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Medium-chain-length polyprenol (C45–C55) formation in chloroplasts of Arabidopsis is brassinosteroid-dependent

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ABSTRACT

Brassinosteroids are important plant hormones influencing, among other processes, chloroplast development, the electron transport chain during light reactions of photosynthesis, and the Calvin-Benson cycle. Medium-chain-length polyprenols built of 9–11 isoprenoid units (C45–C55 carbons) are a class of isoprenoid compounds present in abundance in thylakoid membranes. They are synthetized in chloroplast by *CPT7* gene from Calvin cycle derived precursors on MEP (methylerythritol 4-phosphate) isoprenoid biosynthesis pathway. C45–C55 polyprenols affect thylakoid membrane ultra-structure and hence influence photosynthetic apparatus performance in plants such as Arabidopsis and tomato. So far nothing is known about the hormonal or environmental regulation of CPT7 gene expression. The aim of our study was to find out if medium-chain-length polyprenol biosynthesis in plants may be regulated by hormonal cues.We found that the *CPT7* gene in Arabidopsis has a BZR1 binding element (brassinosteroid dependent) in its promoter. Brassinosteroid signaling mutants in Arabidopsis accumulate a lower amount of medium-chain-length C45–C55 polyprenols than control plants. At the same time carotenoid and chlorophyll content is increased, and the amount of PsbD1A protein coming from photosystem II does not undergo a significant change. On contrary, treatment of WT plants with epi-brassinolide increases C45–C55 polyprenols content. We also report decreased transcription of MEP enzymes (besides C45–C55 polyprenols, precursors of numerous isoprenoids, e.g. phytol, carotenoids are derived from this pathway) and genes encoding biosynthesis of medium-chain-length polyprenol enzymes in brassinosteroid perception mutant *bri1-116*. Taken together, we document that brassinosteroids affect biosynthetic pathway of C45–C55 polyprenols.

1. Introduction

Brassinosteroids (BRs) are plant hormones derived from sterols. There are several alternative biosynthetic pathways leading to active brassinosteroid compounds starting from campesterol, sitosterol, or cholesterol (Fujiyama et al., 2019; Joo et al., 2012; reviewed in Ohnishi, 2018; Bajguz et al., 2020). Brassinosteroids are synthesized in the endoplasmic reticulum in all plant organs and act locally. BRs promote cell growth and elongation by stimulating the transcription of genes encoding proteins implicated in cell-wall synthesis and modification, such as cellulose synthases and hydrolases (reviewed in Vriet et al., 2013). Recently, their new functions in anther maturation, seed development, cell division in the root meristem, root hair determination, and stomata development were discovered (Ye et al., 2010; Jiang et al., 2013; Hacham et al., 2011; Cheng et al., 2014; Gudesblat et al., 2012, reviewed in Nolan et al., 2020). BRs are perceived at the plasma membrane by a receptor kinase BRI1 (Brassinosteroid Insensitive) (Li and Chory, 1997). Upon brassinosteroid binding and dimerization with a co-receptor BAK1, the BRI1 kinase becomes activated and can phosphorylate cytoplasmic targets (Nam and Li, 2002). A cascade of phosphorylation/dephosphorylation events started by BRI1/BAK1 dimerization leads to a brassinosteroid-dependent transcriptional response (Tang et al., 2008). Only two transcription factors: BZR1 and BZR2 (BES1) have been discovered to directly mediate the BR-dependent transcription in Arabidopsis (Wang et al., 2002; Yin et al., 2005; He et al., 2005). However, the number of genes being either positively or negatively regulated by these two transcription factors is enormous. Many of the genes regulated directly by BZR1 or BES1 encode

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transcription factors themselves (reviewed in Nolan et al., 2020). What is more, they interact with promoter regions of the genes simultaneously regulated by BZR1 or BES1 and, sometimes, with the BZR1 protein itself, creating a multi-level network of brassinosteroid-dependent transcriptional responses (Sun et al., 2010; Yu et al., 2011; Guo et al., 2013). ChIP-on-chip experiments and RNA-matrix analysis of genes regulated by BRs enabled the identification of direct and indirect targets of BZR1 and BES1.Besides the role in plant cell growth and differentiation, brassinosteroids influence photosynthesis rate, by improving Calvin-Benson cycle enzyme expression (Yin et al., 2021; Li et al., 2016) and promoting photomorphogenesis of chloroplasts (Tachibana et al., 2022; Zhang et al., 2021). In particular brassinosteroid application rescue chloroplast function under stress condition in model and crop plants (Barros Junior et al., 2021; Pereira et al., 2020). Brassinosteroids also affect architecture of chloroplast inner membranes (Dobrikova et al., 2014; Krumova et al., 2013; Sadura et al., 2020).

Polyprenols (dehydrodolichols), are a group of isoprenoid derived lipids, abundant in chloroplasts in form of free alcohols, phosphates and short-chain fatty acid esters (Surmacz and Swiezewska, 2011). They accumulate during aging of plant organs, their amount increases during vegetative season of a plant (Bajda et al., 2005). Their content increases in stress conditions, both biotic (Bajda et al., 2009) and abiotic (salinity, drought; Milewska-Hendel et al., 2017). Because of these observations they were considered as non-specific factors preventing lipid oxidation and damage of photosynthetic apparatus (Bajda et al., 2009, Komaszylo et al., 2016). Polyprenols are known modifiers of membrane structure and fluidity, promoting hexagonal phase formation in lipid bilayers (summarized in Hartley and Imperiali, 2012). The latter function was recently discovered to play an important role in photosynthetic membranes organization (Akhtar et al., 2017; Buszewicz et al. BiorXiv). Although plants devoid of chloroplast C45–C55 polyprenol synthesis are viable, the photosynthetic parameters in them are strongly decreased (Akhtar et al., 2017).

Polyprenols of medium-length of isoprenoid chain (9–11 isoprenoid units, corresponding to C45–C55 carbons) are synthesized in chloroplast from methylerythritol 4-phosphate (MEP) pathway intermediates through geranylgeranyl diphosphate (GGPP) (Rodríguez-Concepción, 2010; Surmacz and Swiezewska, 2011; Akhtar et al., 2017). It means, that they share important metabolic intermediates with major photosynthetic pigments such as chlorophyll (phytyl side chain) and carotenoids (Rodríguez-Concepción, 2010). Lack of C45–C55 polyprenols decreases photosynthetic efficiency and influences the physicochemical state of the thylakoid membranes in Arabidopsis and tomato (Akhtar et al., 2017; Van Gelder et al., 2018; Buszewicz et al. BiorXiv) in a similar way to brassinosteroid deficiency.

MEP pathway genes, implicated in photosynthetic pigments biosynthesis are regulated by diurnal cycle (reviewed in Hemmerlin et al., 2012), but nothing is known about the regulation of the genes dedicated to the medium-chain-length polyprenol biosynthesis. Several observations indicate that environmental stress factors, light and brassinosteroid play role in their expression. The aim of this study was to elucidate the role of brassinosteroid hormones in chloroplast polyprenol synthesis.

2. Materials and methods

Arabidopsis thaliana Col-0 ecotype was used in this work as control plants. *det2-1* mutant in the same background (carrying a point mutation in steroid-5-alpha-reductase encoded by At2g38050 locus) was used as an example of a mutant in brassinosteroid biosynthesis. *bri1-116* mutant in Col-0 background (carrying a point mutation in At4g39400 locus; this mutation decreases BRI1 receptor kinase stability and leads to fast degradation of the protein) was used as an example of a mutant in brassinosteroid signaling. Plants were grown in a greenhouse in standard long-day conditions (16 h light 23 °C, 8 h darkness 16 °C). Rosette leaves were harvested from at least 4–9 plants per sample. Experiments were performed in three independent biological replicates (independent cultivation). The same plants were used to obtain material for RT-qPCR, protein lysate for protein hybridization by Western blots, and lipid samples for HPLC-UV analysis and photosynthetic pigments measurements.

2.1. Treatment of plants with epi-brassinolide

Plants were grown in soil in standard greenhouse conditions of a long day for 4 weeks and starting from the moment rosette leaves were sprayed with 4 μM f.c. epi-brassinolide in water, containing 0.01% ethanol every second day, as described in (Xia et al., 2009). Matched control plants were sprayed with water solution containing the same concentration of ethanol. After 5 cycles of epi-BR application plants were left for two additional days. Following this period rosette leaves were harvested and frozen in liquid nitrogen. Rosette leaves were harvested from at least 4–9 plants per sample. The same plants were used to obtain material for protein lysate, lipid samples for HPLC-UV analysis and photosynthetic pigments measurements.

2.2. Microscopy

Rosette leaves from 1-month-old plants were harvested and fixed in 2,5 % glutaraldehyde in 0,1 M cacodylate buffer pH = 7,2 overnight at room temperature. Samples were washed in cacodylate buffer three times and stained with 1 % osmium tetroxide in H_2O overnight at room temperature. Samples were washed in H_2O and dehydrated through a graded series of EtOH (30 %, 50 %, 70 %, 80 %, 96 %, absolute ethanol, and acetone). Samples were embedded in epon resin (SERVA) and polymerized for 24 h at 60 °C in an incubator (Agar Scientific, England). Next, 400 nm and 70 nm sections were cut with a diamond knife on RMC MTXL ultramicrotome (RMC Boeckeler Instruments, USA) for light and transmission electron microscopy. The sections on grids were contrasted with UranyLess (Micro to Nano, Netherlands) and lead citrate after 2 min. Samples were analyzed in a LIBRA 120 transmission electron microscope (Carl Zeiss, Germany), at 120 keV. Photographs were made with a Slow-Scan CCD camera (ProScan, Germany), using the EsiVision Pro 3.2 software. Measurements were performed using the analySIS® 3.0 image-analytical software (Soft Imaging Systems GmbH).

2.3. RNA extraction, reverse transcription, and RT-qPCR analysis

Rosette leaves of 1-month-old plants were collected. Plant tissue was homogenized in liquid nitrogen with mortar and pestle. About 65 mg of frozen powder was weighed for RNA extraction. RNA was extracted with GeneJet RNA Purification Kit (Thermo Fisher Scientific) according to the protocol dedicated to the plant material. Reverse transcription was performed with a RevertAid kit (Thermo Fisher Scientific) at 42 °C for 1 h with oligo dT primer using 1 μg of RNA per reaction. For RT-qPCR analysis, reaction mixtures contained 2,9 μL MiliQ water, 5 μL SYBR green (Roche), 0,05 μL of 100 μM forward primer, and 0,05 μL of 100 μM reverse primer. 2 μL of 10-fold diluted cDNA was added to each well and mixed. All samples were prepared in two technical replicates. Reactions were performed for control primer pair and primer pairs designed to genes encoding enzymes from the MEP pathway, chloroplast isoprenoid biosynthesis, and carotenoid biosynthesis. Primer sequences are listed in Supplementary Table S1. Primers were designed in Primer-BLAST (www.ncbi.nlm.nih.gov) to amplify the exon-exon junction region of 100–150 bp length, giving only one specific product located close to the 3′ end of the analyzed gene. Reactions were performed in 384 well plates (Roche) on LightCycler 480 (Roche) equipped with LightCycler 480 S.W 1.5 software. The program for PCR reaction is given in Supplementary Table S2. Mean Cp values obtained for tested genes were normalized to the expression of a reference gene *PP2A*.

2.4. Analysis of polyprenol content by HPLC-UV

Rosette leaves of 1-month-old plants (0.5 g) were homogenized in liquid nitrogen with mortar and pestle. The powder was extracted with chloroform: methanol 1:1 overnight with shaking at room temperature in darkness. The internal standard of 10 μg of Prenol-14 (C70)(Collection of Polyprenols, IBB PAS) was added to each sample. Purification and hydrolysis were performed according to (Akhtar et al., 2017).The organic extract was filtered, evaporated under a nitrogen stream, and subjected to alkaline hydrolysis at 95 °C for 1 h. Released free polyprenols were extracted three times with hexane, and organic phases were washed with water (1:1, by vol.), pooled, and evaporated under a nitrogen stream. Dried lipids were dissolved in a small amount of hexane and further subjected to separation on a silica-gel column (1 mL) equilibrated with hexane. After washing the column with 3 mL of hexane, carotenoids were eluted with 2 % ethyl ether in hexane (6 mL), and subsequently, polyprenols were eluted with 10 % ethyl ether in hexane (10 mL). Compounds eluted with 10 % ethyl ether in hexane were dried under nitrogen stream, dissolved in 50 μL 2-propan-ol, and subjected to HPLC-UV analysis on 4,6 mm × 75 mm x 3,5 μm Zorbax XDB-C18 reverted-phase column on HPLC-UV system (Waters) equipped with dual absorbance detector set to 210 nm wavelength. Lipids were eluted in a gradient (Supplementary Table S3) and retention times of the peaks were compared to a mix of prenol standards (Collection of Polyprenols, IBB PAS) containing a mixture of Prenols 9–25 (C45– C125). Signal integration and quantitative analysis were performed in Empower 2. program (Waters).

2.5. Analysis of photosynthetic pigments

Chlorophylls and carotenoids were analyzed spectrophotometrically as described earlier (Lichtenthaler and Buschman, 2001). Photosynthetic pigments were extracted from fresh plant tissue homogenized in acetone in darkness at room temperature. Diluted samples were scanned at wavelength range 700 nm–400 nm on a UV–Vis spectrophotometer (Varian Cary Bio 50) in a 10 mm quartz cuvette using the CaryWinUV-Scan software. The concentration of pigments was calculated with equations given in (Lichtenhaler and Buschman, 2001) and corrected for dilutions.

2.6. Chloroplast protein analysis

Rosette leaves of 1-month-old plants were homogenized in liquid nitrogen with mortar and pestle. The same amount of frozen powder from each sample was extracted in a buffer containing: 20 mM Tris-Cl pH = 7.5, 100 mM NaCl, 0,1 % β-mercaptoethanol, 1 % sucrose, and protein inhibitor cocktail (ProteaseMini EDTA-free, Roche). After centrifugation for 30 min at 16 000 rpm at 4 °C, protein concentration in the lysates was determined with the Bradford method. Normalized amounts of lysates were run on a standard 10 % denaturing acrylamide gel. Protein bands were transferred onto the PVDF membrane (Immobilon-P, Amersham). The membrane was cut in stripes corresponding to proteins of low and high molecular mass (according to protein standard: PAGE ruler, Thermo Fisher Scientific) and blocked in 5 % non-fat milk in PBS for 1 h. The membrane was incubated with the following primary antibodies (Agrisera) diluted in PBST with non-fat milk: anti-RbcL (AS03 037) 1:2000 and PsbA (AS10 704) 1:400 at 4 °C overnight. Membranes were washed and incubated with secondary anti-rabbit antibodies conjugated with HRP from Sigma Aldrich (A0545) diluted 1:2000 in non-fat milk in PBST for 1 h at room temperature. The signal was visualized with Super Signal WestPico PLUS (Thermo Fisher Scientific) substrate on Amersham Hyperfilm MP (GE Healthcare). Intensity of the signal was measured by ImageJ.

2.7. Data analysis

Obtained numerical data were analyzed and plotted with GraphPad 5.0 software. For statistical analysis one-way ANOVA with Tukey post-test was used.

3. Results

3.1. CPT7 gene promoter region contains binding sites for brassinosteroid-dependent transcription factors

Polyprenols of medium length (9–11 isoprene units) were recently discovered to play important role in photosynthetic apparatus organization in Angiosperm plants, such as Arabidopsis and tomato (Akhtar et al., 2017; van Gelder et al., 2018). These compounds are synthesized in chloroplast by a CPT7 cis-prenyl transferase enzyme by an elongation of geranylgeranyl allylic precursor. Currently, nothing is known about the transcriptional regulation of C45–C55 polyprenol synthesis. To gain some knowledge on the possible regulation of this process by hormonal and environmental signals we analyzed available data on transcription factor binding sites in the promoter region of the *CPT7* gene. A database search (PlantPan 3.0 server; http://plantpan.itps.ncku.edu.tw; Chow et al., 2016) revealed that several putative brassinosteroid-responsive elements are present in the *CPT7* promoter sequence. In particular, it was shown experimentally by the ChIP-on-chip method, that the BZR1 transcription factor binds directly to the *CPT7* promoter (Sun et al., 2010). BZR1 is a transcription repressor that occupies promoter regions in brassinosteroid absence but dissociates from promoters upon BRI1 signaling cascade activation by brassinosteroids. In the *CPT7* gene promoter also several PIF binding sites were found by experimental methods, for example, PIF4 (Oh et al., 2012) (phytochrome interaction factors-transcription repressors acting downstream of BZR1 in brassinosteroid signaling cascade). What is more, BES1 (BZR2) putative binding site is located in the *CPT7* promoter region (Li et al., 2018). Altogether the available data coming from high-throughput experiments raise the possibility, that *CPT7* gene expression is regulated in a brassinosteroid-dependent fashion. To follow the subject of regulation of *CPT7* gene expression by brassinosteroids in Arabidopsis experimentally we have chosen two mutants in brassinosteroid perception or production, both in Col-0 background (Fig. 1). One is *det2-1* (*deetiolated 2*, carrying a point mutation in steroid-α-5-reductase, an enzyme engaged in an early step of brassinosteroid biosynthesis from campesterol – Noguchi et al., 1999; Fujioka et al., 1997). This mutant is relatively not much affected in stature, probably because alternative brassinosteroid biosynthesis pathways (from sitosterol and cholesterol, reviewed in Bajguz et al., 2020) can compensate for the reduction in the main hormone level normally derived on pathways leading through campesterol (Noguchi et al., 1999). The second mutant that we studied is *bri1-116*, a null mutant in the main, ubiquitously expressed brassinosteroid receptor BRI1 (Friedrichsen et al., 2000). Although other brassinosteroid receptors are encoded in the Arabidopsis genome (BRL1, BRL2, BRL3; Fàbregas and Caño-Delgado, 2014; Friedrichsen et al., 2002), due to their limited sites of expression (Caño-Delgado et al., 2004) and lower brassinosteroid binding activity (Caño-Delgado et al., 2004; Kinoshita et al., 2005) the level of brassinosteroid signaling in *bri1-116* is strongly decreased and the phenotype is particularly strong. We also made an attempt to measure C45–C55 polyprenols and photosynthetic pigments in the plants that have up-regulated brassinosteroid signaling, namely in WT and *det2-1* plants treated with epi-brassinolide.

3.2. Transcription of the genes encoding for photosynthetic pigments and C45–C55 polyprenol biosynthesis is downregulated in rosette leaves of brassinosteroid perception mutant

We performed RT-qPCR analysis of transcription of the genes engaged in medium-length polyprenol synthesis in the chloroplast in WT

Fig. 1. Mature rosettes of Arabidopsis WT, *det2-1* and *bri1-116* mutant plants.
Plants grown in soil in a standard long-day conditions for 30 days. *det2-1* plants have a smaller rosette diameter and slightly curled leaves in comparison to WT Col-0 plants. *bri1-116* plants form small, compact rosettes with extremely curled leaves in comparison to WT plants.

and *bri1-116* mature leaves (Fig. 2). These genes were coding for all main enzyme isoforms of methylerythritol 4-phosphate (MEP) pathway synthesizing isoprene units precursors - IPP and DMAPP in the chloroplast (reviewed in Rodríguez-Concepción and Boronat, 2015). Generally, down-regulation of the MEP pathway in *bri1-116* mutant was observed (Fig. 2A), as transcription of most of the analyzed genes is decreased, including *DXR* and *DXS*, genes encoding rate-limiting enzymes (Wang and Dowd, 2018; Hemmerlin, 2013; Rodríguez-Concepción and Boronat, 2015), with the exception of *MCT* gene (Fig. 2A). Alongside, expression of chloroplast localized geranylgeranyl diphosphate (GGPP) synthase encoding gene (*GGPS2* - Nagel et al., 2015; Beck et al., 2013), together with *GGPS11* encoding for the main GGPS isoform responsible for photosynthetic pigments synthesis in Arabidopsis (Ruiz-Sola et al., 2016), is down-regulated on transcriptional level (Fig. 2B). Similarly, the chloroplast-localized cis--prenyltransferase encoding gene, *CPT7*, shown to be responsible for medium-chain-length polyprenol biosynthesis (Akhtar et al., 2017), is considerably down-regulated (Fig. 2B). Finally all the genes encoding carotenoid biosynthesis are also down-regulated (Fig. 2C). Altogether transcriptomic profile of *bri1-116* mutant shows a significant decrease of transcription of nuclear genes coding for chloroplast localized enzymes indispensable for photosynthesis. Reports for other plant species support the notion, that the brassinosteroid signaling pathway is important for the basic metabolism of chloroplasts and that brassinosteroids regulate

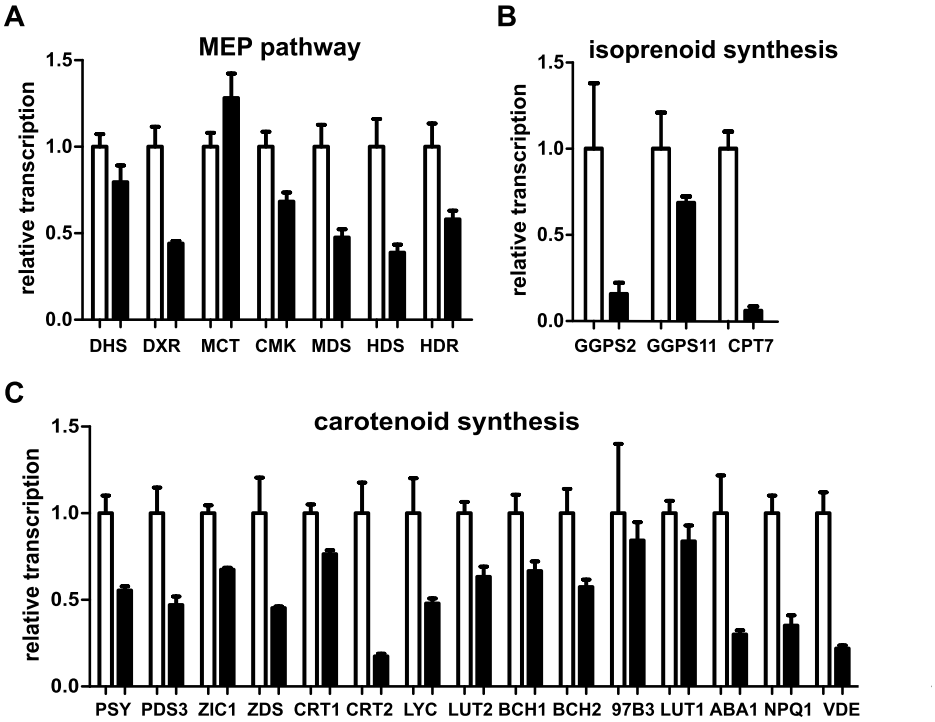

Fig. 2. Relative transcription of genes coding for enzymes engaged in isoprenoid lipids formation in chloroplast of WT and *bri1-116* mutant of Arabidopsis.
Relative transcription of the genes encoding for enzymes from **A)** MEP (methylerithritol phosphate patway, non-mevalonic isoprenoid biosynthesis pathway), **B)** geranylgeranyl diphosphate synthases localized to chloroplast, cis-prenyl transferase 7 and **C)** carotenoid biosynthesis pathway was measured by RT-qPCR method. Codes for gene names are: DXS: 1-Deoxy-D-xylulose 5-phosphate synthase, DXR: 1-Deoxy-D-xylulose 5-phosphate reductoisomerase, MCT: 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, CMK: 4-(Cytidine 5-diphospho)-2-C-methyl-D-erythritol kinase, MDS: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, HDS: 4-Hydroxy-3-methylbut-2-enyl-diphosphate synthase, HDR: 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase, GGPS2: Geranylgeranyl diphosphate synthase 2, GGPS11: Geranylgeranyl diphosphate synthase 11, CPT7: cis-prenyl transferase 7, PSY: phytoene synthase, PDS: phytoene desaturase 3, ZIC: 15-cis-zeta-carotene isomerase, ZDS: zeta-carotene desaturase, CRT1: carotenoid isomerase1, CRT2: carotenoid isomerase2, LYC: lycopene beta-cyclase, LUT2: lycopene epsilon-cyclase, BCH1: carotene hydroxylase1, BCH2: carotene hydroxylase2, 97B3: cytochrome P450, LUT1: carotene hydroxylase1, ABA1: zeaxanthin epoxidase, NPQ1: violaxanthin de-epoxidase, VDE: violaxanthin de-epoxidase-like. Experiment was performed in triplicate and normalized on transcription of a PP2AA3 reference gene. White bars- WT plants, black bars – *bri1-116* plants. Bars show mean ± SD.

the cellular level of enzymes of the Calvin-Benson cycle, MEP pathway, and pigment synthesis encoded in the nuclear genome, but imported to chloroplasts (Yin et al., 2021; Meier et al., 2011).

3.3. Brassinosteroid perception mutant accumulates a negligible amount of C45–C55 polyprenols, while the WT and det2-1 plants treated with epi-brassinolide have increased amount of C45–C55 polyprenols

To further analyze these results we extracted lipids from Arabidopsis plants grown in standard long-day conditions of WT, *det2-1*, *bri1-116* mutants and WT and *det2-1* plants treated with brassinosteroid-epi-brassinolide. We analyzed the C45–C55 polyprenol content and composition by the HPLC-UV method. Polyprenols in tissues of plants always build a „family" of compounds differing in one isoprenoid unit (5 carbons) one from each other, usually one of the compounds is dominating (Surowiecki et al., 2019; Jozwiak et al., 2013; Ciepichal et al., 2007). In isolated chloroplasts of Arabidopsis the family of polyprenols of chain length 9-10-11 isoprene units is dominating, the structures of these compounds we confirmed earlier by mass spectrometry (Kania et al., 2012) and NMR studies (Akhtar et al., 2017). The qualitative analysis of C45–C55 polyprenols in WT, *det2-1* and *bri1-116* plants was performed by comparison with the external standard of polyprenol mixture, the quantitative analysis was performed using the internal standard method. The obtained results are presented in (Fig. 3A, B, C). The amount and composition of C45–C55 polyprenols of WT and *det2-1* plants did not differ significantly (Fig. 3B). However, in *bri1-116* the intensity of peaks corresponding to polyprenols composed of 9, 10, or 11 isoprene units was strongly decreased (Fig. 3A and B). We also performed C45–C55 polyprenol quatification in WT and *det-2* plants treated by foliar application of epi-brassinolide hormone (Fig. 3C). We found, that their content significantly increased in case of WT plants and *det2-1* plants. Additionally we noted, that the amount of polyprenols per g of fresh weight vary between experiments-we suggest that this is due to slightly different seasonal conditions in the greenhouse.

3.4. Photosynthetic pigments are affected adversely to C45–C55 polyprenols in bri1-116 mutant

We measured the fresh weight (Fig. 4G) and photosynthetic pigments level (Fig. 4A–C). The fresh weight of *det2-1* plants was much lower than WT plants, but not affected by epi-brassinolide treatment. *bri1-116* plants were extremely small. The amounts of chlorophyll *a*, chlorophyll *b*, and carotenoids per gram of fresh weight of the plant in *det2-1* plants in comparison to WT plants remained the same (Fig. 4A–C) while it was increased significantly in *bri1-116* plants in comparison to WT (Fig. 4A–C). This result is consistent with earlier reports on brassinosteroid perception and biosynthesis mutants in Arabidopsis (Zhang et al., 2021) where *det2* and *bri1* mutant (*bri1-5* weak mutant) had a slightly increased chlorophylls level but in our case *det2-1* showed chlorophyll content comparable to WT, while *bri1-116* had an increased chlorophyll content. In plants treated with epi-brassinolide the content of chloroplast pigments remained the same as in WT plants.

Additionally, we analyzed the amount of selected chloroplast proteins: RuBisCO L and PsbA D1 in WT, *det2-1, bri1-116* leaf lysates, as well as in plants treated with epi-brassinolide (Fig. 4D–F). Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is one of the most important and abundant proteins in chloroplasts. It catalyzes the rate-limiting reaction in the Calvin-Benson cycle of carbon dioxide

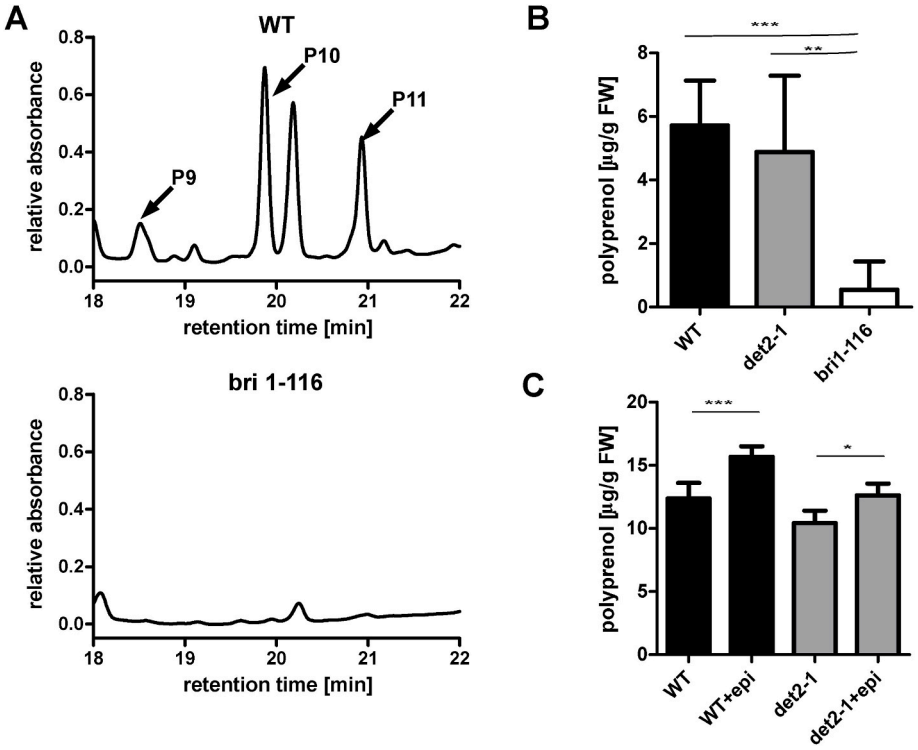

Fig. 3. Qualitative and quantitative determination of medium-chain-length polyprenols C45–C55 in WT, *det2-1* and *bri1-116* Arabidopsis plants and plants treated with foliar applied epi-brassinolide.
A) Medium-chain-length polyprenol composition in WT and *bri1-116* mutant was determined after isolation, alkaline hydrolysis and subfractionation from total lysates of mature rosette leaves by HPLC-UV method, against a standard mixture of polyprenols of known hydrocarbon chain length. Representative chromatograms are shown. Arrows point to the peaks of a retention time corresponding to polyprenols built of 9, 10 and 11 isoprene units. **B)** Quantitative determination of polyprenols (9-10-11 isoprenoid chain length) from WT, *det2-1 and bri1-116* or **C)** WT and *det2-1* plants treated with 4 µM epi-brassinolide (brassinosteroid analoque) was performed by comparison of a known amount of internal standard (prenol 14) added to the plant lysates during initial lipid extraction. Bars represent mean ± SD. Amount of lipid was normalized per g of fresh weigth of plants used for lipid extraction. Data analyzed with one-way ANOVA with Tukey post test. ANOVA $p < 0{,}001$; * denotes $p = 0.05$, ** denotes $p = 0.01$, *** denotes $p = 0.001$ in Tukey post test.

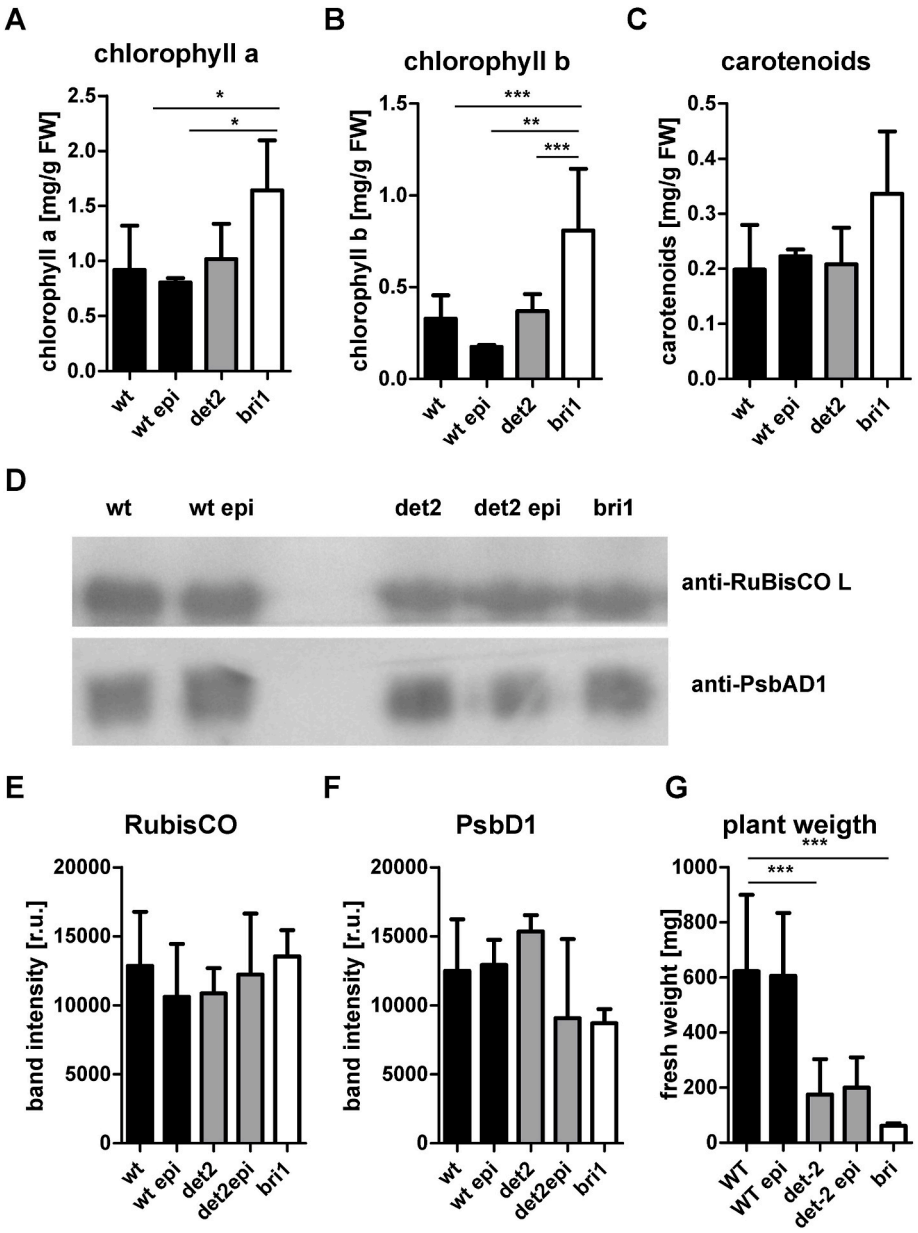

Fig. 4. Quantitative determination of photosynthetic pigments and selected chloroplast proteins in WT, *det2-1 and bri1-116* Arabidopsis plants and plants treated with foliar applied epi-brassinolide.

A) Photosynthetic pigments amount was determined spectrophotometrically in acetone extracts. Experiment was performed in at least triplicate, bars represent mean ± SD. Amount of pigments was normalized per g of fresh weigth of plants used for lipid extraction. **A)** Amount of chlorophyll *a*, one-way ANOVA p = 0,0219; **B)** Amount of chlorophyll *b*, one-way ANOVA p = 0,0002; **C)** Amount of carotenoids, one way ANOVA p = 0,0730. **D)** Chloroplast localized proteins engaged in photosynthesis: RuBisCO L and PsbAD1 were determined by Western blot analysis of total protein lysates isolated from WT, *det2-1, bri1-116* Arabidopsis plants and plants treated with foliar applied epi-brassinolide. Amount of protein loaded to each lane was normalized. Representative result is shown. **E)** quantitative determination of RuBisCO L protein. Densitometry of the membranes was performed in ImageJ, bars show mean of three replicates ± SD for each experiment, one way ANOVA p = 0,8007; **F)** quantitative determination of PsAD1 protein, one way ANOVA p = 0,1700. Densitometry of the membranes was performed in ImageJ, bars show mean of three replicates ± SD for each experiment. **G)** Fresh weight of plants of studied genotypes. At least 20 plants were measured for each genotype. Bars show mean ± SD for each experiment. Data analyzed with one-way ANOVA with Tukey post test, for all graphs * denotes p = 0.05, ** denotes p = 0.01, *** denotes p = 0.001 in Tukey post test.

assimilation and resides in chloroplast stroma (Sharkey, 2022). We did not find a significant difference in this protein amount between all the plants (Fig. 4D and E). Also in a previous study the activation status of RuBisCO but not the total content and transcripts of RuBisCO were closely related to the endogenous BR levels in tomato (Li et al., 2016), however, in cucumber an active hormone epi-brassinolide up-regulated, while the inhibitor, brassinazol, down-regulated, the expression of RuBisCO L encoding gene (Xia et al., 2009). The content of PsbA D1, a protein of photosystem II multi-subunit complex (PS II) that carries the reaction center, was also analyzed (Fig. 4D,F). The PsbA D1 protein is localized in the stroma-exposed thylakoid membrane (Inagaki, 2022), mainly in the densely stacked part of the thylakoid granum (reviewed in Kouřil et al., 2018). We did see a decrease in the amount of PsbA D1 protein between some groups of plants, but it was statistically insignificant (Fig. 4D,F). What is worth noticing, both of these proteins are chloroplast genome encoded, and may therefore undergo different regulation upon brassinosteroid signaling depletion than nuclear-encoded genes.

3.5. Grana structure in chloroplasts of brassinosteroid synthesis/perception mutants is mis-organized

When we observed chloroplast ultra-structure by transmission electron microscopy in WT, *bri1-116*, and *det2-1* plants clear differences were noticed. WT chloroplasts were relatively large, lens-shaped with well-defined thylakoid grana and lamellae (Fig. 5A), large starch deposits and plastoglobules. In the *det2-1* mutant, the size of chloroplasts was not changed, but the internal thylakoid membranes formed more irregularly shaped grana. Also, the number of thylakoids in a stack was increased in comparison to WT, but the chloroplasts contained large starch deposits (Fig. 5B). In *bri1-116* the chloroplasts were smaller, often round-shaped or irregular (Fig. 5C), which confirmed earlier observations of other *bri1* mutants (Zhang et al., 2021). The thylakoids in *bri1-116* were not well organized in grana stacks and lamellae, often transitional structures were visible, and the stacks on average contained more thylakoids (Fig. 5C), a trait observed also by (Zhang et al., 2021). Chloroplast in *bri1-116* rarely contained starch or plastoglobuli (Fig. 5C). Mis-regulation of starch accumulation was earlier observed in plants deficient in brassinosteroid synthesis/signaling by microscopic and biochemical methods (Zhang et al., 2021; Schroder et al., 2014). Moreover, our data were supported by earlier physicochemical analysis by atomic force microscopy that revealed a larger thylakoid area per chloroplast in the *bri1-116* mutant compared to WT (Krumova et al., 2013).

4. Discussion

4.1. Synthesis of photosynthetic pigments and C45–C55 polyprenols in Arabidopsis rosette leaves is regulated by brassinosteroids

Results obtained by us revealed an increased amount of photosynthetic pigments per gram of fresh weight in the Arabidopsis *bri1-116* mutant in comparison to the WT control, while the amount of C45–C55 polyprenols was much lower in the mutant than in the WT control. An interesting explanation would be, that in Arabidopsis GGPP precursor used to synthesize both photosynthetic pigments and C45–C55 polyprenols, is directed towards metabolites considered more important for plant survival (carotenoids and chlorophylls crucial for photosynthesis). In contrast, WT plants and *det2-1* plants treated with epi-brassinolide showed an increase in C45–C55 polyprenol content.

C45–C55 polyprenols are important for photosynthesis efficiency (Akhtar et al., 2017), but still dispensable for plant survival (Akhtar et al., 2017) and hence their synthesis may be less important for plants than chrorophylls and carotenoids. Accordingly, in WT tomato fruits treated with brassinosteroids transcription of carotenoid biosynthesis genes and the carotenoid accumulation in a pericarp was increased in comparison to non-treated control (Nie et al., 2017; Liu et al., 2014; Yin et al., 2021). Other results obtained for brassinosteroid signaling/biosynthesis mutants or plants treated with BRI1 inhibitors or brassinosteroid analogues are not fully compatible between mono- and dicotyledonous plants (Janeczko et al., 2016; Liu et al., 2014; Rothova et al., 2014). Most probably the relative increase/decrease in chlorophyll and carotenoid levels upon plant treatment with brassinosteroids in different experiments depend on plant age, developmental stage, and species-specific factors, for example, increased carotenoid metabolism in tomato fruit, as mentioned earlier (Liu et al., 2014) or C4 type of photosynthesis, as suggested by (Janeczko et al., 2016; Rothova et al., 2014).

4.2. Synthesis of isoprenoid lipids engaged in photosynthesis undergo complex regulation by brassinosteroids and light

Many genes encoding enzymes crucial for photosynthesis have brassinosteroid dependent BRZ1 binding sites in their promoters. Lot of these genes are also regulated by PIF transcription factors, acting

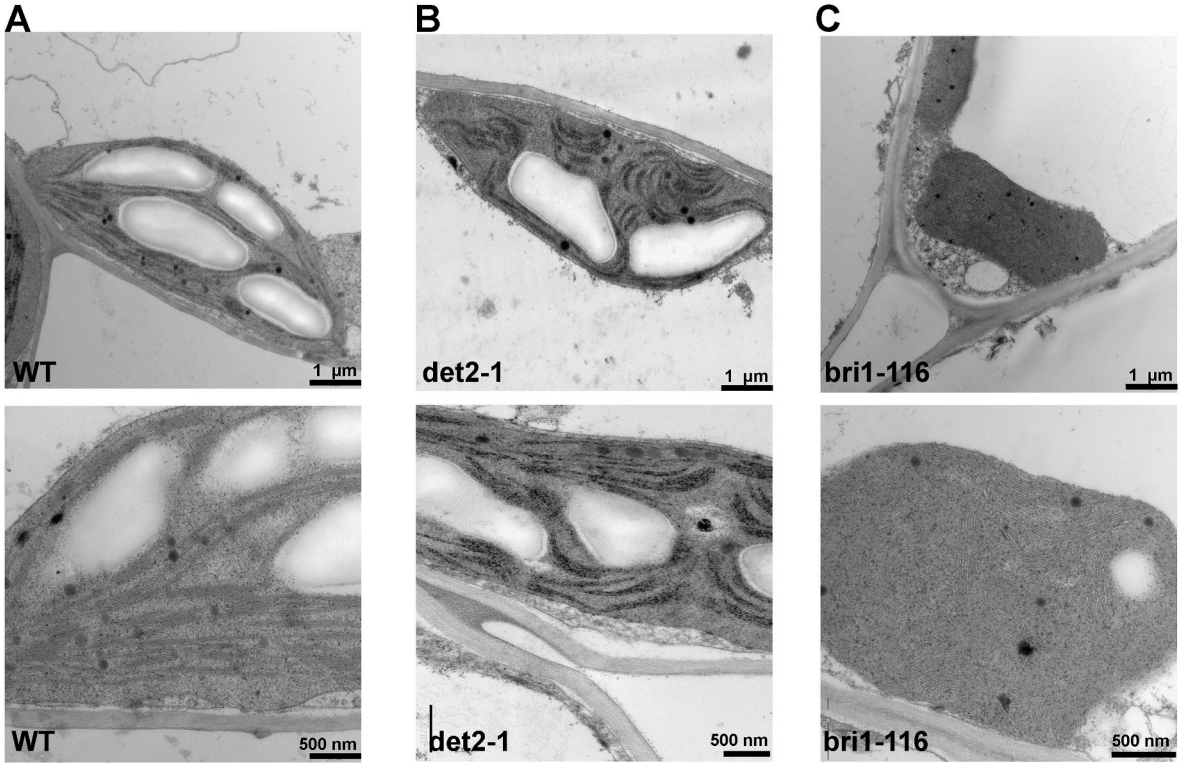

Fig. 5. Ultrastructure of chloroplast coming from mature mesophyll cells of *Arabidopsis* leaves.
A) WT plants, **B)** *det2-1* mutant, **C)** *bri1-116* mutant. Plants were grown in standard long-day conditions and leaves were gathered in the middle of the light period. TEM images were done on material obtained by chemical fixation of leaves. Experiment was performed in duplicate. Scale bars on the electronographs.

downstream of BZR1 and known to be regulated by light intensity (reviewed in Hemmerlin et al., 2012). It was shown that brassinosteroid and light signals (mediated by PIFs) together with gibberellin signaling are integrated by a DELLA/BZR1/PIF4 transcription module (Bai et al., 2012). BZR1 and PIF4 directly interact with each other, forming a complex on common promoters and acting as negative regulators of photomorphogenesis (Oh et al., 2012). If PIF4 is degraded in response to light signal or BZR1 is degraded in brassinosteroid signaling deficient mutant, the complex is not formed, which promotes chloroplast maturation in the dark (Oh et al., 2012). PIFs negatively regulate transcription of *PSY,* a gene encoding phytoene synthase, the branching point enzyme catalyzing condensation of two geranylgeranyl diphosphate molecules to form the first dedicated carotenoid precursor phytoene (Toledo-Ortiz et al., 2010) and chlorophyll biosynthesis genes (Moon et al., 2008; Tachibana et al., 2022).

CPT7*,* an enzyme responsible for C45–C55 polyprenol biosynthesis, also has brassinosteroid-dependent regulatory elements in its gene promoter, most importantly BZR1 binding site (Sun et al., 2010) and PIF7 binding site (Burko et al., 2022). *CPT7* promoter also contains PIF4 binding site in common with *PSY* and *GGPS11* genes (Oh et al., 2012). Some regulatory elements in gene promoters are specific for only *CPT7*, namely BES1 binding site (Li et al., 2018), but are absent in either *GGPS11* or *PSY*. In contrast, some transcription factors seem to regulate both *PSY* and *GGPS11* genes, but not *CPT7* gene expression (Table 1), namely GLK1, and PIF1 (Zhang et al., 2021; Yang et al., 2020). Altogether the genes encoding enzymes crucial for isoprenoid lipid biosynthesis in chloroplast undergo complex regulation by brassinosteroids and light signals. Of note is that in WT Arabidopsis plants the amount of C45–C55 polyprenols, synthesized by CPT7 enzyme, seems to be very low without light stimulation (Bykowski et al., 2020), and negligible in brassinosteroid perception mutant, as shown by us.

4.3. C45–C55 Polyprenol deficiency may be one of the factors influencing thylakoid structure

The increased amount of photosynthetic pigments per gram of fresh weight in *bri1-116* plants may be a consequence of the disturbed organization of thylakoid membranes. It was reported earlier that brassinosteroid imbalance affects photosynthesis, in particular, electron chain transport (Krumova et al., 2013; Sadura and Janeczko, 2021; Dobrikova et al., 2014; Rothova et al., 2014). Literature data also mention changes in thylakoid membrane properties in *bri1-116* interpreted as the disassembly of large PS II complexes into smaller ones (Krumova et al., 2013; Dobrikova et al., 2014). These data go along well with the decrease in the accumulation of recently described chloroplast membrane modifiers such as C45–C55 polyprenols as described by us, or fatty acid composition in chloroplast lipids (Rudolphi-Szydło et al., 2020; Fedina et al., 2017). In a combined biochemical/biophysical experiment, the lipid composition and biophysical properties of the membranes isolated from *det2* and *bri1* mutants of barley was studied (Rudolphi-Szydło et al., 2020). MGDG, a major lipid component of chloroplast membranes, had different composition of fatty acid chains in WT barley and brassinosteroid-related mutants. In particular the ratio of 18:3 fatty acid and also the global ratio of unstarurated vs. saturated fatty acids in these mutants were much lower than in WT plants under ambient temperature (20 °C), but increased above the WT level after cold treatment (Rudolphi-Szydło et al., 2020). These changes in lipid composition correlated with changes in physicochemical parameters of the membrane. Also in the pea leaves, the application of epi-brassinolide caused the change in fatty acid composition of galactolipids (Fedina et al., 2017). Interestingly, we show that in *bri1-116* mutant the amount of PS II proteins is not affected. The findings concerning change of C45–C55 polyprenol pool and fatty acid composition raise the possibility, that PS II organization, but not number, is changed in *bri1* mutants.

The cellular processes that are affected in C45–C55 polyprenol deficient mutant *cpt7* and *CPT7* RNAi silenced lines seem the same as these reported to occur in brassinosteroid perception mutants, i.e. decrease in membrane fluidity (Akhtar et al., 2017; Krumova et al., 2013; Dobrikova et al., 2014; Sadura and Janeczko, 2021), affecting membrane-embedded PS II and LHC II protein complex aggregation (Akhtar et al., 2017; Buszewicz et al. BiorXiv; Krumova et al., 2013; Dobrikova et al., 2014) and decrease of electron transport through photosystem II lowering the effective quantum yield of photosystem II without a decrease in maximum quantum yield (Akhtar et al., 2017; Krumova et al., 2013; Rothova et al., 2014).

Table 1
Transcription factors binding to promotors of *GGPS11*, *PSY* and *CPT7* genes of Arabidopsis. Only transcription factors regulated by brassinosteroid signaling pathway were included.

GGPS11	PSY	CPT7
–	BZR1	BZR1
–	–	BES1/BZR2
BIM1	BIM1	BIM2, BIM3
BEE1, BEE3	BEE1	BEE1, BEE2
–	–	BEH2, BEH3, BEH4
PIF1, PIF4	PIF1, PIF4, PIF7	PIF4, PIF5, PIF7
GLK1	GLK2	–

5. Conclusion

We showed that deficiency in brassinosteroid perception in *Arabidopsis* leads to chloroplast inner membranes deformation, mainly poorly stacked grana and longer stroma thylakoids. In parallel, we showed that the transcription of MEP-pathway encoding genes is decreased in the *bri1-116* mutant, but this does not lead to a significant decrease in photosynthetic pigment accumulation. We showed that the transcription of the *CPT7* gene encoding for cis-prenyltransferase that is responsible for the synthesis of plastidial polyprenols is significantly reduced in *bri1-116* and this result is consistent with a decreased amount of plastidial polyprenols built of 9-10-11 isoprene residues in this mutant. Treatment of the WT plants with epi-brassinolide caused an adversed effect-polyprenol accumulation. According to the literature, the deficiency of plastidial polyprenols built of 9-10-11 isoprene residues leads to decreased photosynthetic electron transport rate (Akhtar et al., 2017; van Gelder et al., 2018). Since the same effect was noticed in studies on brassinosteroid signaling deficiency in various plant species the decreased amount of polyprenols might constitute the molecular background of this phenomenon.

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CRediT authorship contribution statement

Małgorzata Gutkowska: designed and performed experiments (lipid and protein analysis), analyzed the results, wrote the manuscript and obtained funding. **Daniel Buszewicz:** designed and performed the experiments, analyzed the data (RT-qPCR) and obtained funding. **Marta Zajbt-Łuczniewska:** performed the experiments (lipid analysis and RT-qPCR). **Mateusz Radkiewicz:** performed the experiments (pigment analysis and TEM). **Julita Nowakowska:** performed the TEM analysis. **Ewa Swiezewska:** analyzed the data and wrote the manuscript. **Liliana Surmacz:** performed the experiments for the revisions and analyzed the data.

Declaration of competing interest

Authors declare no conflict of interests.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jplph.2023.154126.

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