Title:

Conserved Role of Fructokinase-like Protein 1 in Chloroplast Development
Revealed by a Seedling-lethal Albino Mutant of Pepper

Running title:

FLN1 contributes to chloroplast development

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Word count for the text body: 1195

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Chloroplasts perform photosynthesis and thus, drive the growth, development and reproduction of plants. Mutations in genes that encode chloroplast proteins frequently lead to chlorophyll deficiencies. Studies on chlorophyll-deficient mutants provided major advances to our knowledge of chloroplast- and photosynthesis-associated genes in model plants. Similarly, studies on chlorophyll-deficient mutants will contribute to our understanding of chloroplast development and function in solanaceous crops, such as pepper, and thus, help us to rationally manipulate photosynthesis for the purpose of crop improvement. Pepper is a globally important vegetable that is used as a spice, food and medicine.

Chloroplast genes are transcribed by either the plastid-encoded or nuclear-encoded RNA polymerase (PEP or NEP). PEP is a multisubunit enzyme that consists of core subunits encoded by the plastid-localized genes rpoA, rpoB, rpoC1 and rpoC2. PEP-transcribed genes are essential for efficient photosynthesis and normal growth. Loss-of-function mutants deficient in PEP-associated proteins (PAPs) develop albino or pale-green leaves. Thioredoxin Z (TRXz) and a fructokinase-like protein (FLN) associate with the PEP complex and contribute to the redox regulation of the PEP. Deficiencies in either TRXz or FLN1 inhibit both PEP and photoautotrophic growth. In Arabidopsis, TRXz and FLN1 contribute to PEP-mediated transcription and the accumulation of chlorophyll. In rice, OsTRXz and two PAPs, WLP2/OsFLN1 and HSA1/OsFLN2, form a TRX-FLN complex that regulates PEP-mediated transcription and consequently affects chloroplast development. Although PAPs have been studied in Arabidopsis and rice, few studies on PAPs have been performed with solanaceous crops, such as pepper.

We report the positional cloning of a mutant allele responsible for an albino seedling-lethal phenotype in a miniature pepper cultivar, MiniPep (Capsicum annuum). This mutant was unable to produce true leaves and eventually died at the seedling stage (Fig. 1a). Chlorophyll a and b were barely detectable in the 14-d-old seedlings of the mutant (e1493) relative to wild type (Fig. 1b). Transmission electron microscopy demonstrated that the abnormal shape and ultrastructure of the plastids in e1493 relative to wild type. The plastid in e1493 is round or nearly round, and the thylakoid membranes were barely detectable (Fig. 1c). A genetic analysis indicated that a recessive nuclear gene was responsible for these phenotypes.
To clone the causal gene, we performed a bulked segregant RNA-Seq (BSR) analysis with an F2 population prepared by crossing a plant that was heterozygous for the mutant allele from e1493 with the PC69 cultivar of C. annuum. We extracted RNA from pools of albino and wild-type tissue and used equivalent amounts of tissue from 30 seedlings for each pool. The BSR analysis indicated that the causal gene was located between 6.1 M and 18.9 M on chromosome 12 in the CM334 genome (Fig. 1d, Fig. S1a). To identify the target gene, we re-sequenced the wild type (MiniPep) and e1493. We identified 49 candidate single-nucleotide polymorphisms (SNPs) in the 12.8-Mb interval (Table S1) that were homozygous for G/C to A/T substitutions. After annotation, only one SNP (chr12_12913738) with a ΔSNP index of -0.86 (Table S2) was found to be a missense mutation and was located in CA.PGAv.1.6.scaffold321.29, which we named CaFLN1. We independently demonstrated the presence of this missense mutation in CaFLN1 using Sanger sequencing (Fig. S1b). The combination of BSR, parental resequencing and the criteria for filtering SNPs allowed us to rapidly identify the gene responsible for the albino phenotype in e1394. We expect that this strategy will be generally useful for cloning EMS alleles in crops. A sequence analysis showed that CaFLN1 is homologous to AtFLN1 (AT3G54090) from Arabidopsis, which encodes a fructokinase-like protein. The mutant of AtFLN1 also shows an albino phenotype, and FLN1 interacts with the plastid-localized TRXz to promote chloroplast development. CaFLN1 also showed high similarity with WLP2 (Os01t0851000). wlp2 mutants are also albino. Moreover, WLP2 interacts with OsTRXz to form a TRX-FLN complex that promotes chloroplast development. Similar to the cafln1 mutant, the osfln1 (wlp2) mutant is also an albino and a seedling lethal mutant in rice. A bioinformatics analysis provides evidence that CaFLN1 is targeted to the chloroplast and that CaFLN1 is preferentially expressed in leaves (Fig. S1c-d).

CaFLN1 contains two exons and one intron with a 1392-bp open reading frame that encodes a protein containing 464 amino acid residues. The mutation changes a G to an A at position 481 relative to the first bp of the first exon, which changes a glycine (Gly) to an arginine (Arg) residue in the CaFLN1 protein (Fig. 1d). FLN1 contains the conserved fpkB domain (Fig. S2). Our phylogenetic analysis indicates that there are four fpkB subfamilies: fructokinase (RFKs), fructokinase-like protein (FLN), adenosine kinase (ADK) and other kinases. The FLNs and RFKs
are located on the same branch (Fig. S3). An amino acid sequence alignment of FLN1 orthologues from the Ensembl Plants database indicates that FLN1 orthologues are found in most plants. The Gly-to-Arg substitution in the e1493 mutant is in the highly conserved fpkB domain (Fig. S2). The FLN1 protein-protein interaction network is highly conserved in tomato, Arabidopsis and rice (Fig. S4).

We used the virus induced gene silencing (VIGS) technique to knock down the expression of CaFLN1 in pepper. We also silenced SlFILN1, the orthologue of CaFLN1 in tomato. Consistent with the albino phenotype of the CaFLN1 mutant, both CaFLN1-silenced pepper plants and SlFILN1-silenced tomato plants developed chlorotic leaves (Fig. 1e, 1f). Using qRT-PCR we demonstrated that the expression of CaFLN1 and SlFILN1 was significantly reduced in the VIGS plants (Fig. 1g). The chlorophyll content of the silenced plants was also significantly decreased, although the tomato VIGS plants accumulated normal levels of chlorophyll a (Fig. 1h). Our results demonstrated that CaFLN1 contributes to the accumulation of chlorophyll in pepper and that the biological function of FLN1 is conserved in pepper and tomato.

To gain mechanistic insight, we compared the transcriptomes in the cotyledon tissue of MiniPep and e1493. The correlation coefficient among the three biological replicates reached 0.99-1 (Fig. S5a). Meanwhile, PC1 could explain 95.8% of the total variance (Fig. S5b). A total of 5524 differentially expressed genes (DEGs) were identified, including 2648 up-regulated and 2876 down-regulated genes (Fig. S5c). A Gene Ontology (GO) term enrichment analysis indicated that the missense mutation in CaFLN1 led to changes in the expression of genes associated with many GO terms including membrane and chloroplast development (Fig. S6a; Table S3). A KEGG pathway analysis indicated that CaFLN1 is closely associated with photosynthesis (Fig. S6b; Table S4). An independent quantification of the relative expression of eight chloroplast-related DEGs using qRT-PCR validated the data from the RNA-Seq experiment (Fig. S7a-b). We conclude that the missense mutation in the CaFLN1 gene affects chloroplast development by disrupting PEP-mediated transcription.

In Arabidopsis and rice, FLN1 is an important subunit of the PEP complex and promotes chloroplast development6,8. Multiple lines of evidence, such as high levels of sequence similarity; a
highly conserved pfkB domain; phenotypic characterizations of mutants in rice, Arabidopsis, tomato and pepper; a predicted protein-protein interaction network that is conserved; and our VIGS experiments are all consistent with FLN1 performing a conserved biological function in plants that is essential for chloroplast development. We speculate that the PEP complex is impaired in our pepper mutant because of a missense mutation in CaFLN1 that consequently affects chloroplast biogenesis. Nevertheless, the exact mechanism remains to be elucidated.
Fig. 1 Conserved role of fructokinase-like protein 1 in chloroplast development revealed by the seedling-lethal albino mutant of pepper, *e1493*. (a) Phenotypes of 14-d-old wild-type (MiniPep) and mutant (*e1493*) seedlings. Scale bar, 1 cm. (b) Chlorophyll content of the cotyledons from Miniprep and *e1493*. Error bars indicate standard deviation. *** P < 0.000005, Student’s t-test. (c) Ultrastructure of chloroplasts from the cotyledons of Miniprep and *e1493* observed using transmission electron microscopy. SG, starch grain; TGS, thylakoid grana stack. (d) Mapping of a missense allele in *CaFLN1*. The mutant allele was mapped to chromosome 12 (circos plot) and to an interval between 6.1 M and 18.9 M using bulked segregant RNA-Seq (BSR). The causative SNP (chr12_12913738) is presented inside the circle. The horizontal thick line represents an intron. The light and navy blue boxes represent untranslated regions (UTRs) and exons, respectively. The translational start codon (ATG), translational stop codon (TGA), and the G-to-A missense mutation are indicated with vertical lines. The missense mutation is located at 481 bp relative to the first bp of the translational start codon and changes a glycine (Gly) to Arginine (Arg) residue in the *CaFLN1* protein. (e) VIGS of *CaFLN1* in pepper. (f) VIGS of *SiFLN1* in tomato. Plants inoculated with an *Agrobacterium* strain containing the pTRV2 empty vector (pTRV2 (EV)) were used as a negative control. For a positive control, plants were inoculated with an *Agrobacterium* strain containing the pTRV2-PDS vector, which contains a fragment of the phytoene desaturase (*PDS*) gene from either pepper (e) or tomato (f). Scale bar, 2 cm. (g) Relative expression of *FLN1* in pepper and tomato plants subjected to VIGS. Thirty-d-old plants were analyzed. Gene expression was normalized to the expression of *UBI-3* in pepper and *SlFRG37* in tomato. (h) Chlorophyll content in the chlorotic leaves from pepper and tomato plants subjected to VIGS. Error bars indicate standard deviation (n = 3). *P < 0.05, **P < 0.01, Student’s t-test.

Acknowledgments

We thank Prof. Feng Liu from Hunan Agricultural University for kindly providing the miniature pepper accession MiniPep, Dr. Rugang Chen from Northwest A&F University for providing the VIGS vectors, and our dedicated colleague, Prof. Robert Larkin, for editing our manuscript. This research was funded by the National Key Research and Development Program (2018YFD1000800, 2016YFD0101704), and the National Natural Science Foundation of China (31972416, U1906205).

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B.O. and C.S. designed the research and wrote the manuscript; C.S. and X.S performed most of the experiments, Z.Z., Y.Z. and C.S. constructed the pepper EMS mutant collection C.S. analyzed data, R.C., J.L. and Y.T. contributed to the VIGS experiment. F.L and Y.L provided valuable suggestions and modified the manuscript.

Data availability

The RNA-seq and Resequencing data of MiniPep and e1493 are available at the NCBI repository (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA770097)

Conflict of interest

The authors declare no competing interests.

Supplementary information

Supplementary Document S1: Supplementary Materials and Methods; References for Supplementary Materials and Methods; Supplementary Figures and Legends

Supplementary Document S2: Supplemental Tables S1-S7.

References

1. Börner, T., Aleynikova, A.Y., Zubo, Y.O. & Kusnetsov, V.V. Chloroplast RNA polymerases: role in chloroplast biogenesis. Biochim. Biophys. Acta 1847, 761-769 (2015).

2. Demarsy, E., Courtois, F., Azevedo, J., Buhot, L. & Lerbs-Mache, S. Building up of the plastid transcriptional machinery during germination and early plant development. Plant Physiol. 142, 993-1003 (2006).

3. Yu, Q.B. et al. TAC7, an essential component of the plastid transcriptionally active chromosome complex, interacts with FLN1, TAC10, TAC12 and TAC14 to regulate chloroplast gene expression in Arabidopsis thaliana. Physiol. Plant. 148, 408-421 (2013).

4. Chang, S.H. et al. pTAC10, a key subunit of plastid-encoded RNA polymerase, promotes chloroplast development. Plant Physiol. 174, 435-449 (2017).

5. Riggs, J.W. & Callis, J. Arabidopsis fructokinase-like protein associations are regulated by ATP. Biochem J. 474, 1789-1801 (2017).

6. Wimmelbacher, M. & Börnke, F. Redox activity of thioredoxin z and fructokinase-like protein
1 is dispensable for autotrophic growth of *Arabidopsis thaliana*. *J. Exp. Bot.* **65**, 2405–2413 (2014).

7. Arsova, B. et al. Plastidial thioredoxin z interacts with two fructokinase-like proteins in a thiol-dependent manner: evidence for an essential role in chloroplast development in *Arabidopsis* and *Nicotiana benthamiana*. *Plant Cell* **22**, 1498-1515 (2010).

8. Lv, Y. et al. White leaf and panicle 2, encoding a PEP-associated protein, is required for chloroplast biogenesis under heat stress in rice. *J. Exp. Bot.* **68**, 5147-5160 (2017).

9. He, L. et al. Fructokinase-like protein 1 interacts with TRXz to regulate chloroplast development in rice. *J. Integr. Plant Biol.* **60**, 94–111 (2018).

10. Shirasawa, K., Hirakawa, H., Nunome, T., Tabata, S. & Isobe, S. Genome-wide survey of artificial mutations induced by ethyl methanesulfonate and gamma rays in tomato. *Plant Biotechnol. J.* **14**, 51-60 (2016).