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ORIGINAL ARTICLE

Domain of Unknown Function 143 Is Required for the Functioning of PEP-associated Protein DG238 in the Chloroplast

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Abstract Plastid-encoded plastid RNA polymerase (PEP) is essential for chloroplast development and plastid gene expression in *Arabidopsis thaliana*. However, PEP is a large complex, and many proteins in this complex remain to be identified. We previously reported that Delayed Greening 238 (DG238) interacts with PEP subunit protein FLN1 and may function as a PEP-associated protein and participate in early chloroplast development and PEP-dependent plastid gene expression. DG238 contains Domain of Unknown Function 143 (DUF143), whose function is currently unknown. Here, we found that a deficiency of the DUF143 domain in DG238 affected its localization, which resulted in abnormal interactions with PEP-associated proteins in the chloroplast. Furthermore, DG238 lacking the DUF143 domain or DG238 with only this domain failed to function. Interestingly, the lack of conserved amino acids 193–217 of the DUF143 domain in DG238 also affected its function. In addition to FLN1, DG238 also interacts with other PEP-associated proteins, including FSD2, FSD3, MRL7-L, and MRL7, to regulate plastid gene expression. These results suggest that the DUF143 domain is necessary for the functioning of the PEP-associated protein DG238 in chloroplasts.

Keywords: Arabidopsis, Chloroplast, DG238, DUF143, PEP, Plastid gene expression

Introduction

Chloroplast biogenesis in higher plants is a complex process whose regulatory mechanism remains largely unknown (Pogson et al. 2015). Plant chloroplasts have an endosymbiotic origin: after a long period of evolution, many photosynthetic cyanobacterium-like organisms became engulfed by various heterotrophic unicellular eukaryotes, and numerous prokaryotic genes were transferred into the host nucleus, thereby forming new organisms, the photosynthetic eukaryotes (Martin et al. 2002). In general, chloroplasts develop from proplastids as follows: After induction by light, the inner envelope of the proplastid becomes enfolded to form a vesicle. Subsequently, the vesicles fuse into a lamellar structure with a well-developed thylakoid membrane, which develops into grana stacks, thereby forming a mature chloroplast (Lopez-Juez 2007; Jarvis and Lopez-Juez 2013; Osteryoung and Pyke 2014).

Chloroplasts are semi-autonomous organelles that contain their own genomes encoding approximately 100 genes. However, nearly 2,000 proteins are located in the chloroplast. Most of these proteins are encoded by nuclear genes and are transported into chloroplasts through their chloroplast transmembrane signal peptides (Abdallah et al. 2000). These nucleus-encoded chloroplast proteins often play important roles in photosynthesis, plastid gene expression, photosynthetic pigment biosynthesis and metabolite biosynthesis (Luo et al. 2012; Zhou et al. 2012; Luo et al. 2013). Therefore, proper functioning of the chloroplast requires coordination between nucleus- and chloroplast-encoded genes.

Chloroplast development, a process vital for plant growth, is regulated by numerous proteins (Pogson et al. 2015). Plastid-encoded plastid RNA polymerase (PEP) is a large complex, containing many protein subunits, such as pTAC6, pTAC12, TRXz, FLN1, FSD3, and so on, that regulates plastid gene transcription and chloroplast development in plants (Pfalz et al. 2006; Myouga et al. 2008; Arsova et al. 2010; Pfalz and Pfannschmidt 2013; Yu et al. 2014). The PEP complex also includes other associated proteins, many of which have recently been identified, such as HSP21, FTRc, PRDA1, and MRL7. All of these associated proteins are located in the chloroplast nucleoid, can interact with PEP subunit proteins, and regulate PEP-dependent gene expression

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and chloroplast development in plants (Qiao et al. 2011; Qiao et al. 2013; Zhong et al. 2013; Wang et al. 2014). A potential PEP-associated protein, Delayed Greening 238 (DG238), was recently found to participate in the regulation of PEP-dependent gene expression and early chloroplast development in *Arabidopsis thaliana* (Wang et al. 2016). Iojap, the homologous protein of DG238 in maize, is involved in regulating chloroplast function, but its roles remain unclear (Han et al. 1992).

In this study, we found that DG238 can interact with the PEP-associated proteins FSD2, FSD3, MRL7-L and MRL7, and it cooperates with FLN1 to regulate plastid gene expression. Furthermore, DG238 contains a DUF143 domain whose deficiency affects the localization of DG238 and its interactions with PEP-associated proteins in the chloroplast, as well as the functioning of DG238 itself. These results suggest that the DUF143 domain plays important roles in the functioning of DG238.

Results

The DUF143 Domain Is Vital for the Localization of DG238 and Its Interaction with PEP-associated Proteins

Bioinformatics analysis showed that DG238 contains a DUF143 domain (Fig. 1A) (Wang et al. 2016); however, the function of this domain remains unclear. To explore the function of the DUF143 domain, we constructed vectors to express DG238$^{\Delta DUF143}$ (deficiency of the DUF143 domain) and DG238^{DUF143} (having only the DUF143 domain) (Fig. 1A). Since DG238 is localized to the chloroplast nucleoid, we first investigated whether the DUF143 domain affects the localization of DG238. When we transformed Arabidopsis protoplasts with the DG238$^{\Delta DUF143}$-GFP construct, the fluorescent signals co-localized with chloroplasts in general rather than being restricted to the nucleoid (Fig. 1B). In protoplasts transformed with DG238^{DUF143}-GFP, fluorescent signals similar to those for DG238-GFP were observed (Fig. 1B), suggesting that the DUF143 domain is essential for the correct localization of DG238.

We previously reported that DG238 interacts with PEP subunit protein FLN1; however, the interacting region of DG238 was unclear (Wang et al. 2016). Since DG238 contains the DUF143 domain, which affects the localization of DG238, we explored whether this domain is necessary for the interaction between DG238 and FLN1. Bimolecular fluorescence complementation (BiFC) assays showed that DG238$^{\Delta DUF143}$-GFP interacted with FLN1, with yellow fluorescent signals dispersed throughout the chloroplast (Fig. 2A). However, we failed to detect obvious signals between DG238^{DUF143} and FLN1 (Fig. 2A). These results indicate that

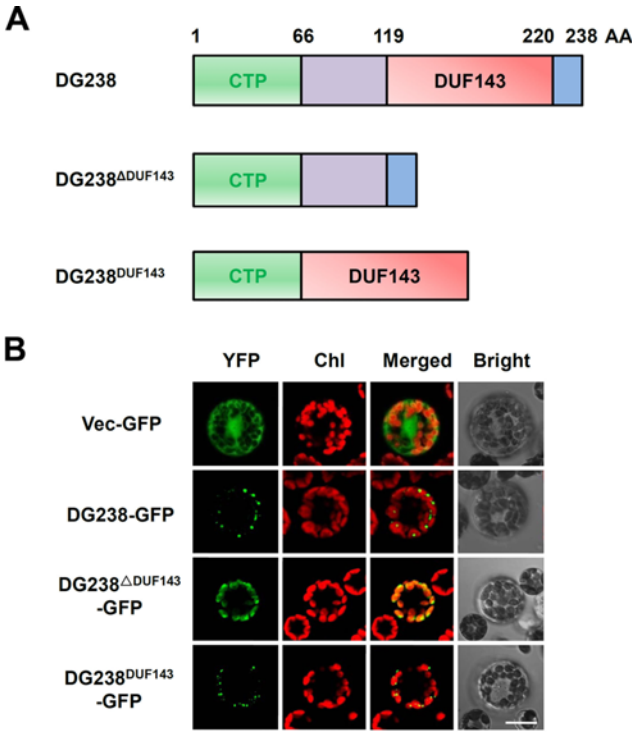

Fig. 1. The DUF143 domain affects the chloroplast nucleoid localization of DG238. (A) Domain structure of DG238. DG238$^{\Delta DUF143}$, DG238 without the DUF143 domain; DG238^{DUF143}, DG238 containing only the DUF143 domain. (B) The effect of the DUF143 domain on the chloroplast nucleoid localization of DG238, as determined by GFP assays. Vec-GFP, empty vector control; DG238-GFP, DG238-GFP fusion; DG238$^{\Delta DUF143}$-GFP, DG238-GFP fusion without the DUF143 domain; DG238^{DUF143}-GFP, DG238-GFP fusion containing only the DUF143 domain. Bars = 10 μm.

the DUF143 domain is not the interaction region for FLN1, although the loss of the DUF143 domain led to an abnormal interaction between DG238 and FLN1 (Fig. 2A). To further investigate the function of the DUF143 domain, we transformed *dg238-2* mutant lines with the DG238$^{\Delta DUF143}$ and DG238^{DUF143} sequences fused to a C-terminal 2×Flag sequence and successfully obtained *dg238-2* transgenic lines *35S:DG238$^{\Delta DUF143}$-Flag/dg238-2* and *35S:DG238^{DUF143}-Flag/dg238-2*, respectively (Fig. 2B and Fig. S1). Neither *35S:DG238$^{\Delta DUF143}$-Flag* nor *35S:DG238^{DUF143}-Flag* complemented the normal functioning of DG238, as all of the transformed plants showed low photosynthetic efficiency similar to that of *dg238-2* plants (Fig. 2B).

The Functions of Conserved Amino Acids in the DUF143 Domain

To further investigate the function of the DUF143 domain, we compared its sequences in various organisms using ClustalW and found that the DUF143 domain of DG238 (residues 119–220) shares sequence homology with Iojap

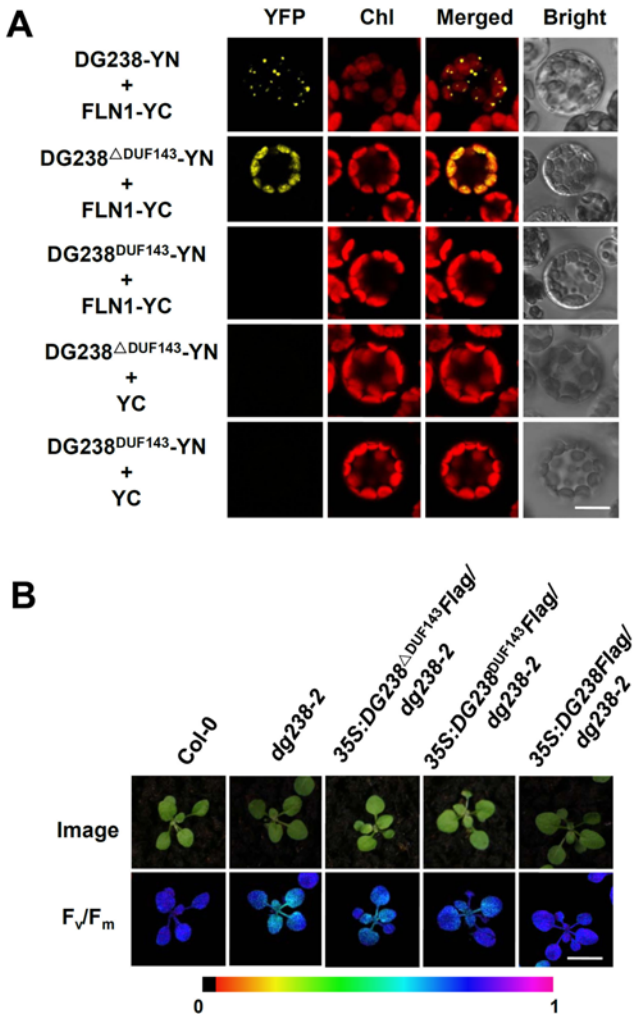

Fig. 2. Analysis of the role of the DUF143 domain in the interaction between DG238 and FLN1. (A) BiFC assay of Arabidopsis protoplasts transformed with different combinations of the constructs and visualized using confocal microscopy. Bars = 10 μm. (B) Photographs and chlorophyll fluorescence images of *dg238-2* plants grown for 2 weeks on soil. The chlorophyll fluorescence color ranges from black (0) to red, yellow, green, blue, and purple (1), as indicated at the bottom. Bars = 1 cm.

from *Zea mays* (residues 116–213), which is involved in regulating chloroplast development (Han et al. 1992); YbeB from *E.coli* (residues 1–104), which associates with the large subunit of the bacterial ribosome (Hauser et al. 2012); C7orf30 from human (residues 93–194), which is required for the biogenesis of the large subunit of the mitochondrial ribosome (Rorbach et al. 2012); and ATP25 from *Saccharomyces cerevisiae* (residues 104–246), which is required for the expression and assembly of the Atp9p subunit of mitochondrial ATPase (Zeng et al. 2008) (Fig. 3A). Furthermore, sequence analysis showed that conserved amino acids of DUF143 is D138 (residue 138, aspartic acid), I156 (residue 156, isoleucine), S161 (residue 161, serine), W193 (residue 193, tryptophan), D197 (residue 197, aspartic acid), R211 (residue 211, arginine), L216 (residue 216, leucine), and E217 (residue 217, glutamic acid) (Fig. 3A). To explore the functions of these conserved amino acids in the DUF143 domain, we performed an amino acid mutation assay to mutate these conserved amino acids to alanine (Fig. 3B). Since W193, D197, R211, L216, and E217 are closely linked in the DUF143 domain, we constructed a vector to express DG238$^{\Delta 193\text{-}217}$ (the DG238 protein lacking amino acids 193–217) (Fig. 3B). Our BiFC analysis showed that neither the single amino acid mutations described above nor the lack of amino acids 193–217 affected the interaction between DG238 and FLN1 (Fig. 3C), further confirming that the DUF143 domain is not the interaction region for FLN1. We also transformed the *dg238-2* mutant with these vectors and found that all of the mutant proteins expressed in the *dg238-2* background successfully complemented its mutant phenotype, except for DG238$^{\Delta 193\text{-}217}$, which lacked amino acids 193–217 (Fig. 3D and Fig. S2). These results suggest that amino acids 193–217 are required for the functioning of DG238.

The Interactions of DG238 with Other PEP-associated Proteins

When *DG238* was co-expressed with *FLN1* in Arabidopsis, DG238 interacted with the PEP subunit FLN1, suggesting that DG238 functions as a PEP-associated protein, which together with FLN1, regulates the early stage of chloroplast development and PEP-dependent plastid gene expression in a synergistic manner (Arsova et al. 2010; Wang et al. 2016). To further investigate the cooperative relationship between DG238 and FLN1, we performed Virus Induced Gene Silencing (VIGS) to repress *FLN1* expression in both wild-type (Col-0) and *dg238-2* mutant Arabidopsis plants to obtain VIGS-FLN1 (Col-0) and VIGS-FLN1 (*dg238-2*) plants, respectively. Plants not expressing *DG238* and *FLN1*, i.e., VIGS-FLN1 (*dg238-2*), showed retarded growth and more severe phenotypes in young leaves compared with VIGS-FLN1 (Col-0) and VIGS-GFP (*dg238-2*) plants, in which only one of these genes was not expressed (Fig. 4A). To explore PEP-dependent gene expression in VIGS-FLN1 (*dg238-2*), we investigated the expression of PEP-dependent genes *psaA*, *psbA*, *psbK*, and *rbcL*, along with *psaE* as a representative nucleus-encoded gene (as a control). The expression of the PEP-dependent genes was reduced in VIGS-FLN1 (Col-0), VIGS-GFP (*dg238-2*), and VIGS-FLN1 (*dg238-2*), especially *psaA* and *rbcL* expression in VIGS-FLN1 (*dg238-2*), compared with VIGS-FLN1 (Col-0) and VIGS-GFP (*dg238-2*) plants (Fig. 4B). These results suggest that DG238 and FLN1 function cooperatively in regulating PEP-dependent gene expression.

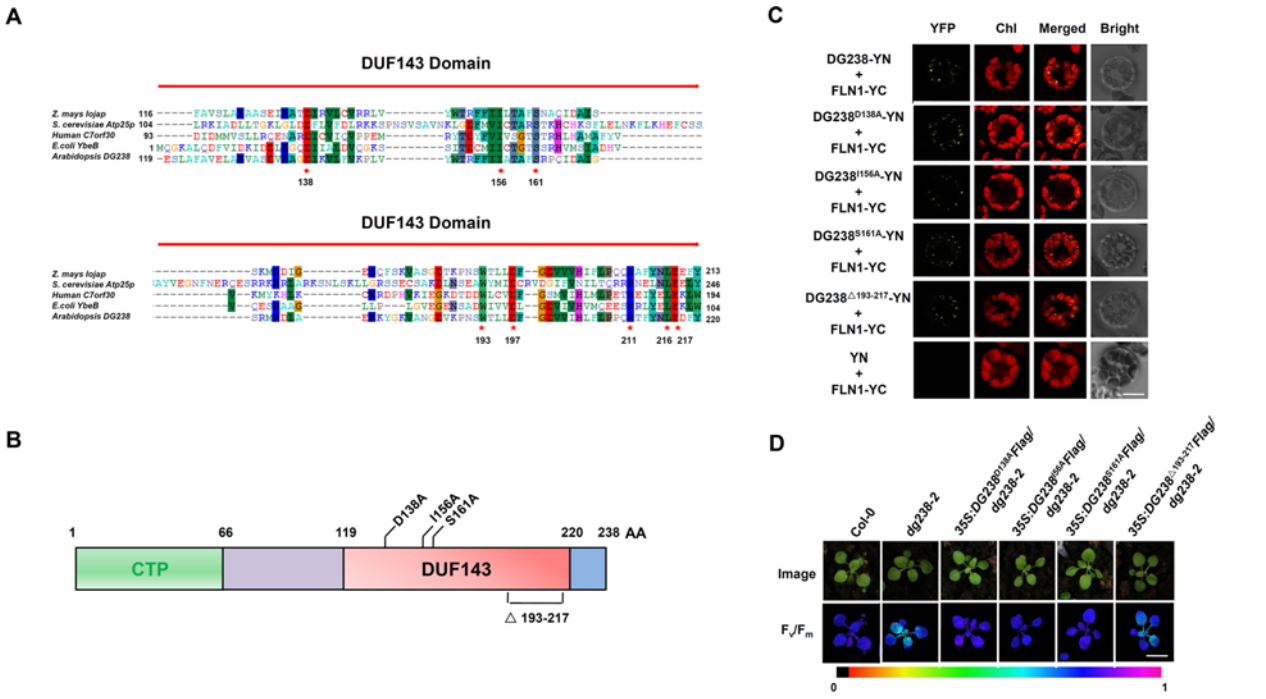

Fig. 3. Identification of the functions of conserved amino acids of the DUF143 domain. (A) The amino acid sequence of the DUF143 domain with homologous sequences from various species, including *Zea mays* (No. NP_001105495), human (NP_612455), *E. coli* (NP_752658), and *Arabidopsis thaliana* (NP_566439.1). Asterisks indicate conserved amino acids. The sequences were aligned with ClustalW. (B) Diagram of the DUF143 domain of DG238. D138A, I156A, and S161A represent the replacement of amino acids 138, 156, and 161 of DG238 with alanine, respectively; Δ193-217 represents DG238 without amino acids 193–217. D, Asparticacid; I, Isoleucine; S, Serine; H, Histidine; A, Alanine. (C) BiFC assay of Arabidopsis protoplasts transformed with different combinations of the constructs and visualized using confocal microscopy. Bars = 10 μm. (D) Photographs and chlorophyll fluorescence images show genetic material grown for 2 weeks on soil. Bars = 1 cm.

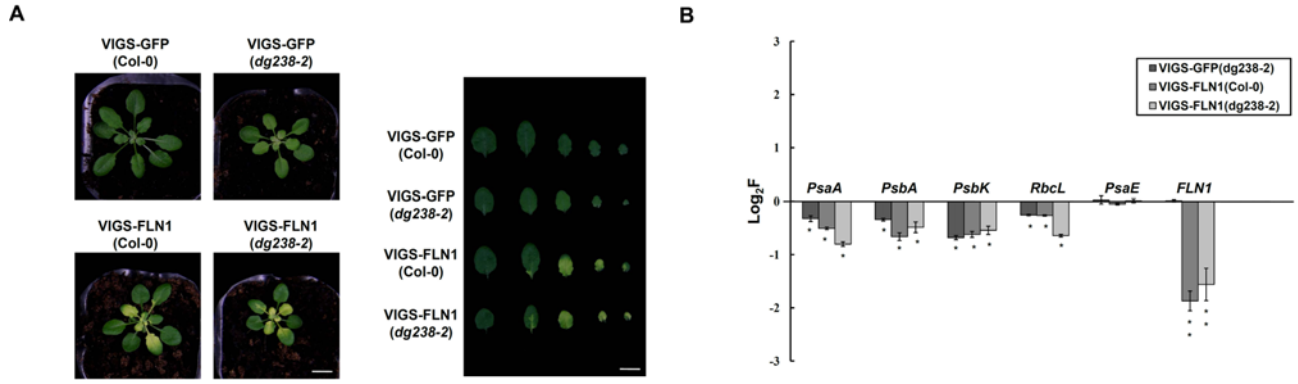

Fig. 4. The genetic interaction between DG238 and FLN1. (A) Phenotypes of VIGS-FLN1(*dg238-2*) seedlings. Photographs show VIGS-GFP(Col-0), VIGS-GFP(*dg238-2*), VIGS-FLN1(Col-0), and VIGS-FLN1(*dg238-2*) plants grown for 4 weeks on soil. Bars = 1 cm. (B) qRT-PCR analysis of *psaA*, *psbA*, *psbK*, and *rbcL* transcript levels in VIGS-GFP(Col-0), VIGS-GFP(*dg238-2*), VIGS-FLN1(Col-0), and VIGS-FLN1(*dg238-2*) plants. F values were calculated based on the ratio of signal intensities of VIGS-GFP(*dg238-2*), VIGS-FLN1(Col-0), and VIGS-FLN1(*dg238-2*) versus VIGS-GFP(Col-0) plants. The $\log_2$F value is given, where 3.32 corresponds to 10-fold upregulation and -3.32 corresponds to 10-fold downregulation, in VIGS-GFP(*dg238-2*), VIGS-FLN1(Col-0), and VIGS-FLN1(*dg238-2*) plants relative to VIGS-GFP(Col-0) plants. *UBQ4* was used as a reference. The data represent means ± SD of three biological replicates. Significant differences in VIGS-GFP(*dg238-2*), VIGS-FLN1(Col-0), and VIGS-FLN1(*dg238-2*) relative to VIGS-GFP(Col-0) plants were calculated using Student's t-test and are indicated by * $P \leq 0.05$ and ** $P \leq 0.01$.

Through bioinformatics analysis, we also found that not only is *DG238* co-expressed with *FLN1*, but it is also co-expressed with *MRL7-L* (Fig. S3), encoding the PEP-associated protein MRL7-L. In addition, MRL7 is a paralogous protein of MRL7-L in Arabidopsis, and MRL7 interacts with PEP subunit protein FSD2 (Qiao et al. 2011; Qiao et al. 2013). In Arabidopsis, FSD2 interacts with FSD3, which might function together to regulate PEP-dependent plastid gene expression

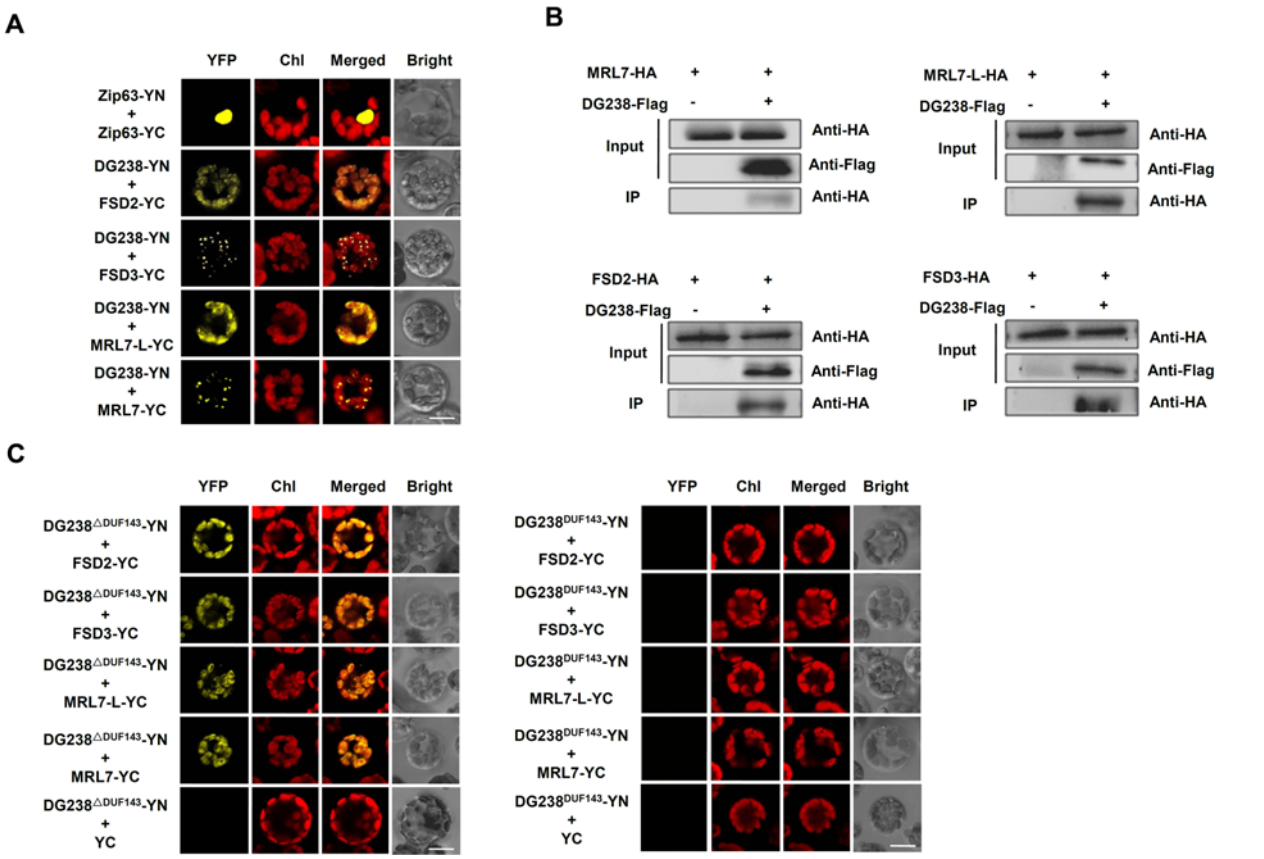

Fig. 5. The interactions between DG238 and other PEP-associated proteins. (A) BiFC assay of Arabidopsis protoplasts transformed with different combinations of the constructs and visualized using confocal microscopy. Zip63, a nuclear protein that interacts with itself, was used as a positive control. Bars = 10 μm. (B) Co-IP assay of DG238-Flag and FSD2-HA, FSD3-HA, MRL7-L-HA, and MRL7-HA co-expressed in Arabidopsis protoplasts. Total proteins were immunoprecipitated by anti-Flag affinity gel and detected using anti-HA antibody. (C) BiFC assay of Arabidopsis protoplasts transformed with different combinations of the constructs and visualized using confocal microscopy. Bars = 10 μm.

(Myouga et al. 2008). These findings imply that some interactions may occur among DG238, MRL7-L, MRL7, FSD2, and FSD3. Through BiFC assays, we found that DG238 can interact with MRL7, MRL7-L, FSD2, and FSD3, whereas we did not observe obvious fluorescent signals between DG238-YN and PTAC6-YC, PTAC12-YC, TRXz-YC, or FTRc-YC, which served as negative controls. Co-immunoprecipitation (Co-IP) analysis confirmed the interaction of DG238 with MRL7-L, MRL7, FSD2, and FSD3 (Fig. 5A and Fig. 5B). Notably, like the interaction between DG238 and FLN1, the interaction area of DG238 with MRL7, and FSD3 is localized solely in the chloroplast nucleoid. However, the interaction area of DG238 with MRL7-L and FSD2 was dispersed throughout the chloroplast (Fig. 5A). Likewise, DUF143 domain also did not interact with MRL7-L, MRL7, FSD2, and FSD3 (Fig. 5C). Interestingly, the interaction area of DG238$^{\Delta DUF143}$ with MRL7, and FSD3 was altered to be dispersed throughout the chloroplast, but the interaction area of DG238 with MRL7-L and FSD2 was not affected by the lack of DUF143 domain (Fig. 5C).

Discussion

Chloroplast development is an essential process for plant growth that is regulated by many proteins, especially nucleus-encoded chloroplast proteins (Pfalz and Pfannschmidt 2013). PEP is a large complex containing numerous proteins, many of which are encoded by the nucleus. This complex is essential for plastid gene expression and chloroplast development (Yu, Huang et al. 2014). We previously identified DG238 as a potential PEP-associated protein that functions together with FLN1 to regulate plastid gene expression and early chloroplast development (Wang et al. 2016). In the current study, we used VIGS to silence *FLN1* gene expression in the *dg238* mutant and obtained VIGS-FLN1 (*dg238-2*) plants, in which *DG238* and *FLN1* were not expressed. Our results show that simultaneous lack of expression of *FLN1* and *DG238* led to more severe phenotypes in young leaves (Fig. 4A) and lower transcripts levels of PEP-dependent genes *psaA* and *rbcL* compared with VIGS-FLN1 (Col-0) and VIGS-GFP (*dg238-2*) plants, in which one of these genes was silenced or mutated

(Fig. 4B). Together, these results point to the functional redundancy or synergy of DG238 and FLN1 in regulating PEP-dependent plastid gene expression and chloroplast development.

Bioinformatic analysis revealed that DG238 contains a DUF143 domain, the function of which is unknown (Wang et al. 2016). In *Saccharomyces cerevisiae*, ATP25, the homolog of DG238, is involved in the expression and assembly of the Atp9p. However, the N-terminus (containing the DUF143 domain) and C-terminus of ATP25 differ from those of DG238: the C-terminus of ATP25 is sufficient to maintain the mRNA stability of *ATP9* and to recover the biosynthesis of Atp9p. The N-terminus of ATP25 may function in the oligomerization of Atp9p, but its detailed function is still unclear (Zeng et al. 2008). C7orf30, the homologous protein of DG238 in human, functions in the biogenesis of the large subunit of the mitochondrial ribosome. Overexpression of $C7orf30^{H185A}$ (in which residue 185 [histidine] is mutated to alanine) in HEK239T cells affected the profile of the large subunit of the mitochondrial ribosome but had no effect on $C7orf30^{D178A}$ (with residue 178 [aspartic acid] mutated to alanine) cells: both amino acids are located in the DUF143 domain (Rorbach et al. 2012). The functions of the DUF143 domain in ATP25 and C7orf30 suggest that it plays important roles in various species.

In plants, It is found that DG238 from *Arabidopsis thaliana* participates in plastid gene expression and chloroplast development (Wang et al. 2016), and the homolog of DG238, Iojap from *Zea mays* is involved in chloroplast development (Han et al. 1992). However, the function of DUF143 in plants is still unknown. In this study, we successfully constructed $DG238^{\Delta DUF143}$ and $DG238^{DUF143}$ vectors and found that the loss of the DUF143 domain affected the nucleoid localization of DG238 in the chloroplast (Fig. 1B), whereas the localization of $DG238^{DUF143}$ matched that of wild-type protein DG238 (Fig. 1B). These results suggest that the DUF143 domain is essential for the proper localization of DG238 in the chloroplast of plants. Furthermore, the interaction area of DG238 with FLN1, MRL7 and FSD3 was also altered by the lack of DUF143 domain and it was dispersed throughout the chloroplast rather than being localized solely in the chloroplast nucleoid, further confirming that the DUF143 domain affects the localization of DG238 in the chloroplast (Fig. 1B) and ultimately affects the DG238 and partners interaction area (Fig. 2A). DG238, like FSD2, is a chloroplast stroma-localized protein (Myouga et al. 2008; Wang et al. 2016). DUF143 contains conserved amino acids found in various species, but our results show that the mutation of these amino acids did not affect the function of DG238, except when amino acids 193–217 were deleted (Fig. 3C and Fig. 3D); however, additional studies are needed to identify the key conserved amino acids in this region that affect the functioning of DG238.

In this study, we found that, in addition to being co-expressed with *FLN1*, *DG238* is also co-expressed with *MRL7-L* (Fig. S3), which encodes the PEP-associated protein MRL7-L. In addition, we also found that DG238 can interact with PEP-associated proteins MRL7, MRL7-L, FSD2, and FSD3 (Fig. 5A and Fig. 5B). These findings provide further evidence that DG238 is a PEP-associated protein that, together with other PEP-associated proteins, participates in PEP-dependent plastid gene expression (Fig. S5). However, the connections among these proteins require further study. Furthermore, the lack of the DUF143 domain in DG238 led to abnormal interactions among these proteins (Fig. 5C). Interestingly, the interaction of DG238 with FSD2 and MRL7-L was not specific to the chloroplast nucleoid (Fig. 5A), suggesting that DG238, FSD2, and MRL7-L may possess additional functions in chloroplast of plants. Indeed, LOW SULFUR UPREGULATED (LSU) network hubs integrate abiotic and biotic stress responses via interactions with the superoxide dismutase FSD2; this interaction area is not specific to the chloroplast nucleoid (Garcia-Molina et al. 2017). In maize, the loss of Iojap, the homolog of DG238, results in the production of defective chloroplast ribosomes (Shumway and Weier 1967). The diverse functions of FSD2 and Iojap suggest that DG238 might also play additional roles in Arabidopsis.

Materials and Methods

Plant Materials and Growth Conditions

The *Arabidopsis thaliana dg238-2* (SALK_121803) mutant lines in the Col-0 background were obtained from the Arabidopsis Biological Resource Center (ABRC). The ecotype Col-0 was used as the wild type in this study. For soil cultivation, the plants were grown in a growth chamber with 120 μmol $m^{-2}s^{-1}$ of light, a 12 h light/12 h dark cycle, and 60–70% humidity at 22°C. For plants grown on agar plates, the seeds were surface sterilized using 75% ethanol for 60 s, followed by 15% bleach for 20 min, and sown on 1/2MS medium (Phyto Technology Laboratories) supplemented with 1% (w/v) sucrose and 0.8% (w/v) agar. The seeds were then vernalized in darkness at 4°C for 3 d and transferred to a growth chamber.

GFP and BiFC

The Arabidopsis protoplast were prepared as previously described (Zhai et al. 2009), and subcellular localization of GFP fusion proteins and BiFC were performed as previously described (Zhang et al. 2011).

Plasmid Construction and Plant Transformation

To obtain the transgeneic plants, *DG238*, *DG238* with amino acid mutations, and *DG238* with various amino acid deletions were used to amplify the corresponding coding sequences fused with the 2×Flag sequence at the C-terminus. The open reading frame of *DG238* was cloned into pRi35S, excised with HindIII/SalI, and inserted into pCAMBIA1301 to generate the *35S:DG238-Flag*, *35S:DG238$^{\Delta DUF143}$-Flag*, *35S:DG238^{DUF143}-Flag*, *35S:DG238^{D138A}-Flag*, *35S:DG238^{I156A}-*

Flag, *35S:DG238^{S161A}-Flag*, and *35S:DG238$^{\Delta 193\text{-}217}$-Flag* constructs. The constructs were then transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation. The cells were transformed into Arabidopsis *dg238-2* mutant plants using the floral dip method (Clough and Bent 1998). Transgenic plants were screened on 1/2MS agar plates supplemented with hygromycin (50 μg/mL). Obviously resistant plants were transferred to soil and grown in a growth chamber to produce seeds. The successful production of complementation lines were confirmed by western blot analysis.

Protein Extraction and Western Blotting

Total proteins were extracted from plant leaves using extraction buffer (50 mM HEPES [pH 7.5], 1 mM EDTA, 1 mM dithiothreitol, 150 mM KCl, 1% Triton X-100, and protease inhibitor cocktail). The extracted proteins were separated using 15% SDS-PAGE, blotted onto PVDF (polyvinylidene difluoride) membranes (Millipore), and probed using antibodies specifically directed against individual enzymes. Signals were detected using the SuperSignal West Pico Chemiluminescent Substrate (Thermo).

Co-IP Assays

Arabidopsis protoplasts (4 mL) were cotransfected with 400 μg of plasmid DNA from two constructs or 400 μg of plasmid DNA from one construct. Total proteins were extracted in 500 μL of protein extraction buffer (50 mM HEPES [pH 7.5], 1 mM EDTA, 1 mM dithiothreitol, 150 mM KCl, 1% Triton X-100, and protease inhibitor cocktail). Before incubation, 20 μL of protein extract was used as an input. The total cell extracts were further incubated with 20 μL of anti-FLAG affinity gel (Sigma-Aldrich) for 5 h at 4°C with 60 rpm rotation. After incubation, the FLAG affinity gel was washed at least six times with ice-cold TBS buffer (20 mM Tris-HCl [pH 7.4] and 150 mM NaCl) to remove impurities. The specifically bound proteins were eluted by boiling the gel in 20 μL of SDS-PAGE sample buffer without *B*-mercaptoethanol and subjected to 10% SDS-PAGE, followed by immunoblotting.

Chlorophyll Fluorescence Parameters

Chlorophyll fluorescence parameters were measured using the MAXI version of the Imaging-PAM M-Series Chlorophyll fluorescence system. Before measurement, the plants were dark-adapted for 20 min. The F_v/F_m was determined as described (Jin et al. 2014).

VIGS Assay

The plasmids pTRV1 and pTRV2 are VIGS vectors based on the Tobacco rattle virus. *FLN1* was amplified using the primers described in Table S1 and inserted into the pTRV2 vector to prepare the *pTRV2-FLN1* vectors. The *pTRV2-GFP* vector was used as a negative control. The pTRV1and pTRV2 derivatives were introduced into Arabidopsis plants by *Agrobacterium tumefaciens* infiltration as described previously (Wang et al. 2013).

RNA Isolation and qRT-PCR

Total RNA was extracted from Arabidopsis leaves using a Plant RNA Kit (Magen). Total RNA (5 μg) was used for cDNA synthesis with a PrimeScript RT Reagent kit (Takara) according to the manufacturer's instructions. The qRT-PCR was performed using SYBR® Premix ExTaq™ (Takara) via real-time amplification in a LightCycler480 system (Roche). *Ubiquitin4* (*UBQ4*) was used as a reference gene. The primers used for qRT-PCR analyses are listed in Table S1.

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Authors' Contributions

H.L.J., and M.W. designed experiments and wrote the paper. M.W. and L.J. performed the experiments. H.B.W., J.W., Q.D., J.L. and D.F. revised the article. All authors read and approved the final manuscript.

Supporting Information

Fig. S1. Identification of transgenic lines harboring *35S:DG238$^{\Delta DUF143}$ Flag/dg238-2* and *35S:DG238^{DUF143}Flag/dg238-2*.
Fig. S2. Identification of the DG238 amino acid mutation and amino acid deletion transgenic lines.
Fig. S3. Co-expression analysis of *DG238*.
Fig. S4. Negative controls for the BiFC assay.
Fig. S5. The interactions between DG238 and PEP complex-associated proteins.
Table S1. Sequences of the oligonucleotide primers used in this study.

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