesting that the activity of FAD5 desaturase was compromised. In the mutant, the *FAD*2 and *FAD*3 genes were downregulated but levels of 18:3-PC were increased, suggesting posttranscriptional regulation for the ER-localized fatty acid desaturases. The Toc120 or Toc159 receptor is likely to compensate for a defective Toc132 receptor. In the cold-acclimated mutant, the *TOC*159 gene was repressed ca. 300-fold, whereas the *TOC*120 gene was repressed 7-fold relative to the non-acclimated wild type. Thus, the *TOC*159 gene is more sensitive to cold-stress and might not compensate for defect in the *TOC*132 gene under these conditions. Overall, these data show that a mutation in the *TOC*132 gene results in decreased 16:3 levels, indicating the need for an intact Toc132/Toc120 receptor, presumably to facilitate the import of the FAD5 preprotein into chloroplasts.

1. Introduction

The majority of proteins required for chloroplast biogenesis are nuclear-encoded and posttranslationally imported into the plastids. Most of the enzymes that function in photosynthesis and lipid synthesis are nuclear-encoded. The development of thylakoid membranes requires monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), which accounts for more than 70% of total thylakoid lipids (Dörmann, 2007; Kobayashi et al., 2007). Photosystems with their associated proteins, chlorophylls and carotenoids are embedded in the thylakoid membranes. These pigments are required for the assembly and provide stability to the light harvesting complex (LHC) apoproteins (Dörmann, 2007; Pogson et al., 1998).

In chloroplasts, the nuclear-encoded pyruvate dehydrogenase uses the photosynthate to make acetyl-CoA available for the synthesis of fatty acids (Tovar-Mendez et al., 2003). Acetyl-CoA may also be imported into plastids from the mitochondria (Harwood, 1996; Ohlrogge and Browse, 1995). In the stroma, fatty acid synthesis yields palmitic acid (16:0), which is elongated to stearate (18:0) by 3-ketoacyl-ACP synthase II (KAS II) (Harwood, 1996; Martz et al., 2006; Ohlrogge and Browse, 1995). Stearate is desaturated into oleate (18:1) by a stromal enzyme, stearoyl-ACP desaturase. In the plastids, palmitic and oleic acids are transferred from acyl carrier protein (ACP) to glycerol-3-phosphate and yields phosphatidate. The latter is a precursor in the synthesis of MGDG, DGDG, sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) (Ohlrogge and Browse, 1995). Palmitate and oleate are also exported to the ER and used in the synthesis of

Abbreviations: MGDG, monogalactosyldiacylglycerol; 16:3, hexadecatrienoic acid; FAD5, fatty acid desaturase 5.

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phosphatidylcholine (PC), phosphatidylethanolamine (PE) and other phospholipids (Wallis and Browse, 2010). Diacylglycerol (DAG) that is derived from PC is transported to chloroplasts and used in the synthesis of MGDG and DGDG (Benning, 2008). The MGD1 synthase, uses both the plastid and ER-derived DAG to synthesize MGDG (Awai et al., 2001; Benning and Ohta, 2005; Dörmann, 2007; Kobayashi et al., 2007). By contrast, DGD1 synthase, an enzyme that synthesizes the bulk of DGDG prefers eukaryotic MGDG as a substrate (Benning and Ohta, 2005; Kobayashi et al., 2007). It is well documented that DGDG contains more than 70% of α-linolenic acid (18:3). In *Arabidopsis*, hexadecatrienoic acid (16:3) and 18:3 account for ca. 30 and 58% of the total fatty acids in MGDG (Hendrickson et al., 2006; Kim et al., 2010). The plastid-derived 16:0-MGDG is desaturated by the FAD5 enzyme to yield 16:1Δ^7-MGDG (Heilmann et al., 2004). The activities of FAD6 and FAD7/8 desaturases result in the synthesis of 16:3$\Delta^{7,10,13}$ in MGDG. Thus, 16:3 is contributed exclusively by the prokaryotic pathway and levels of 16:3 in MGDG are good indicators of the activities of FAD5, FAD6, and FAD7/8 desaturases.

Enzymes that are encoded by *MGD*1 and *FAD*5 genes contain chloroplast targeting sequences, which suggest their import might be mediated by the general protein import pathway. The nuclear-encoded FAD6, FAD7 and FAD8 desaturases lack chloroplast transit peptides (Falcone et al., 1994; Gibson et al., 1994; Iba et al., 1993), and it is not known if they engage the plastid import machinery. A recent study suggests that superoxide dismutase, which lacks a chloroplast transit peptide, requires an ortholog of atToc120 receptor for its import into pea chloroplasts (Chang et al., 2014). Nevertheless, enzymes that catalyze the synthesis and desaturation of glycerolipids are nuclear-encoded and posttranslationally imported into the plastids.

Plastids evolved from the photosynthetic bacteria that had an endosymbiotic relationship with a eukaryote host cell. Majority of the bacterial genes were transferred to the nucleus of ancestral eukaryote cell. Hence, the biogenesis of plastids requires ca. 3000 proteins that are nuclear-encoded (Jarvis, 2008; Kessler and Schnell, 2009). Consequently, plants evolved mechanisms to coordinate the expression of nuclear genes with the developmental and functional state of the plastids (Inaba et al., 2011; Jarvis and Lopez-Juez, 2013). The cytoplasm-synthesized proteins are imported into the plastids in a process that is mediated by translocons or organized protein complexes at the outer and inner envelope membranes of chloroplasts called TOC/TIC complexes (Jarvis, 2008; Kessler and Schnell, 2009). In *Arabidopsis*, the *TOC*159, *TOC*132, *TOC*120 and *TOC*90 genes encode the Toc159 protein family, and the Toc159 and Toc132/Toc120 receptors recognize different types of proteins (Jarvis, 2008; Kessler and Schnell, 2009). The Toc132 and Toc120 receptors are found in the same protein complex and are functionally redundant (Ivanova et al., 2004). Details of the ultrastructure revealed that a mutant of *Arabidopsis* that is null for the *TOC*132 or *TOC*120 gene has normal chloroplasts, indicating that the loss of either the *TOC*132 or *TOC*120 gene does not affect the biogenesis of plastids (Ivanova et al., 2004; Kubis et al., 2004). The *toc*132 (homozygous) *toc*120 (heterozygous) had smaller chloroplasts and less developed thylakoids, indicating the need for at least one intact allele of either *TOC*132 or *TOC*120 in the biogenesis of plastids (Ivanova et al., 2004; Kubis et al., 2004). The *toc*132*toc*120 double homozygotes had a severe albino phenotype (Kubis et al., 2004) or were embryo lethal (Ivanova et al., 2004), which indicate the need for an intact *TOC*132 or *TOC*120 allele for seedlings to be viable (Ivanova et al., 2004). Studies suggest that the atToc159 receptor does recognize and imports photosynthetic proteins (Bauer et al., 2000; Smith et al., 2004), while the atToc132/atToc120 receptor imports non-photosynthetic proteins (Ivanova et al., 2004; Kubis et al., 2004).

In our study, we tested whether the *toc*132 (homozygous) *toc*120 (heterozygous) mutant, henceforth referred to as *toc*132*toc*120+/− mutant, was capable of accumulating normal levels of fatty acids and glycerolipids. We measured the levels of mRNA for fatty acid desaturases and galactolipid synthesis enzymes. Since the atToc132/atToc120 receptor recognize and facilitates the import of non-photosynthetic proteins, we speculated that this receptor might mediate the import of lipid synthesizing enzymes into chloroplasts.

2. Methods

2.1. Plant material and growth conditions

The *toc*132*toc*120+/− mutant of *Arabidopsis thaliana* ecotype Columbia was described by Kubis et al. (2004) and seeds were a kind gift by Paul Jarvis (University of Oxford, United Kingdom). The wild type and mutant seeds were sterilized and plated as described previously (Afitlhile et al., 2015). The 10-day-old wild type and mutant seedlings were transferred onto autoclaved soil and watered when the top soil was dry. Plants were fertilized with Miracle-Gro (20-20-20) once in 3 weeks. For cold acclimation, 4-week-old wild type and the *toc*132*toc*120+/− mutant plants were transferred and grown at 4 °C for 5 d under continuous white light.

2.2. Extraction of plant pigments

Leaf tissues were harvested from 4-week-old wild type and *toc*132*toc*120+/− mutant plants. Plant pigments were extracted in 80% acetone and tissue extracts were centrifuged briefly. The supernatant was decanted and absorbance measured at 470, 646 and 663 nm using Spectrophotometer (Spectronic 20D+, Thermo Scientific). The concentration of chlorophylls and carotenoids were determined as described previously (Wellburn, 1994).

2.3. Extraction and separation of polar lipids

The harvested leaves were frozen in liquid N_2 and pulverized. Lipids were extracted and processed as described previously (Afitlhile et al., 2015; Miquel and Browse, 1992). Separation of lipid classes by TLC was carried out as described by Wang and Benning (2011). Lipid samples were heated at 80 °C in 2.5% H_2SO_4 in methanol and fatty acid methyl esters in isooctane were analyzed by GC-FID as described previously (Afitlhile et al., 2015).

2.4. RNA extraction and cDNA synthesis

We followed the protocol in RNeasy Plant Mini kit (Qiagen) to extract total RNA from leaves of the wild type and *toc*132*toc*120+/− mutant plants. The cDNA was synthesized as described previously (Afitlhile et al., 2013, 2015).

2.5. Primer design and quantitative real-time PCR

Primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The gene-specific primer pairs to cDNA were as follows: *DGD*1 (At3g11670) 5′-CGTACCACCTCACGTTCACA-3′ (forward) and 5′-AAGCAATAACTGGGTGGCCT-3′ (reverse), *FAD2* (At3g12120) 5′-GTCCATATTGCGTTTGCGGG-3′ (forward) and 5′-CATCAGAGTGGGACTGGCTC-3′ (reverse), *TOC*120 (At3g16620) 5′-ACAGAGGAGCAGGGCAAGTA-3′ (forward) and 5′-CCATATTGCATTTGCTCAGGGG-3′ (reverse), *TOC*132 (At2g16640) 5′-GAACAATAGAGGAGCAGGGCA-3′ (forward) and 5′-GTCCATATTGCGTTTGCGGG-3′ (reverse), and *TOC*159 (At4g02510) 5′-GCGGACAGATCACAGTCAGA-3′ (forward) and 5′-TGTACTTGTCGTTCGTCGCT-3′