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An *ACCUMULATION AND REPLICATION OF CHLOROPLASTS 5* gene mutation confers light green peel in cucumber

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Research Article

Abstract The peel color of fruit is an important commercial trait in cucumber, but the underlying molecular basis is largely unknown. A mutant showing light green exocarp was discovered from ethyl methane sulfonate (EMS) mutagenized cucumber line 406 with dark green exocarp. Genetic analysis showed the mutant phenotype is conferred by a single recessive gene, here designated as *lgp* (light green peel). By re-sequencing of bulked segregants, we identified the candidate gene *Csa7G051430* encoding ACCUMULATION AND REPLICATION OF CHLOROPLASTS 5 (ARC5) that plays a vital role in chloroplast division in *Arabidopsis*. A single nucleotide polymorphism (SNP) causing amino acid alteration in the conserved GTPase domain of Csa7G051430 showed co-segregation with the altered phenotype. Furthermore, the transient RNA interference of this gene resulted in reduced number and enlarged size of chloroplasts, which were also observed in the *lgp* mutant. This evidence supports that the non-synonymous SNP in *Csa7G051430* is the causative mutation for the light green peel. This study provides a new allele for cucumber breeding for light green fruits and additional resource for the study of chloroplast development.

Keywords: *ARC5*; *Cucumis sativus*; ethyl methane sulfonate mutant; fruit peel color; whole genome re-sequencing
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INTRODUCTION

Cucumber (*Cucumis sativus* L.) is an economically important vegetable crop worldwide (Qi et al. 2013). Peel color of immature fruits is an important external quality trait in cucumber. It exhibits a wide spectrum of colors from light to dark green in various cucumber accessions. In the green peel of cucumber, the major pigment is chlorophyll, followed by the tenfold decrease of carotenoids, and even less anthocyanins (Lancaster et al. 1997; Sun et al. 2003).

Both chloroplast development and chlorophyll biosynthesis are correlated with organ coloration. A rice chlorophyll synthase gene mutant exhibits a delayed chloroplast development, and thus results in yellow green leaf (Wu et al. 2007). In tomato, the mutants with less and abnormally enlarged chloroplasts show pale green fruit phenotype at the mature green stage and accumulates less chlorophyll than the dark green wild-type (Forth and Pyke 2006). The *Arabidopsis* ACCUMULATION AND REPLICATION OF CHLOROPLASTS 5 (ARC5) is a member of the family of dynamin-like proteins (DLPs) and has a role in chloroplast division (Gao et al. 2003). The *Arabidopsis arc5* mutant shows a reduction in chloroplast number and an increase in chloroplast size in mesophyll cells; however, it appears normal in morphological coloration and growth vigor (Pyke and Leech 1994). In cucumber, no gene responsible for organ coloration has been reported to our knowledge.

In this study, we discovered a cucumber mutant that exhibits predominantly light green color on fruit peel by screening an ethyl methane sulfonate (EMS) mutagenized library. Using whole genome re-sequencing and genetic mapping, we demonstrated a non-synonymous mutation in the cucumber *ARC5* homolog (*CsaARC5*) underlying the mutant phenotype. In agreement with the role of *ARC5* in chloroplast division, we observed abnormal chloroplast development in fruit exocarp protoplasts in the mutant line.

RESULTS

Inheritance of the *lgp* mutant

The *lgp* mutant showing light green peel was identified from an EMS mutant library of the cucumber inbred line 406 that has dark green peel color (Figure 1). All the F_1 plants derived from the cross of *lgp* mutant and wild type 406 had normal dark green peel. Among the 94 F_2 individuals, 20 displayed light green and 74 normal dark green, which fitted the 1:3 segregation ratio ($\chi^2 = 0.51$, $P = 0.47$). These results suggested a single recessive gene underlying the mutated light green phenotype.

Figure 1. Immature fruits of wild type 406, *lgp* mutant and F_1 plant

Identification of the candidate gene for *lgp*

The pooled DNA from 20 F_2 plants showing light green peel (*lgp* pool) and DNA from wild type 406 was re-sequenced respectively with a depth of approximately 11× using Illumina HiSeq 2000 sequencer. The consensus sequences of *lgp* pool and 406 were compared, yielding 1,131 SNPs. Trait-unrelated SNPs are shared among mutant lines of the same origin (Zuryn et al. 2010; Abe et al. 2012). Through looking into the sequencing data of other 12 mutants generated in parallel with the *lgp* mutant, such common SNPs were eliminated and 690 SNPs remained. It was expected that the SNP responsible for the recessive phenotype was homozygous in the *lgp* pool. This reduced the number of candidate SNPs to 23 SNPs (Table 1). All 23 SNPs displayed G/C to A/T transitions, which was consistent with canonical alkylation-induced mutation (Drake and Baltz 1976).

Among the 23 SNPs, 14 were located on chromosome 7, implying that chromosome 7 harbors the causative mutation (Tables 1, 2; Figure 2A). One SNP, SNP7G3286953 displaying C to T transition between the wild type and *lgp* pool, caused a non-synonymous change within the gene *Csa7G051430* (R213K) encoding a dynamin-like protein (DLP). To infer the function of Csa7G051430, we performed a phylogenetic analysis of the putative DLPs in cucumber and the reported DLPs in *Arabidopsis*, human, yeast etc. Csa7G051430 was clustered into a unique branch including the DLPs involved in chloroplast division in plants and algae, suggesting that Csa7G051430 could play a role in cucumber chloroplast division (Figure S1). In addition, Csa7G051430 was distinct from other putative cucumber DLPs that gathered with microtubule-associated proteins that participated in the cell plate formation during cytokinesis, mitochondrial and peroxisomal division or vesicle trafficking. The phylogenetic results suggested that Csa7G051430 should be the only DLP involved in cucumber chloroplast division. Furthermore, the R213K mutation within Csa7G051430 was located in the conserved GTPase domain of DLP (Praefcke and McMahon 2004) (Figure 2C) and the wild-type residue was highly conserved across plants, fungi, animals and human (Figures 2D, S2), implying that the amino acid alteration at this residue may influence the protein function and thus result in abnormal chloroplast phenotype. Therefore, SNP7G3286953 was considered the candidate causative mutation of *lgp* mutant.

To determine the correlation between SNP7G3286953 and the mutant phenotype, we performed genetic linkage analyses for the mutant phenotype in the F_2 population using this SNP and other two flanking SNPs (SNP7G2145087 and SNP7G5654155), which were suitable for developing derived cleaved amplified polymorphic sequence (dCAPS) markers (Figure 2B; Table 3). As expected, SNP7G3286953 co-segregated with the *lgp* locus. There were five recombinants between SNP7G2145087 and the *lgp* locus and four between SNP7G5654155 and the *lgp* locus, which delimited *lgp* into a 3.5-Mb interval. Besides SNP7G3286953, only one SNP (SNP7G3309643) was identified within the interval that is a silent intronic mutation. These results indicate that SNP7G3286953 is the causative mutation of the light green peel phenotype.

Table 1. Genomic distribution of single nucleotide polymorphisms (SNPs) between 406 and *lgp* pool

Chr	No. total SNP[a]	No. homozygous SNP[b]	Intergenic[b]	Intron[b]	Exon[b]	
					Synonymous	Non-synonymous
1	113	2	2	0	0	0
2	97	3	2	1	0	0
3	127	3	2	1	0	0
4	75	1	1	0	0	0
5	108	0	0	0	0	0
6	102	0	0	0	0	0
7	68	14	6	4	0	4
Total	690	23	13	6	0	4

[a]All SNPs between 406 and *lgp* pool. [b]SNPs that are homozygous in *lgp* pool.

Table 2. Genomic homozygous single nucleotide polymorphisms (SNPs)

Chr	Position	*lgp* pool /406	Type	Gene	Annotation
1	2,899,407	A/G	Intergenic		
1	20,631,096	G/T	Intergenic		
2	2,659,777	T/C	Intronic		
2	11,199,219	T/C	Intergenic		
2	18,300,741	A/G	Intergenic		
3	13,106,992	A/G	Intergenic		
3	34,411,487	A/G	Intergenic		
3	39,470,073	T/C	Intronic		
4	13,585,908	T/C	Intergenic		
7	579,241	T/C	Non-synonymous	*Csa7G009170*	calcium-binding protein
7	1,307,108	T/C	Intergenic		
7	1,569,525	A/G	Intergenic		
7	2,145,087	T/C	Intergenic		
7	3,286,953	T/C	Non-synonymous	*Csa7G051430*	Dynamin-like protein
7	3,309,643	T/C	Intronic		
7	5,654,155	T/C	Intergenic		
7	7,344,033	T/C	Intergenic		
7	8,088,977	T/C	Intergenic		
7	8,819,080	T/C	Non-synonymous	*Csa7G239600*	Cyclin-like protein
7	9,614,939	T/C	Intronic		
7	11,784,891	T/C	Intronic		
7	14,889,405	A/G	Non-synonymous	*Csa7G392400*	RNA polymerase II
7	15,060,726	A/G	Intronic		

Abnormal chloroplast morphology in *lgp* mutant and *CsaARC5* transient RNAi plant

Phylogenetic analysis indicated that *Csa7G051430*, designated as *CsaARC5* in this study, was the ortholog of *Arabidopsis ARC5* in cucumber (Figure S1), and their protein sequences showed 75% identity. ARC5 is an essential component of chloroplast division apparatus (Gao et al. 2003) and the mutant *arc5* plant contains fewer and larger chloroplasts than the wild-type (Pyke and Leech 1994; Robertson et al. 1996). We compared the phenotype of chloroplasts in protoplasts isolated from immature fruit peel of wild-type 406 and the *lgp* mutant. Each protoplast isolated from the 406 line contained more than 20 chloroplasts, whereas in *lgp* mutant it was less than 7 (Figure 2E, F). In addition, chloroplasts had enlarged size and some showed irregular shape in *lgp* mutant (Figure 2E, F). Such chloroplast morphology was similar to that observed in *Arabidopsis arc5*, suggesting that *lgp* mutant could be an *arc5* mutant in cucumber.

To further confirm *CsaARC5* function, transient RNA interference (RNAi) was performed on cotyledons of the wild type cucumber line 406. The expression of *CsaARC5* from RNAi sample was reduced about 30% compared to that in the control sample (Figure 3A). Accordingly, the proportion of protoplasts with reduced chloroplast number from RNAi sample was 2.3 times more than that from the control sample (Figure 3B). Compared to in control sample, protoplasts in cotyledons with transient RNAi of *CsaARC5* had fewer chloroplasts (Figure 3C, D), which was similar to the morphology of chloroplasts in immature fruit peel of *lgp* mutant. The results indicated that the reduced expression of *CsaARC5* might block chloroplast division in cucumber to some extent, and supported that *CsaARC5* as the gene underlying the *lgp* mutant.

DISCUSSION

In this study, by whole genome re-sequencing of the wild-type parental line and pooled F_2 progenies with mutated phenotype, we delimited the causative mutation to an interval with enrichment of homozygous mutated SNPs. This was accomplished with the aid of sequencing of other 12 EMS mutants in parallel, which remarkably improve the power of filtering irrelevant SNPs (Zuryn et al. 2010; Abe et al. 2012).

By whole genome re-sequencing and genetic analysis, a mutation in *CsaARC5*, the *Arabidopsis ARC5* ortholog in cucumber, was revealed to underlie the *lgp* mutant. ARC5 has been demonstrated to be responsible for abstricting chloroplasts in chloroplast division, as a result, the *Arabidopsis arc5* mutant contains larger and fewer chloroplasts (Gao et al. 2003). We also observed larger and fewer chloroplasts in peel protoplasts of *lgp* mutant. Furthermore, the chloroplast phenotype in *lgp* mutant was very similar to that in *CsaARC5* transient RNAi plants, supporting that the mutation in *CsaARC5* underlies the chloroplast defects. This evidence suggests that the mutations in *Arabidopsis arc5* and cucumber *lgp* might both affect the chloroplast development but may act in different modes. GTPase domain is essential for dynamin function of DLPs (Roux et al. 2006; Chappie et al. 2010). In *Arabidopsis arc5*, the mutation results in a premature stop codon behind the GTPase domain (Gao et al. 2003). In *lgp* mutant, the mutation led to an amino acid substitution (R213K) at a highly conserved residue of the GTPase domain (Figures 2D, S2). Presumably the mutations occurring at different sites affect the function of ARC5 in different modes.

The fruit peel color of cucumber is an important economic trait for consumption, and the fruits with light green peel

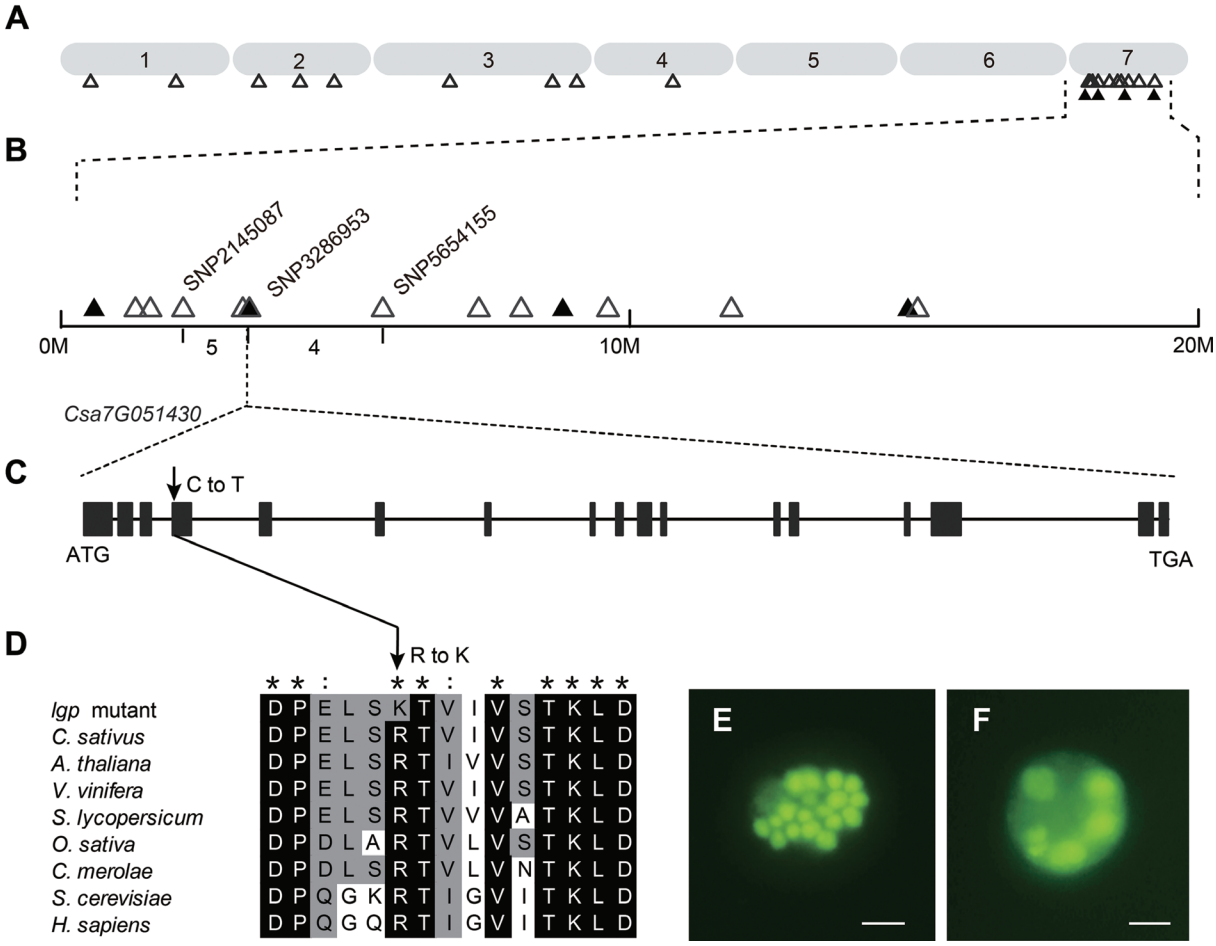

Figure 2. Identification of causative mutation and analysis of chloroplast morphology in wild type 406 and *lgp* mutant
(A) Genomic distribution of homozygous single nucleotide polymorphisms (SNPs). Seven cucumber chromosomes are designated by numbers one through seven. Hollow triangles indicate synonymous SNPs; solid triangles indicate non-synonymous SNPs. **(B)** Homozygous SNPs on chromosome 7. Three SNPs from which derived cleaved amplified polymorphic markers (dCAPS) markers were derived are shown above the triangles, and number of recombinants between SNP markers and the mutated phenotype is indicated below the line. **(C)** Gene structure of predicted *Csa7G051430*. Rectangles represent exons; solid lines represent introns; arrow indicates the position of SNP3286953. **(D)** Alignment of a segment of the GTPase domain of Csa7G051430 and its orthologous sequences from different species. The full view of proteins alignment is shown in Figure S2 and accession numbers for the sequences are listed in Table S1. **(E, F)** Chloroplast morphology of the protoplast isolated from 406 (E) and *lgp* mutant (F) fruit peels. Scale bar = 10 μm.

Table 3. Primers and digestion information for derived cleaved amplified polymorphic sequence (dCAPS) markers

SNP	Primer[a]	Recognition site	Product size (bp)
SNP7G2145087	F: AAGATGTATGGACAAAATGGGC R: GAAATTTGGAGAATGAGATCAGTTC	GGCC	156
SNP7G3286953	F: AGTTTGGTTGATACAATCACAGGC R: TGACTTTGTCATCAGTTCTTTATTT	AGGCCT	158
SNP7G5654155	F: AACCCTTCGATGAAGTCTATCGTCGAGT R: CGTATGAATATAGAGATACATTGGAT	GANTC	169

[a]F, forward primer; R, reverse primer.

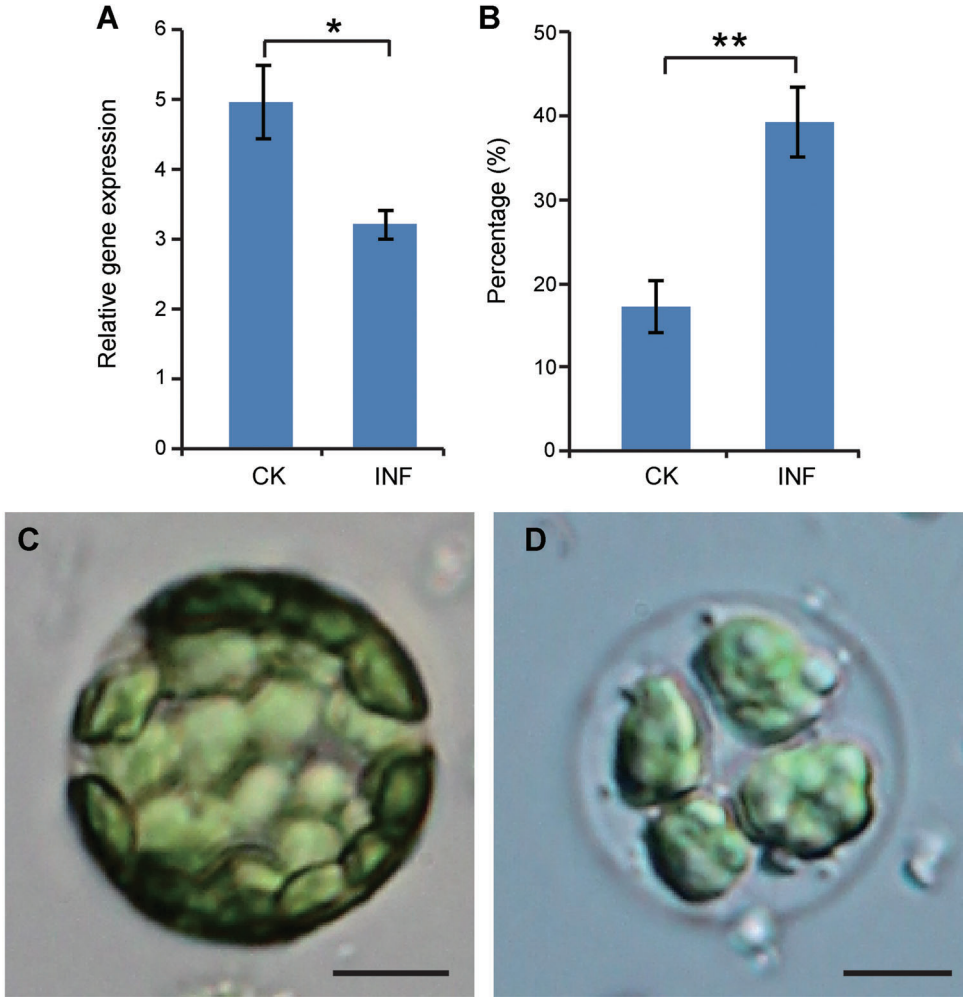

Figure 3. Transient silencing of *CsaARC5* in cucumber cotyledons
(A) Transient gene expression of *CsaARC5* in cotyledons infiltrated with agrobacterium. CK, sample infiltrated with empty vector PBI121; INF, sample infiltrated with PBI121-*CsaARC5*. **(B)** Quantitative proportion of protoplasts with abnormal chloroplast number in cotyledons infiltrated with agrobacterium. For each biological replicate 50 protoplasts were counted and an abnormal protoplast was defined if it harbored less than 10 chloroplasts. **(C, D)** Chloroplast morphology of the representative protoplasts isolated from CK (**C**) and INF (**D**). Scale bar = 10 μm. Error bar indicates standard error. * stands for significant difference ($P < 0.05$), ** stands for significant difference ($P < 0.01$).

appear much more tender and fresher compared to normal green fruits. There is no obvious difference between the *lgp* mutant and the wild type 406 in fruit production and flavor. The *lgp* mutant would be a novel material for fruit quality improvement in cucumber breeding, and the identified dCAPS markers could facilitate this process.

MATERIALS AND METHODS

Plant materials

A north China type cucumber (*Cucumis sativus* L.) inbred line 406 was used in mutageneis experiments. Seeds of 406 were treated with 1% EMS (Sigma M0880) (Tadmor et al. 2007). M1 plants were self-pollinated and the *lgp* mutant was identified in the M2 population. An F_2 segregating population was generated by crossing *lgp* mutant with wild type 406. All plants were grown in the greenhouse in Hunan Vegetable Research Institute, Changsha, China.

Whole genome re-sequencing

Genomic DNA was extracted from fresh leaves using standardized CTAB protocol (Dellaporta et al. 1983). DNA of 20 plants showing mutant phenotype in F2 population was mixed equally to construct the *lgp* pool. For Illumina paired-end sequencing, the sequencing library with the insert size of 500 bp was constructed according to the standard protocol. Then the library was sequenced with the read length of 100 bp using Illumina Hi-Seq 2000 sequencer. The sequencing generated 41 million paired-end reads for 406 and 43 million

for *lgp* pool. In both cases, more than 92% of bases possessed an Illumina phred quality score higher than 30.

SNP calling and filtering

The short reads of 406 and *lgp* pool were aligned against the reference genome of cucumber inbred line 9930 (Huang et al. 2009; Li et al. 2011) using software SOAP (Li et al. 2009b). The output of paired-end alignment files was processed with SOAPsnp (Li et al. 2009a) software with default parameters to generate consensus genome sequences. SNP calling was carried out by directly comparing the consensus sequences of 406 and *lgp* pool base-for-base using a custom python script.

The output of variant sites was introduced into a filter pipeline to minimize false positives caused by sequencing or alignment errors. The pipeline included several criteria: (i) A reliable SNP should be bi-allelic between 406 and *lgp* pool. SNPs showing allele frequency of wild-type genotype lower than 0.9 in 406 or higher than 0.7 in *lgp* pool were discarded as possible false positives; (ii) Quality scores of both sequencing and read mapping should be higher than 30. The number of uniquely mapped reads should be more than 3 and less than 45 at any SNP site; for a SNP heterozygous in *lgp* pool, each allele should be covered by at least two reads. Besides *lgp*, we also sequenced another 406 EMS mutant lines to remove the phenotype-irrelevant SNPs.

Sequence alignments and phylogenetic analysis

The genome sequences and annotation of cucumber inbred line 9930 is accessible in the GenBank under the accession ACHR00000000 and the version described in this article is ACHR02000000. The sequence alignment was performed with the ClustalW and the phylogenetic tree was constructed with the neighbor-joining method using the MEGA5.05 program. The accession numbers of listed proteins were provided in Table S1.

Development of dCAPS markers

Polymerase chain reaction (PCR) primers for dCAPS markers were designed using the web-based software dCAPS FINDER 2.0 (Neff et al. 1998). PCR was carried out in a total volume of 20 μL containing 10 ng template DNA, 2 μL 10× PCR buffer, 0.15 mM of each dNTP, 1U *Taq* DNA polymerase, and 0.25 μM of each forward and reverse primers using the following protocol: an initial DNA denaturation for 4 min at 94 °C was followed by 35 cycles of 20 s DNA denaturation at 94 °C, 20 s annealing at 59 °C, and 40 s elongation at 72 °C, then a final 5 min elongation at 72 °C. The PCR products were digested with appropriate restriction enzymes and subsequently subjected to electrophoresis in 8% polyacrylamide gels. The primers, restriction sites and product sizes are summarized in Table 3.

Chloroplast phenotype analysis

Protoplast isolation was performed according to the reported protocol (Yoo et al. 2007) with minor modifications. Cucumber peels were removed from flesh and cut into fine strips (~0.5 mm) with a blade. The strips were immediately transferred to enzymolysis solution (1% cellulose (Yakult, Tokyo, Japan), 2% pectinase (Yakult), 0.4 M mannitol, 10 mM $CaCl_2$, 20 mM KCl, and 20 mM MES), then gently shaken in weak light for 4–6 h until the protoplasts were released into the solution. After digestion, the enzymolysate was filtered through nylon membrane (100 mesh), centrifuged at 100 *g* for 15 min, and then resuspended in collecting solution (154 mM NaCl, 125 mM $CaCl_2$). The protoplasts of cucumber peel were observed with epi-fluorescence microscope condenser III RS, Zeiss 400 color film, PI filter.

Transient gene silence in cucumber cotyledons

Transient RNAi system was performed on cotyledons of cucumber according to the protocol by (Shang et al. 2014). A pair of reverse complementary specific fragments (258 bp) of *CsaARC5* was amplified from cDNA of wild type line 406 using the primers listed in Table S2. Then the pair of fragments was separately constructed into BamH I site and Sac I site of the binary vector pBI121 by In-Fusion (Clontech, Otsu, Japan). The control sample was empty vector pBI121. Constructs were separately transformed into *A. tumefaciens* strain EHA105. Then the agrobacterium suspension was infiltrated into cotyledons of wild type line 406 seedlings. Five days after injection, the cotyledons were cut off for chloroplast observation and quantitative real-time PCR (qPCR) analysis (Table S3). Three biological replicates were conducted in this experiment.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Phylogenetic analysis of Csa7G051430

Phylogenetic tree of reported dynamin-like proteins (DLPs) in Arabidopsis, human, yeast, alga and putative DLPs in cucumber. Nodes are labeled with the percentage of bootstrap iteration.

Figure S2. Alignment of Csa7G051430 with homologs from different species

Arrow indicates the position of SNP3286953 in cucumber *lgp* mutant. The dark line below the alignment indicates the GTPase domain.

Table S1. Gene accessions for alignment and phylogenetic analysis

Table S2. Primers used for vector construct in RNAi system

Table S3. Primers used for qPCR analysis of *CsaARC5*