A chloroplast envelope membrane protein containing a putative LrgB domain related to the control of bacterial death and lysis is required for chloroplast development in Arabidopsis thaliana

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Received: 16 June 2011
Accepted: 10 August 2011


Key words: carbon partitioning, chloroplast development, leaf senescence, LrgB domain, membrane permeability, starch metabolism.

Summary

• A protein encoded by At1g32080 was consistently identified in proteomic studies of Arabidopsis chloroplast envelope membranes, but its function remained unclear. The protein, designated AtLrgB, may have evolved from a gene fusion of lrgA and lrgB. In bacteria, two homologous operons, lrgAB and cidAB, participate in an emerging mechanism to control cell death and lysis.
• We aim to characterize AtLrgB using reverse genetics and cell biological and biochemical analysis.
• AtLrgB is expressed in leaves, but not in roots. T-DNA insertion mutation of AtLrgB produced plants with interveinal chlorotic and premature necrotic leaves. Overexpression of full-length AtLrgB (or its LrgA and LrgB domains, separately), under the control of CaMV 35S promoter, produced plants exhibiting veinal chlorosis and delayed greening. At the end of light period, the T-DNA mutant had high starch and low sucrose contents in leaves, while the 35S:AtLrgB plants had low starch and high sucrose contents. Metabolite profiling revealed that AtLrgB appeared not to directly transport triose phosphate or hexose phosphates. In yeast cells, AtLrgB could augment nystatin-induced membrane permeability.
• Our work indicates that AtLrgB is a new player in chloroplast development, carbon partitioning and leaf senescence, although its molecular mechanism remains to be established.

Introduction

In Arabidopsis and many other plant species, triose phosphates, the direct products of photosynthetic carbon assimilation, are partitioned between starch and sucrose (Geiger & Servaites, 1994; Gibon et al., 2004; Smith et al., 2005). Starch is synthesized in the plastid stroma during the day and degraded at night to satisfy ongoing energy requirements. Sucrose is synthesized in the cytosol and used for photosynthetic cells themselves and transported to other nongreen tissues (Preiss, 1982; Smith & Stitt, 2007; Zeeman et al., 2007; Stitt et al., 2010). Recently, metabolite profiling of 94 Arabidopsis accessions has revealed that starch is a major integrator in the regulation of plant growth (Sulpice et al., 2009). Circadian control of starch turnover to maltose and glucose to support plant growth at night has been found to be necessary for maintaining plant productivity (Graf et al., 2010).

Plastid inner envelope phosphate translocators comprise the best-characterized transporter family related to carbon partitioning. Until now, four distinct subfamilies of phosphate translocators have been characterized: the triose-phosphate/phosphate translocator (TPT), the glucose 6-phosphate/phosphate translocator (GPT), the phosphoenolpyruvate/phosphate translocator (PPT), and the xylulose 5-phosphate/phosphate translocator (XPT) (Flügge, 1995, 1999; Weber, 2004; Weber et al., 2005; Linka & Weber, 2010). Besides the phosphate translocators, plastids...
have a maltose transporter MEX1, a novel Plantae-specific protein that is unrelated to other hexose transporters (Niittyla et al., 2004; Tyra et al., 2007).

Proteomics is a very powerful approach to reveal the function of plastid envelope membranes (Rolland et al., 2003; Bräutigam & Weber, 2009; Joyard et al., 2009; Ferro et al., 2010). Proteomic studies of Arabidopsis chloroplast envelope membranes have identified many uncharacterized proteins (Ferro et al., 2002, 2003; Froehlich et al., 2003; Kleffmann et al., 2004; Mitra et al., 2007; Zybalov et al., 2008). In these surveys, a novel protein encoded by At1g32080 was consistently identified. The abundance of this protein in plastid envelope membranes was found to be as high as the TPT (Ferro et al., 2003; Bayles, 2007; Rice & Bayles, 2008). Null mutations of lrgA in Staphylococcus aureus produced increased extracellular murein hydrolase activity, while overexpression of LrgA and LrgB in wild-type cells resulted in a significant decrease in extracellular murein hydrolase activities (Groicher et al., 2000). The LrgA protein shares many characteristics with bacteriophage antiholins. Holins and antiholins are often different only in a few amino acids at the N-terminus, and they control the formation of channels for tiholins are often different only in a few amino acids at the C-terminus and a low-similarity LrgA domain in the N-terminus (Fig. 1).

In bacteria, an emerging mechanism that controls cell death and lysis has been illuminated recently by studies of terminus (Fig. 1).

Based on a set of cell biological, biochemical and genetic results, we suggest that AtLrgB is a new player in chloroplast development, carbon partitioning and leaf senescence, although its molecular function remains to be established.

Materials and Methods

Plant materials and growth condition

Arabidopsis thaliana ecotype Columbia-0 was used as a source of wild-type plant material. Seeds were surface-sterilized and cultured aseptically on 9 cm Petri dishes containing Gamborg’s B5 medium with 1% (w/v) sucrose and 1% (w/v) agar. The plates were maintained at 4°C for 2 d, and then transferred to a culture room (23°C, 80 μM m⁻² s⁻¹ irradiance with a 16 h photoperiod, 30–40% RH).

Isolation of T-DNA insertion mutants

Screening of T-DNA insertion mutants was accomplished by genomic PCR using gene-specific primers AtLrgB-F1 and AtLrgB-R0, and T-DNA left border (LB) primer LB-P (Table S1, Fig. S2). Reverse transcription polymerase chain reaction (RT-PCR) of the AtLrgB transcript was performed using primers AtLrgB-F1 and AtLrgB-R2 targeted for the 5' and 3' ends of the AtLrgB coding sequence (Table S1).

Vector construction and plant transformation

We used the GATEWAY™ system for vector construction. Entry vectors were created using the pENTR™/D-TOPO kits (Invitrogen). The PCR primers for construction of entry vectors are listed in Table S1. Each entry clone was confirmed by DNA sequencing. To generate an AtLrgB: EGFP (enhanced green fluorescent protein) fusion, the AtLrgB coding sequence was amplified by PCR using an AtLrgB cDNA clone (stock number RAFL05-04-H06, RIKEN-BRC), as the template, with the primers AtLrgB-

Fig. 1 Schematic diagram of the lrgAB operon and the AtLrgB protein. (a) Organization of the lrgAB operon of Staphylococcus aureus. The direction of transcription is indicated by arrows. Approximate sizes of lrgA transcripts and protein sizes of LrgA and LrgB are shown. (b) Organization of the AtLrgB protein of Arabidopsis thaliana, indicating the chloroplast transit peptide (CTP), the LrgA domain and the LrgB domain. The length of each part of AtLrgB is given as the number of amino acids (aa). (c) Schematic representation of the predicted membrane topology of AtLrgB.
F1 and AtLrgB-R1. For promoter activity analysis, a c. 1 kb fragment upstream of the AtLrgB translational start codon was PCR-amplified from genomic DNA with the primers AtLrgB-P1 and AtLrgB-P2. For overexpression of full-length AtLrgB, the coding sequence was amplified by PCR with the primers AtLrgB-F1 and AtLrgB-R2, using the AtLrgB cDNA clone as the template. For overexpression of truncated forms of AtLrgB, the AtLrgB-D1 fragment was amplified by PCR using the primers AtLrgB-F1 and AtLrgB-R3, and the AtLrgB-D2 fragment was amplified by overlapping PCR using the primers AtLrgB-F1, AtLrgB-OV1, AtLrgB-OV2 and AtLrgB-R2. To generate the AtTic21-EYFP (enhanced yellow fluorescent protein) fusion, AtTic21 coding sequence was cloned by RT-PCR using primers AtTic21-F1 and AtTic21-R1.

GATEWAY®-compatible destination vectors (Karimi et al., 2002) for protein subcellular localization (pH7FWG2.0), promoter analysis (pHGWF5.0), overexpression (pH7WG2D.1) and hairpin RNA interference (pH7GW1WG2.0) were purchased from the Functional Genomics Division, VIB-Ghent University (Ghent, Belgium). The LR reaction was conducted to generate different expression vectors.

For the complement experiments, the ccdB fragment was PCR-amplified using pH7FWG2.0 as a template, with the primers ccdB-F and ccdB-R, containing an introduced SacI and SpeI site, respectively. The ccdB fragment was then digested to replace the 35S promoter sequence in the 35S:AtLrgB, 35S:AtLrgB-D1 and 35S:AtLrgB-D2 vectors, thus forming three new destination vectors. The AtLrgB promoter fragment from the corresponding entry vector was then recombined into these destination vectors by the LR reactions.

All of the expression vectors were electroporated into Agrobacterium tumefaciens strain GV3101. Plants were transformed using the vacuum infiltration method (Bechtold et al., 1993). Transgenic plants were selected on B5 plates with 12.5 μg ml⁻¹ hygromycin. Single-locus and homozygous transgenic lines were characterized as described previously (She et al., 2010). For the complementation experiments, the expression levels of transgenes were checked by RT-PCR. The primers AtLrgB-F1 and AtLrgB-R3 were used to amplify the AtLrgB-D1 fragment, and the primers AtLrgB-F1 and AtLrgB-R2 were used to amplify the AtLrgB-D2 fragment and the full-length AtLrgB.

Microscopic analysis

For the localization of fluorescence fusion proteins, a confocal microscope system (Zeiss LSM510) was used. Mesophyll protoplast isolation and polystyrene glycol-mediated transformation were performed according to the methods described by Yoo et al., 2007.

For transmission electron microscopy, cotyledons and leaves were cut into 1–3 mm pieces, vacuum-infiltrated, fixed in 2% glutaraldehyde overnight, and then transferred into 2% osmium tetroxide overnight. The samples were dehydrated using an acetone series and embedded in Spurr’s resin. Thin sections (100 nm) were cut and stained with uranyl acetate and lead citrate, and the sections were observed with a Hitachi H-7650 transmission electron microscope.

Chloroplast isolation and protease sensitivity assay

Chloroplasts were isolated, Percoll-purified and quality control-tested by methods described by Seigneurin-Berny et al. (2008). For each sample, 10 g (FW) of 4-wk-old rosette leaves were collected.

Protease sensitivity assay was performed as described by Fitzpatrick & Keegstra (2001). Chloroplasts (25 μg chlorophyll) of 35S:AtLrgB:EGFP plants were incubated with 0, 20, 50 or 100 μg ml⁻¹ trypsin (10350 BAEE units mg⁻¹), on ice in the dark for 1 h. The reactions were quenched by adding a protease inhibitor solution containing 0.1 mg ml⁻¹ soybean trypsin inhibitor (Bio Basic Inc., Markham, Ontario, Canada) and 5 mM phenylmethylsulphonyl fluoride (PMSF) for 10 min. Chloroplasts were resolated over a 40% (v/v) Percoll cushion. The resolated chloroplasts were lysed in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. SDS-PAGE and subsequent Western blot analysis were conducted as described previously (Bian et al., 2002). The AtLrgB:EGFP fusion protein was probed with an anti-EGFP monoclonal antibody (1 : 1000 dilutions, GenScript, Piscataway, New Jersey, USA). As controls of chloroplast envelope inner and outer membrane protein, Tic40 and ToC75 were probed, respectively, with anti-Tic40 and anti-ToC75 polyclonal antibodies (1 : 1000 dilutions, Agisera, Antibodies, Vannas, Sweden).

Histochemical detection of beta-glucuronidase (GUS) expression

Seeds and young seedlings at different developmental stages and different parts from mature transgenic plants were collected and used for histochemical detection of GUS expression. They were stained at 37°C overnight in 1 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid (X-Gluc), 1 mM potassium ferricyanide, 0.1% Triton X-100, and 0.1 M sodium phosphate buffer, pH 7.0, with 10 mM EDTA. Samples were washed in 70% ethanol to remove chlorophyll.

RNA isolation, northern blotting, and quantitative RT-PCR analysis

For RNA blot analysis, tissues were frozen and ground to powder in liquid nitrogen and total RNA was extracted by
TRIZol reagent (Invitrogen). Total RNA (30 μg) was separated on 1.0% agarose-formaldehyde gels and transferred to a nylon membrane to hybridize with digoxigenin (DIG)-labelled DNA probes. Probe labelling, hybridization and detection were performed according to the DIG Application Manual for Filter Hybridization (Roche, Penzberg, Germany). The probe was amplified by PCR using the primers AtLrgB-PR1 and AtLrgB-PR2 (Table S1). Equal loading was confirmed by staining the rRNA with methylene blue.

For semiquantitative RT-PCR analysis, 3 μg of total RNA extracted from leaves was reverse-transcribed with oligo(dT) 18 primers according to the manufacturer’s instructions (RNA PCR kit; Takara, Shiga, Otsu, Japan). PCR was performed for 28 cycles. The Actin2 gene (At3g18780) of Arabidopsis was amplified using the primers Actin2-FW and Actin2-RV as an internal control for quantifying relative amounts of cDNA template.

For real-time RT-PCR analysis, total RNA extracted from seedlings was treated with RNase-free DNaseI (Takara). 1 μg of treated RNA was used for the first-strand cDNA synthesis using PrimeScript RT Reagent Kit (Takara). The PCR was performed using the LightCycler 480 (Roche) with the SYBR Premix Ex Taq Kit (Takara). Relative transcript abundances were normalized using Actin2 as a standard. The primers used for RT-PCR were described in Table S1.

Starch and sucrose measurements

Qualitative starch assays were carried out by decolorizing the tissue in 80% ethanol at 80°C, then staining with I2-KI as described previously (Caspar et al., 1985). For quantitative measurements of starch and sucrose, 100 mg (FW) leaves from 10-d-old seedlings were harvested, ground in 80% ethanol and extracted three times at 80°C. The insoluble pellet was used to measure the starch content by enzymatic hydrolysis of the starch to glucose as described by Smith & Zeeman (2006). The supernatant was combined, dried under vacuum, and redissolved in water for sucrose measurement (Jones et al., 1977).

Metabolite profiling

Metabolite intermediates, triose phosphate (TP), glycerate-3-phosphate (3-PGA), glucose 6-phosphate (Glc6P), glucose 1-phosphate (Glc1P), fructose 1-phosphate (Fru1P), fructose 1,6-biphosphate (Fru1,6P2), glucose and fructose in leaf tissues were determined enzymatically in neutralized perchloric acid extracts as described by Stitt et al., 1989 and Häusler et al., 2000. The formation or consumption of NAD(P)H coupling with the enzymatic reaction was quantitated by high-performance liquid chromatography (HPLC) analysis (Sporty et al., 2008). For each leaf tissue sample, 300 mg (FW) of leaves from 10-d-old seedlings was harvested. All of the samples were taken at the end of the light period; each treatment contained at least three independent samples.

Yeast transformation and nystatin treatment

The Saccharomyces cerevisiae line BY4742 (MATα his3 leu2 lys2 ura3) was used. Cells were cultured in YPD (1% yeast extract, 2% peptone, and 2% dextrose), SD/Gal/Raf/-Ura (Synthetic Defined/Galactose/Raffinose/-Uracil, inducible) or SD/Glu/Ura (uninducible) medium (Clontech Laboratories, Mountain View, California, USA) according to experimental design.

For construction of yeast expression vectors, the AtLrgB coding sequence exclusive of its putative chloroplast transit peptide was amplified by PCR using the primers AtLrgBA13-F1 and AtLrgBA13-R1 (Table S1). The PCR product was digested with Hind III and XbaI I, then cloned into yeast-inducible expression vector pYES2, which harbours a GAL1 promoter, and an Ura3 selection marker. Yeast transformation was performed by the lithium acetate method and positive transformants were confirmed by RT-PCR analysis as described previously (Zheng et al., 2007).

For the nystatin-induced growth inhibition test (Cirillo et al., 1964; Marty & Finkelstein, 1975), yeast cells were grown until the early stationary phase in liquid SD/Glu/Ura medium. They were diluted in a 10-fold series with an initial OD600 of 0.5 (Eppendorf BioPhotometer, Hamburg, Germany), and then a 5 μl aliquot of each dilution was spotted on SD/Gal/Raf/-Ura plates containing 0, 0.4 and 0.6 μg ml⁻¹ of nystatin. Photographs were taken after 4 d.

For the membrane permeabilization assay by flow cytometry, yeast cells were harvested and suspended (OD600 = 1.0) in the SD/Gal/Raf/-Ura medium followed by the addition of 5 μg ml⁻¹ nystatin and shaken at 200 rpm for 30, 60, 90, and 120 min. They were harvested and resuspended in PBS, pH 7.4, and stained with 5 mg ml⁻¹ propidium iodide (PI) in the dark at room temperature for 30 min. PI fluorescence was measured by Cytomics FC500 flow cytometry in the FL3 channel. Two thousand to 10 000 cells were measured for each analysis, and each experiment was repeated three times.

Results

Evolution of the AtLrgB gene

The AtLrgB gene, encoding a protein of 512 amino acids, was present as a single copy in the Arabidopsis genome. BLAST searches in GenBank revealed that many LrgB orthologues existed in bacteria, plants and two subphyla of fungi (Pezizomycotina and Saccharomycotina). In bacteria, lrgA and lrgB are different genes in the same operon...
Chloroplasts isolated from 35S assay was conducted (Fitzpatrick & Keegstra, 2001). indicating that AtLrgB was a chloroplast envelope [60x138]cence was colocalized with the EYFP fluorescence, shown in the lower panels of Fig. 2(a), the EGFP fluores-

(60x366)(Schwacke 2011 The Authors AtLrgB as a chloroplast envelope membrane protein Bioinformatics analysis using other programs also classified stably transformed Location of the fusion protein in mesophyll protoplasts of 35S:AtLrgB:EGFP AtLrgB and the EGFP protein (C-terminal translational fusion between the full-length C-terminal LrgB domain (SMART, http:// smart.embl-heidelberg.de/, Fig. 1c). Amino acid sequence alignment of LrgB domains showed that they were conserved from prokaryotes to eukaryotes (Fig. S1a). Alignment of the N-terminal domain of plant LrgB proteins with the bacterial LrgA proteins revealed that they share some similarity, although this similarity was lower than that observed for the LrgB domain (Fig. S1b). In the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) database, plant LrgB proteins were predicted to have both the LrgA domain and the LrgB domain. These results suggested that plant LrgB gene may have evolved from gene fusion of bacterial lrgA and lrgB.

Subcellular localization of AtLrgB protein and the expression pattern of the AtLrgB gene Previous proteomic studies had identified AtLrgB as a chloroplast inner envelope membrane protein (Ferro et al., 2002, 2003). Protein location program ChloroP (Emanuelsson et al., 1999) predicted a chloroplast transit peptide of 13 amino acids in the AtLrgB protein. Bioinformatics analysis using other programs also classified AtLrgB as a chloroplast envelope membrane protein (Schwacke et al., 2003).

To check the subcellular localization of the AtLrgB protein, we generated a transgenic construct where the Cauliflower mosaic virus (CaMV) 35S promoter drove a C-terminal translational fusion between the full-length AtLrgB and the EGFP protein (35S:AtLrgB:EGFP). Location of the fusion protein in mesophyll protoplasts of stably transformed Arabidopsis plants was examined by confocal microscopy. As shown in the upper panels of Fig. 2(a), EGFP fluorescence was identified at the periphery of chloroplasts. These protoplasts were transiently transformed with a chloroplast inner envelope membrane protein control (35S:AtTic21:EYFP, Teng et al., 2006). As shown in the lower panels of Fig. 2(a), the EGFP fluorescence was colocalized with the EYFP fluorescence, indicating that AtLrgB was a chloroplast envelope membrane protein.

To further confirm the location of AtLrgB in the inner membrane of the chloroplast envelope, a protease sensitivity assay was conducted (Fitzpatrick & Keegstra, 2001). Chloroplasts isolated from 35S:AtLrgB:EGFP plants were treated with various concentrations of trypsin. As shown in Fig. 2(b), when outer membrane protein Toc75 was degraded by trypsin, the inner membrane protein Tic40 remained resistant. The fusion protein AtLrgB:EGFP was resistant to trypsin digestion in the absence of Triton X-100, confirming its inner envelope membrane location.

To assess the expression pattern of the AtLrgB gene, we generated transgenic lines harbouring an AtLrgB promoter reporter construct, in which a c. 1 kb DNA fragment upstream of the ATG start codon of AtLrgB, as predicted by AtcisDB (Davuluri et al., 2003), was fused with the GUS gene (PromAtLrgB:GUS). Histochemical staining revealed that GUS activity was present at all developmental stages tested, from seed germination to flowering (Fig. 2c). AtLrgB was expressed mainly in leaves, stems and flowers, but not in roots, and the expression was strong in younger tissues but weak in mature tissues. During leaf maturation, loss of GUS activity proceeded progressively from leaf tip to leaf base (Fig. 2c). This expression pattern suggested that the AtLrgB promoter was regulated in a manner that showed resemblance to the sink-to-source transition of leaves (Turgeon, 1989; Carvalho et al., 2006).

Light is one of the most important signals influencing chloroplast development. To examine the effects of light on the expression of AtLrgB, the accumulation of AtLrgB transcripts during a 16 h light/8 h dark photoperiod was investigated (Fig. 2d). AtLrgB transcripts accumulated at low levels in the dark, increased after illumination for 4 h, reached maximal levels after 16 h of illumination, and returned to the starting level at the end of the dark period. These results indicated that the expression of AtLrgB was subjected to diurnal regulation.

Characterization of a T-DNA insertion mutant of AtLrgB To analyse the function of AtLrgB in Arabidopsis, we obtained T-DNA and transposon insertion lines from the Arabidopsis Biological Resource Center (ABRC), the Nottingham Arabidopsis Stock Centre (NASC), and the Rikagaku Kenkyusho Bioresources Center (RIKEN-BRC). A mutant termed as atlrgB-1 (SALK_053469) from the ABRC stocks was identified, containing a T-DNA at the first intron of the AtLrgB gene (Figs 3a, S2). RT-PCR was performed and no AtLrgB transcript was detected in homozygous seedlings of this mutant (Fig. 3b). Two additional lines from NASC and RIKEN-BRC were also identified, which harboured T-DNA and transposon insertions at positions – 204 (N436293, atlrgB-2) and + 2784 (16-1657-1, atlrgB-3), respectively, relative to the start codon of AtLrgB (Fig. 3a). However, RT-PCR analysis revealed that these insertions did not impair the expression of AtLrgB (data not shown).

The homozygous atlrgB-1 mutant was chosen for further analysis. As shown in Fig. 3(c), the atlrgB-1 seedlings displayed pale-green cotyledons and leaves. In the extreme range of the phenotype, c. 4 ± 0.5% of the atlrgB-1 seedlings exhibited variegated cotyledons. The seedlings –
especially those with variegated cotyledons – were smaller than the wild-type. Another obvious abnormality in the atlrgB-1 mutant was that the cotyledons and juvenile leaves displayed a vein pattern on the pale-green laminas (Fig. 3c), appearing as a weak reticulate mutant where the leaf vascular network can be clearly distinguished (Gonzalez-Bayon et al., 2006).

When atlrgB-1 seedlings grew on Petri dishes on B5 medium containing 1% sucrose for c. 8 d, necrosis began to appear at the tips of the cotyledons (Fig. 3c). After another 5–6 d, necrosis began to appear at the apical margins of the first true leaves (Fig. 3d). When atlrgB-1 seedlings were grown in soil from the time of germination, the necrosis phenotype was slightly delayed (Fig. S3).
The\textit{atlrgB-1} mutant was crossed with wild-type. In the F2 generation, the genotype (wild-type, heterozygous or homozygous mutation) of individual seedlings was determined by a diagnostic PCR analysis (Fig. S2). The results revealed that the mutation phenotype was cosegregated with the T-DNA insertion and that the mutation was recessive ($n = 73$, 3 : 1 segregation, chi-squared test).

To further confirm a relationship between\textit{AtLrgB} perturbation and the leaf phenotype, we generated transgenic plants in which a hairpin RNA of the\textit{AtLrgB} transcript was overexpressed under the control of a constitutive 35S promoter (35S:\textit{AtLrgB}-RNAi). The semiquantitative RT-PCR results showed a visible decrease in the\textit{AtLrgB} mRNA in two of the RNAi lines (Fig. 3e). Consistent with the\textit{atlrgB-1} mutant,\textit{AtLrgB}-RNAi lines showed pale-
green cotyledons and leaves, although the phenotypes were weak compared with the T-DNA insertion mutant (Fig. 3c).

Finally, we generated a transgenic construct where the wild-type AtLrgB gene was driven by its native promoter (PromAtLrgB:AtLrgB). The construct was used to transform atlrgB-1 plants. The expression level of AtLrgB in transgenic lines was checked by RT-PCR analysis (Fig. S4). Results showed that expression of AtLrgB under its native promoter was able to complement the atlrgB-1 mutant, since all transgenic lines (n = 14) displayed wild-type phenotypes (Fig. 3d).

Overexpression of AtLrgB led to a veinal chlorosis and delayed greening phenotype

To further examine the function of AtLrgB in Arabidopsis development, we generated transgenic lines overexpressing AtLrgB under the control of the 35S promoter (35S:AtLrgB). Northern blot analysis revealed a strong accumulation of the AtLrgB transcripts in homozygous transgenic seedlings (Fig. 4a).

We found that overexpression of AtLrgB severely influenced shoot development in comparison with knock-out of AtLrgB (Fig. 4b–i). Most transgenic lines exhibited chlorotic cotyledons and leaves (Fig. 4c). Some seedlings of strong lines exhibited albino cotyledons and eventually arrested in growth (Fig. 4d). In contrast to the atlrgB-1 mutant, the vasculature tissues of 35S:AtLrgB plants appeared pale compared with the mesophyll tissues, displaying a veinal chlorosis phenotype (Fig. 4e,f). In adult 35S:AtLrgB plants, young leaves and the basal parts of middle-aged leaves were veinal chlorotic, whereas old-aged leaves had become nearly as green as the wild-type (Fig. 4g,h), indicating that the chlorosis induced by AtLrgB overexpression was reversible to a certain extent during leaf maturation. The 35S:AtLrgB plants were retarded in flowering and silique production, and both silique number and seed set were reduced (Fig. 4i,j).

The 35S:AtLrgB::EGFP plants had a strong interveinal chlorosis phenotype (Fig. 4k–m). Possibly, the fusion of AtLrgB protein to EGFP caused a dominant negative effect.

Chloroplast ultrastructure of atlrgB-1 and 35S:AtLrgB plants

The atlrgB-1 mutant and 35S:AtLrgB plants exhibited differential abnormality in leaf chlorosis, indicating that chloroplast development in these plants might have been differentially affected. We then evaluated the ultrastructure of chloroplasts in both of the mesophyll cells and the bundle sheath cells of wild-type, atlrgB-1 and 35S:AtLrgB plants by transmission electron microscopy.

In the chloroplasts of mesophyll cells of 5-d-old atlrgB-1 pale-green cotyledons, the thylakoid membrane organization was less abundant, and they appeared to have larger starch grains in comparison to wild-type plants (Fig. 5a,b). Chloroplasts from the green region of variegated cotyledons of atlrgB-1 mutant also appeared to have more, or larger, starch grains (Fig. S5).

Chloroplasts from the mesophyll cells of 35S:AtLrgB plants contained less abundant thylakoids membranes, and the starch grains in these chloroplasts vanished or became smaller than the wild-type (Fig. 5c). In a more extreme phenotype, the internal membrane structures of chloroplasts from the albino cotyledons were completely destroyed (Fig. 5d).

In the chloroplasts of mesophyll cells of 4-wk-old atlrgB-1 plants growing in soil, the thylakoid membrane organization in young leaves was similar to that observed in wild-type plants (Fig. 5e,f). By contrast, in chlorotic parts of 35S:AtLrgB plants, a dramatically reduced number of granal thylakoids and stacked thylakoids were observed (Fig. 5g).

Nevertheless, in chloroplasts from the recovered region of 35S:AtLrgB plants, the thylakoid membrane organizations were restored to a certain extent, but the starch grains appeared to be quite small (Fig. 5h).

In the bundle sheath cells associated with the veins, chloroplasts from 5-d-old atlrgB-1 plants and the wild-type plants were of similar size and had approximately the same amount of granal thylakoid membranes (Fig. 5i,j), coinciding with the generally normal veins of the atlrgB-1 mutant. However, chloroplasts from the 35S:AtLrgB plants were smaller and had fewer numbers of granal thylakoid membranes (Fig. 5k), which coincided with the veinal chlorosis phenotype. Taken together, ultrastructural results strongly suggested that normal expression of the AtLrgB gene was required for chloroplast development.

Carbon partitioning between starch and sucrose was altered in atlrgB-1 and 35S:AtLrgB plants

In the above chloroplast ultrastructure assessment, we found an increase in starch grain size or number in atlrgB-1 mutants, and a decrease in starch grain size or number in 35S:AtLrgB plants. To understand these results more precisely, starch staining by I2-KI was conducted for the 7-d-old seedlings (Fig. 6a). Results showed that the atlrgB-1 mutant accumulated more starch than the wild-type during the day, while the 35S:AtLrgB plants had reduced starch accumulation.

The starch content was then measured in wild-type, atlrgB-1 and 35S:AtLrgB plants during a 12 h photoperiod. In all lines, starch contents increased progressively throughout the day and decreased during the darkness period. Noticeably, at the end of the light period, the atlrgB-1 plants displayed a 24% increase of starch contents com-
pared with wild-type, while 35S:AtLrgB plants had a 18% reduction compared with wild-type (P < 0.01, t-test, Fig. 6b). The different starch content between atlrgB-1 and wild-type at the end of the night was marginal (Fig. 6b), indicating that the overaccumulation of starch in the atlrgB-1 was different from the starch excess mutants, in which the phenotype was usually caused by the inefficiency of starch degradation during the night (Yu et al., 2001; Kotting et al., 2009). As the rate-limiting step of starch biosynthesis is catalysed by ADP-glucose pyrophosphorylase (Stark et al., 1992), the expression levels of subunit genes of ADP-glucose pyrophosphorylase in wild-type, atlrgB-1 and 35S:AtLrgB plants were compared using real-time RT-PCR. The expression levels of 4 APL and 2 APS genes in atlrgB-1 and 35S:AtLrgB plants seemed similar to the wild-type, indicating that the changes in starch contents did not result from the altered expression levels of genes encoding ADP-glucose pyrophosphorylase (Fig. S6).

The sucrose concentrations in three kinds of plants were also measured (Fig. 6c). In the atlrgB-1 mutant, the sucrose

Fig. 4 Phenotype characteristics of Arabidopsis thaliana plants overexpressing AtLrgB. (a) Northern blot analysis of AtLrgB expression in wild-type (WT) plants and 35S:AtLrgB transgenic lines. Total RNA was hybridized with digoxigenin-labelled DNA probes for the AtLrgB gene. The 25S rRNA band was stained using methylene blue. (b–j) Phenotype comparison of 35S:AtLrgB plants with the WT. (b) Cotyledons of 5-d-old WT plants. (c) Chlorotic cotyledons of 5-d-old 35S:AtLrgB plants. (d) Albino cotyledons of 5-d-old 35S:AtLrgB plants. (e) Four-week-old WT plants. (f) Four-week-old 35S:AtLrgB plants. (g) Leaf of 4-wk-old WT plants. (h) Leaf of 4-wk-old 35S:AtLrgB plants. (i) Siliques of WT plants and 35S:AtLrgB plants. (j) Seven-week-old WT plants and 35S:AtLrgB plants. (k–m) Phenotype comparison of 35S:AtLrgB:GFP plants with the WT grown in soil. (k) Four-week-old WT plants. (l) Four-week-old 35S:AtLrgB:GFP transgenic plants. (m) Leaves of 4-wk-old WT plants and 35S:AtLrgB:GFP transgenic plants. Bars, 2 mm (b–d); 5 mm (e,f, k–l).
content remained lower than that of wild-type plants during the light period, indicating that a change of carbon partitioning in favour of starch biosynthesis in the light had occurred. However, during the first 2 h of the dark period, the sucrose content of this mutant increased sharply and became higher than that of the wild-type. This transitory increase indicated that the \textit{atlrgB-1} mutant had more starch for degradation at the beginning of the dark period and thus the biosynthetic rate of sucrose was higher than that of the wild-type for this duration (Fig. 6b,c). For the \textit{35S:AtLrgB} plants, sucrose concentrations were similar to the wild-type at the beginning of the light period, but 9% higher than the wild-type at the end of the light period (Fig. 6c).

To investigate the effect of exogenous sucrose on the development of \textit{atlrgB-1} and \textit{35S:AtLrgB} plants, seeds were germinated on media supplemented with different concentrations of sucrose. The \textit{atlrgB-1} seedlings on 2% sucrose displayed alleviative phenotypes compared with those grown on control media with 1% sucrose – namely, leaf necrosis was suspended and the necrotic region became smaller (Fig. 6d). The \textit{35S:AtLrgB} seedlings on 2% sucrose displayed more deleterious phenotypes than those on control media – namely, petioles and the proximal parts of young leaves bleached after 2 wk of treatment (Fig. 6d). In addition, we also found that exogenous glucose had a similar effect on the \textit{atlrgB-1} and \textit{35S:AtLrgB} plants as exogenous sucrose (Fig. S7). These results indicated that the abnormal phenotypes caused by the overexpression of \textit{AtLrgB} were associated with carbohydrate availability.

No evidence of a role for \textit{AtLrgB} in direct transport of TP or hexose phosphates

Metabolite profiling was undertaken to examine whether \textit{AtLrgB} perturbation affected specific metabolite intermediates. Since the literature on metabolite profiling of isolated \textit{Arabidopsis} chloroplasts is currently lacking, we used \textit{Arabidopsis} leaf tissues for the experiment. Such a strategy was adopted in studies of \textit{Arabidopsis} TPT mutants, and showed that the \textit{tpt-1} mutant had significantly higher contents of TP, 3-PGA and Glc6P at the end of light period in comparison to the wild-type (Schneider \textit{et al.}, 2002; Walters \textit{et al.}, 2004). As shown in Table 1, at the end of light period of 10-d-old leaves, the \textit{atlrgB-1} mutant had 36% higher contents of 3-PGA and 27% lower contents of Glc6P relative to the wild-type ($P < 0.05$, $t$-test). However, no significant difference was seen for the TP contents between the \textit{atlrgB-1} mutant and the wild-type (Table 1, (a) (b) (c) (d) (e) (f) (g) (h) (i) (j) (k).
The 35S:AtLrgB plant had 35% higher contents of TP but 24% lower contents of Glc6P relative to the wild-type (Table 1, P < 0.05, t-test). Comparing metabolic profiles of atrlb-1 and 35S:AtLrgB with tpt-1, we found no evidence for a role of AtLrgB in the direct transport of TP. For the contents of Glc1P, Fru6P, and Fru1,6P2, there were no significant differences among the wild-type, atrlb-1, and 35S:AtLrgB plants (Table 1). In addition, the atrlb-1 mutant and the 35S:AtLrgB plants had similar contents of Glc6P (Table 1). Therefore, AtLrgB also appeared not to transport hexose phosphates directly.

AtLrgB could augment nystatin-induced membrane permeability in yeast cells

As an approach to functionally characterize the AtLrgB protein, we expressed this protein in budding yeast (S. cerevisiae). Bioinformatic analysis revealed that the yeast genome does not contain the homologues of cidAB/lrgAB genes. The putative chloroplast transit peptide of the AtLrgB protein was excluded in yeast expression. The polyene macrolide antibiotic nystatin is known to interact with membrane components to form pores in fungal plasma membranes (Marty & Finkelstein, 1975). Control yeast cells and those cells transformed with AtLrgB

Fig. 6 Starch and sucrose determination in wild-type, atrlb-1 and 35S:AtLrgB Arabidopsis thaliana plants. (a) Starch staining of 7-d-old plants using I2-KI. Plants were grown under a 16 : 8 h, light : dark photoperiod for 7 d. After 0, 3, 6, and 12 h illumination, whole plants were stained by I2-KI. Starch (b) and sucrose contents (c) in leaves of 10-d-old wild-type (WT, diamonds), atrlb-1 (squares) and 35S:AtLrgB (triangles) plants. Plants were grown under a 12 : 12 h, light : dark photoperiod for 10 d. Rosette leaves were harvested at 2–4 h intervals for 24 h from the end of the night period. (d) Phenotypes of seedlings growing on B5 media with two concentrations of sucrose for 2 wk. The arrows indicate the necrotic tissues on leaves. Bars, 5 mm. Values in (b) and (c) represent the mean of three different experiments ± SD.
were grown on agar media with different concentrations of nystatin. As shown in Fig. 7(a), yeast cells expressing AtLrgB were more sensitive to nystatin-induced growth inhibition than the control cells. Membrane permeability of yeast cells grown in liquid media was determined by PI staining and flow cytometric analysis. AtLrgB expression significantly increased the proportion of PI-permeable cells, and the proportion of PI-permeable cells increased with time (Fig. 7b, P < 0.05, t-test). These results indicated that AtLrgB could augment nystatin-induced membrane permeability in yeast cells.

Both the N-terminal LrgA domain and the C-terminal LrgB domain are required for the function of AtLrgB

We were interested in the individual role of the N-terminal LrgA domain and the C-terminal LrgB domain in the function of AtLrgB, and generated two transgenic constructs. AtLrgB-D1 (amino acids 1–267) was a truncated form of AtLrgB, lacking the C-terminal LrgB domain. AtLrgB-D2 (amino acids 1–267 plus amino acids 259–512), lacked the N-terminal LrgA domain (Fig. 8a). The transgenes were expressed under control of the 35S promoter (35S:AtLrgB-D1 and 35S:AtLrgB-D2). Overexpression of transgenes in transgenic plants was confirmed by northern blot analysis (Fig. 8b). The lack of 1.8 k band (the native AtLrgB transcript) in 35S:AtLrgB-D2 plants may result from a transgene-mediated cosuppression (Fig. 8b). In RT-PCR analysis, lower abundance of native AtLrgB transcript was detected in 35S:AtLrgB-D2 plants in comparison with 35S:AtLrgB-D1 plants (data not shown).

35S:AtLrgB-D1 and 35S:AtLrgB-D2 plants exhibited veinal chlorosis phenotypes similar to the 35S:AtLrgB plants overexpressing the full-length gene (Fig. 8c). These results indicated that both parts of the AtLrgB protein were able to perturb chloroplast development possibly in a dominant negative manner. We then generated transgenic constructs in which the truncated forms of AtLrgB were expressed under the control of its native promoter (PromAtLrgB: AtLrgB-D1 and PromAtLrgB:AtLrgB-D2). We transformed atrgB-1 plants with these constructs. RT-PCR analysis showed that the expression of the truncated forms of AtLrgB in the transgenic lines had returned to the similar levels of the native AtLrgB (Fig. S4). As shown in Fig. 8(d), the truncated forms of AtLrgB could not complement the atrgB-1 mutation, indicating that the accurate function of AtLrgB depended on the existence of the entire protein.
Discussion

In bacteria, the cidAB/lrgAB system represents an emerging mechanism to control cell death and lysis. The cidA and lrgA genes encode homologous hydrophobic proteins proposed to function as a holin and antiholin, respectively, to regulate membrane permeability (Bayles, 2007; Ranjit et al., 2011). The cidB and lrgB genes also encode homologous hydrophobic proteins whose molecular functions are unclear (Rice & Bayles, 2008). In the mitochondria of eukaryotic cells, Bax and Bcl-2 are part of a large family of homologous hydrophobic proteins that promote and inhibit programmed cell death, respectively, through the regulation of membrane permeability (Bayles, 2007; Wang et al., 2009). Since the chloroplasts of eukaryotes are evolved from endosymbiotic cyanobacteria, it is an intriguing prospect to explore: do chloroplasts have a pathway analogous to the CidAB/lrgAB or Bax/Bcl-2 systems?

In fact, AtLrgB, a protein containing a putative LrgB domain in its C-terminus (Fig. S1a), has been repeatedly identified in proteomic studies of Arabidopsis chloroplast envelope membranes (Ferro et al., 2002, 2003; Froehlich et al., 2003; Kleffmann et al., 2004; Mitra et al., 2007; Zybailov et al., 2008). We confirmed the location of AtLrgB to the chloroplast envelope inner membrane (Fig. 2a,b). Alignment of its N-terminal region with the bacterial LrgA proteins revealed that they share some similarity (Fig. S1b). These results suggested that the lrgA gene and lrgB gene may have been fused over the course of evolution and that the LrgA domain evolved more quickly than the LrgB domain. Under selective pressure, gene fusion, especially of genes encoding proteins of functional associations or physical interactions, is a major contributor to the evolution of multidomain proteins (Enright et al., 1999; Snel et al., 2000; Yanai et al., 2001; Pasek et al., 2006).

Ultrastructural results strongly suggested that normal expression of the AtLrgB gene was required for chloroplast development (Fig. 5). Measurement of the contents of photoassimilates revealed that, at the end of the light period, the atlrgB-1 mutant accumulated more starch and less sucrose, while 35S:AtLrgB plants accumulated less starch and more sucrose, indicating a role for AtLrgB in carbon partitioning (Fig. 6). The phenotypes of the atlrgB-1 mutant and the 35S:AtLrgB plants may relate to the disrupted balance between sucrose and starch. In the atlrgB-1 mutant, the decrease of sucrose synthesis failed to meet the demand for the early development of mesophyll cells, thus resulting in the pale-green and variegated cotyledons and leaves (Fig. 3). This explanation was supported by the fact that additional sucrose and glucose can alleviate the necrotic
The enzymes responsible for peptidoglycan synthesis do not of the complete carbohydrate metabolism (Groicher et al., 2009). The decreased sucrose content in.attrgB-1 leaves made them highly sensitive to the sink–source transition. This may explain why necrosis always began at the apical margin of leaves (Fig. 3c,d).

A threshold model has been used to explain the formation of a chlorotic tissue phenotype in maize: accumulation of a chloroplast byproduct, probably sucrose, above a threshold concentration induces the formation of a chlorotic tissue phenotype (Braun et al., 2006; Baker & Braun, 2008), since it is proposed that a low starch content and high concentrations of sucrose during the day may lead to feedback inhibition of photosynthetic gene expression and chlorophyll synthesis. As for 35S:AtLrgB plants, the excess sucrose transported through the veins may be responsible for the interveinal chlorosis phenotype. During the progress of the sink–source transition, mature leaves can export excess sucrose to alleviate the stress, thus returning to exhibit normal green tissues (Fig. 4).

The phenotype of the attrgB-1 mutant and the 35S:AtLrgB plant was distinctive, but the molecular function of the AtLrgB protein remained to be established. According to current knowledge, carbon partitioning between sucrose and starch during light periods is mainly controlled by TPT, which exports TP from stroma to the cytosol (Flügge, 1995, 1999). The Arabidopsis genome contains only one TPT gene. The tpt-1/ape2 mutant, in which the TP transport activities were reduced to below 5% of the wild-type, demonstrated clearly increased starch accumulation and decreased sucrose content during the day (Schneider et al., 2002; Walters et al., 2004). The similar features of starch and sucrose contents in the tpt-1/ape2 and attrgB-1 mutants, and the similar abundance of AtLrgB and TPT proteins (Ferro et al., 2003; Froehlich et al., 2003; Zybaïlov et al., 2008), remind us of the possibility that AtLrgB might be involved in transportation of TP. However, metabolite profiling revealed no evidence for AtLrgB’s role in the direct transport of TP or hexose phosphates (Table 1). In addition, the tpt-1/ape2 mutants show severe alterations in carbon metabolism, but they do not show substantial chloroplast development phenotypes (Schneider et al., 2002; Walters et al., 2004). Thus, the mechanism of AtLrgB may be different from TPT.

Early studies in S. aureus revealed that the cidAB/lrgAB operons control cell death and lysis by modulating the access of the murein hydrolases to murein in response to carbohydrate metabolism (Groicher et al., 2000; Rice et al., 2003; Bayles, 2007; Rice & Bayles, 2008). BLAST searches of the complete Arabidopsis genome revealed that many enzymes responsible for peptidoglycan synthesis do not exist; therefore, the Arabidopsis chloroplast should be absent of peptidoglycans (Garcia et al., 2008). In addition, the Arabidopsis genome lacks genes to encode murein hydrolase. Thus, the substrate or mechanism of AtLrgB in Arabidopsis should be somewhat different from that observed for S. aureus.

Recent analysis of lrgAB/cidAB homologues in Bacillus anthracis and Streptococcus mutans revealed that inactivation of lrgAB/cidAB did not appear to affect murein hydrolase and the molecular function of LrgAB/CidAB proteins in these bacterial species remained unclear (Chandramohan et al., 2009; Ahn et al., 2010). In B. anthracis, the lrgAB/cidAB orthologues play important roles in the control of cell death and sporulation (Chandramohan et al., 2009), while in S. mutans, the lrgA and cidA operons modulate autolysis and virulence traits in response to oxygen availability and glucose concentrations (Ahn et al., 2010).

Herein, our work indicates that AtLrgB is a new player in carbon partitioning and leaf senescence, analogous to the physiological role of CidAB/LrgAB in control of bacterial death and carbon metabolism. Although the molecular mechanism of AtLrgB in the chloroplast envelope inner membrane should be different from that of bacterial LrgAB/CidAB homologues in the plasma membrane, they may still have some connections. Two lines of evidence were in favour of this hypothesis. First, we found that AtLrgB could augment nystatin-induced membrane permeability in yeast cells (Fig. 7). Secondly, both the LrgA domain and the LrgB domain of AtLrgB were capable of inducing dominant phenotypes, but neither was able to complement the T-DNA mutant (Fig. 8). In bacteria, despite multiple attempts with a variety of vectors and genetic backgrounds, researchers were unable to complement the cidA/cidB mutations in S. aureus (Rice et al., 2003; Rice & Bayles, 2008) and S. mutans (Ahn et al., 2010). The reason for the lack of success of the complement test in bacteria and Arabidopsis remains unknown, but it indicates that the interactions of these protein domains are intricate. Future genetic work to suppress the phenotypes of attrgB-1 and 35S:AtLrgB, and proteomic work using isolated chloroplasts of attrgB-1 and 35S:AtLrgB, could help to determine the substrate and the mechanism of AtLrgB.

Recent studies have uncovered the significance of transitory starch for plant growth and plant productivity (Sulpice et al., 2009; Graf et al., 2010; Stitt et al., 2010). We identify herein a new component in the regulation of starch metabolism and leaf development. As the LrgB gene is highly conserved in plants, research on the AtLrgB and its plant homologues should provide new knowledge on chloroplast development and carbon partitioning, and shed new light on the bioengineering field of crop improvement. In addition, since AtLrgB is a novel chloroplast envelope inner membrane protein, it is an interesting question whether or...
not and how it participates in the plastid signalling pathways. After extensive studies over the years, no plastidial transmembrane component involved in plastid signalling has been identified (Kleine et al., 2009; Pfannschmidt, 2010). Genetic interaction analysis of AtLrgB and other chloroplast development and plastid signalling genes is required in the future to address these questions.

Acknowledgements

We thank Dr Keke Yi for his valuable comments, Xinhang Jiang for HPLC analysis, and the VIB-Ghent University for the original vectors. We are also grateful to ABRC, NASC and RIKEN-BRC for the distribution of Arabidopsis materials. This work was supported by funding from the National Natural Science Foundation of China (grant nos 31170211 30470923 and 60533050 to J.W.), the National High Technology Research and Development Program (grant no. 2007AA10Z141 to M.Z.), and the Natural Science Foundation of Zhejiang Province (grant no. R304098 to J.W.).

Reference


**Supporting Information**

Additional supporting information may be found in the online version of this article.

**Fig. S1** Amino acid sequence alignment of *AtLrgB* with orthologues from various species.

**Fig. S2** Diagnostic PCR analysis for detection of T-DNA insertion in the *AtLrgB* gene.

**Fig. S3** Phenotypes of the *atlrgB-1* mutant plants grown in soil.

**Fig. S4** RT-PCR analysis of the expression levels of transgenes in the complementation experiments.

**Fig. S5** Chloroplast ultrastructures of variegated cotyledons from the *atlrgB-1* mutant.

**Fig. S6** Detection of the transcripts of the *ADP-glucose pyrophosphorylase (AP)* genes in wild-type (WT), *atlrgB-1* and *35S:AtLrgB* plants by real-time RT-PCR.

**Fig. S7** Phenotypes of seedlings growing on B5 media with different glucose contents for 2 wk.

**Table S1** PCR primer sequences used in this study

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