Giant Chloroplast Development in *ethylene response1-1* Is Caused by a Second Mutation in *ACCUMULATION AND REPLICATION OF CHLOROPLAST3* in *Arabidopsis*

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The higher plants of today array a large number of small chloroplasts in their photosynthetic cells. This array of small chloroplasts results from organelle division *via* prokaryotic binary fission in a eukaryotic plant cell environment. Functional abnormalities of the tightly coordinated biochemical event of chloroplast division lead to abnormal chloroplast development in plants. Here, we described an abnormal chloroplast phenotype in an ethylene insensitive *ethylene response1-1* (*etr1-1*) of *Arabidopsis thaliana*. Extensive transgenic and genetic analyses revealed that this organelle abnormality was not linked to *etr1-1* or ethylene signaling, but linked to a second mutation in *ACCUMULATION AND REPLICATION3* (*ARC3*), which was further verified by genetic complementation analysis. Despite the normal expression of other plastid division-related genes, the loss of *ARC3* caused the enlargement of chloroplasts as well as the diminution of a photosynthetic protein Rubisco in *etr1-1*. Our study has suggested that the increased size of the abnormal chloroplasts may not be able to fully compensate for the loss of a greater array of small chloroplasts in higher plants.

INTRODUCTION

Chloroplasts in the higher plants of today provide strong evidence for endosymbiosis between prokaryotic and eukaryotic cells (Kutschera and Niklas, 2005; Margulis, 1970; McFadden, 2001; Sagan, 1967). Whole-genome sequencing of *Arabidopsis* and other plant species has further revealed that a large portion of the organelle genome was transferred to the nuclear genome or lost after the first symbiosis event (The *Arabidopsis* Genome Initiative, 2000).

Genetic factors that originated in prokaryotic genomes have many roles in eukaryotic cellular activities. For example, a bacterial two-component system (Hoch, 2000; Stock et al., 1989) contributes to intracellular signaling pathways for plant hormones such as cytokinin (Ferreira and Kieber, 2005; Hwang et

al., 2002) and ethylene (Mason and Schaller, 2005; Mount and Chang, 2002). Many of these genes also encode machinery proteins for organelle division and coordinate the biological activities of the two merged organisms (Leon et al., 1998). In mesophyll cells of mature leaves of higher plants, chloroplasts are propagated from 10-15 plastid progenitors into 50-120 organelles through prokaryotic binary fission (Sakamoto et al., 2008). Any functional alteration of the proteins involved in plastid division leads to the generation of extremely large chloroplasts that often occupy a considerable portion of the cytoplasm. This abnormal plastid phenotype has been termed *Accumulation and Replication of Chloroplast* (*ARC*) and is used to screen for regulatory molecules involved in organelle division (Marrison et al., 1999; Pyke and Leech, 1994).

ARC proteins are involved in positioning of plastid division sites and pressing against chloroplast membranes to execute division process (Supplementary Fig. S1; Aldridge et al., 2005; Maple and Møller, 2010; Osteryoung and McAndrew, 2001). A Ca^{2+}-dependent ATPase, ARC11 is the *Arabidopsis* equivalent of bacterial MinD (Fujiwara et al., 2004). ARC3 and ARC5, respectively, locate at the stroma side and the cytosolic side of chloroplasts (Gao et al., 2003; Maple et al., 2007; Shimada et al., 2004). Both ARC6 and PARALOG of ARC6 (PARC6) are found at the inner membrane of plastids (Glynn et al., 2008; 2009). PLASTID DIVISION (PDV) 1 and PDV2 are constituents of plastid division–associated protein complexes at the outer membrane of chloroplasts (Miyagishima et al., 2006; Okazaki et al., 2009).

In spite of abnormal chloroplast shapes and arrangements, *arc* mutants sustain a relatively normal life span (Sakamoto et al., 2008). Thus, it has been proposed that the enlarged size of the chloroplasts of *arc* mutants may compensate for the loss of organelle numbers and maintain a constant chloroplast volume for normal plant growth and development (Pyke and Leech, 1994; Pyke et al., 1994; Stokes et al., 2000). Recently, this view was challenged, because *arc6* and other *arc* mutations affect light-harvesting capacity and cause the loss of adaptability toward light alteration (Ii and Webber, 2005). The decreased

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A

WT   *etr1-1*   *etr1-2*   *ctr1-1*

ein2-1   *ein3-1*   F1   *arc3-2*

B

AIR

C_2H_4

WT   *etr1-1*   WT x *etr1-1*   *etr1-1* x WT

C

WT   *etr1-1*   *etr1-7*

D

WT   *etr1-1*   *etr1-7*
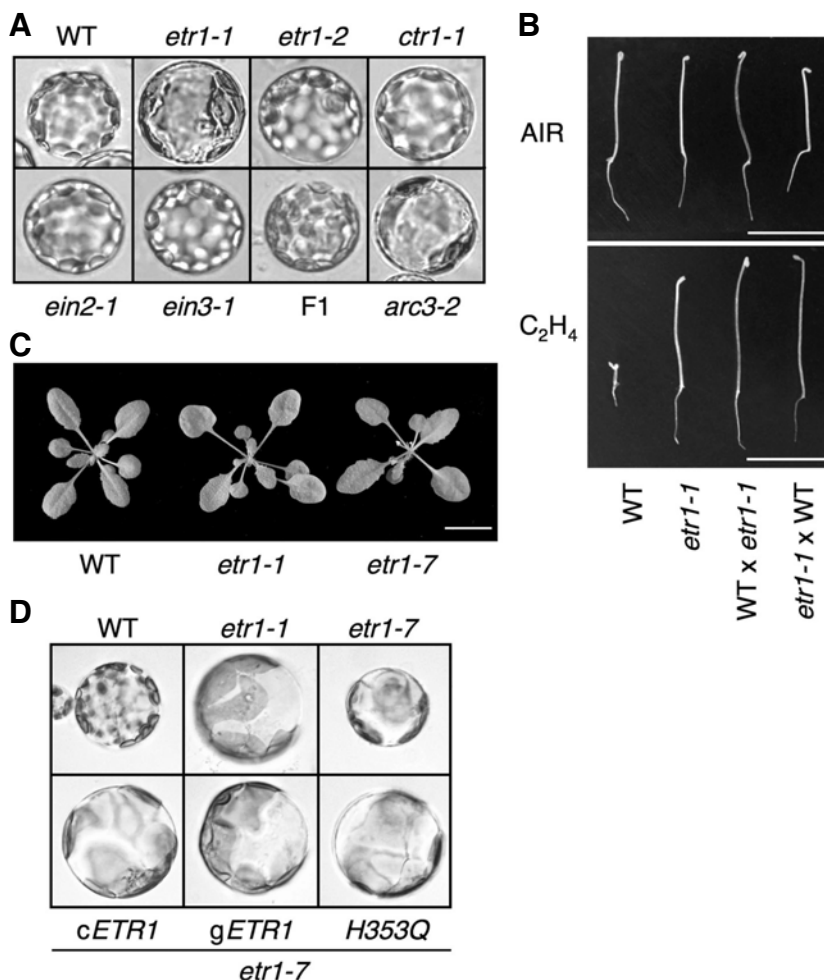
cETR1   *gETR1*   *H353Q*

etr1-7

Fig. 1. Accumulation and replication of chloroplast phenotype in *etr1-1*. (A) Chloroplast phenotypes in leaf mesophyll protoplasts isolated from different genetic backgrounds. F1 indicates the F1 lines of *etr1-1* × WT (Col). Image was taken under a microscope (200×). (B) Analysis of triple response manifested by apical hook formation, hypotocyl and root growth inhibition and hypocotyl thickening for 3.5-day-old etiolated seedlings in the absence and presence of 1-aminocylopropane-1-carboxylic acid (ACC, 10 μM). Scale bar, 10 mm. (C) *Arabidopsis* WT, *etr1-1*, and *etr1-7* plants grew normally in soil at 23°C under a 13 h photoperiod (60 μmol/m²/s). Scale bar, 10 mm. (D) Chloroplasts in leaf mesophyll protoplasts of *etr1-1*, *etr1-7*, and transgenic *etr1-7* expressing wild-type *ETR1* (*cETR1* or *gETR1*) or His-kinase inactive *ETR1* (*H353Q*).

photosynthetic efficiency in *arc6* was partly explained by changes in thylakoid architecture.

In this study we described a giant chloroplast phenotype in *etr1-1*, a dominant ethylene-insensitive mutant of *ETHYLENE RESPONSE1* (*ETR1*; AT1G66340). The abnormal chloroplast phenotype was also observed in *etr1-7* that was an intragenic-suppressor of *etr1-1*, but not in other ethylene response mutants. Here, we elucidated that neither ETR1 nor ethylene signaling was relevant to the abnormal chloroplast phenotype; rather, a second mutation on *ARC3* was responsible for the abnormal chloroplast phenotype in *etr1-1* and *etr1-7*. Although other plastid-division related genes were expressed normally, the loss of *ARC3* caused impaired plastid division and generated giant chloroplasts in *etr1-1*.

MATERIALS AND METHODS

Plant materials and isolation of mesophyll protoplasts
Plants were grown in soil at 23°C for 20 to 22 days under 60 μmol/m²/s with a 13 h photoperiod. Protoplast isolation was carried out as previously described (Yoo et al., 2007). *Arabidopsis thaliana* Columbia served as the wild-type and *etr1-1*, *etr1-2*, *etr1-7*, *ctr1-1*, *ein2-1*, *ein3-1*, and *arc3-2* (SALK_057144) mutants were used for experiments.

Fine mapping of the second mutation in *etr1-1*
To map the second mutation in *etr1-1* that was responsible for enlarged chloroplasts, F2 population was generated from a cross between L*er* and *etr1-1*. Among 183 F2 plants, 44 mutants were selected based on the chloroplast phenotype under microscopy and subjected for further molecular marker analysis.

Gene expression analysis
For gene expression analysis, seedlings were grown in B5 plates (Duchefa) containing 1% sucrose under a 16 h photoperiod. Total RNA was isolated by the Trizol method (Invitrogen) and 1 μg of total RNA was used for cDNA synthesis. Semiquantitative reverse transcriptase PCR analysis was carried out with gene-specific primers for *ARC3*, *PDV1*, and *PDV2*. Detailed primer sequence information can be found in Supplementary Table S1. Experiments were repeated three times with consistent results.

RESULTS AND DISCUSSION

Accumulation and replication of chloroplast phenotype in *etr1-1*
Leaf mesophyll cells of higher plants typically contain about 50 to 120 small chloroplasts (Sakamoto et al., 2008), as shown in *Arabidopsis* wild-type (Col) protoplasts freshly isolated from mature leaf tissue (Fig. 1A). In contrast, we observed an ab-
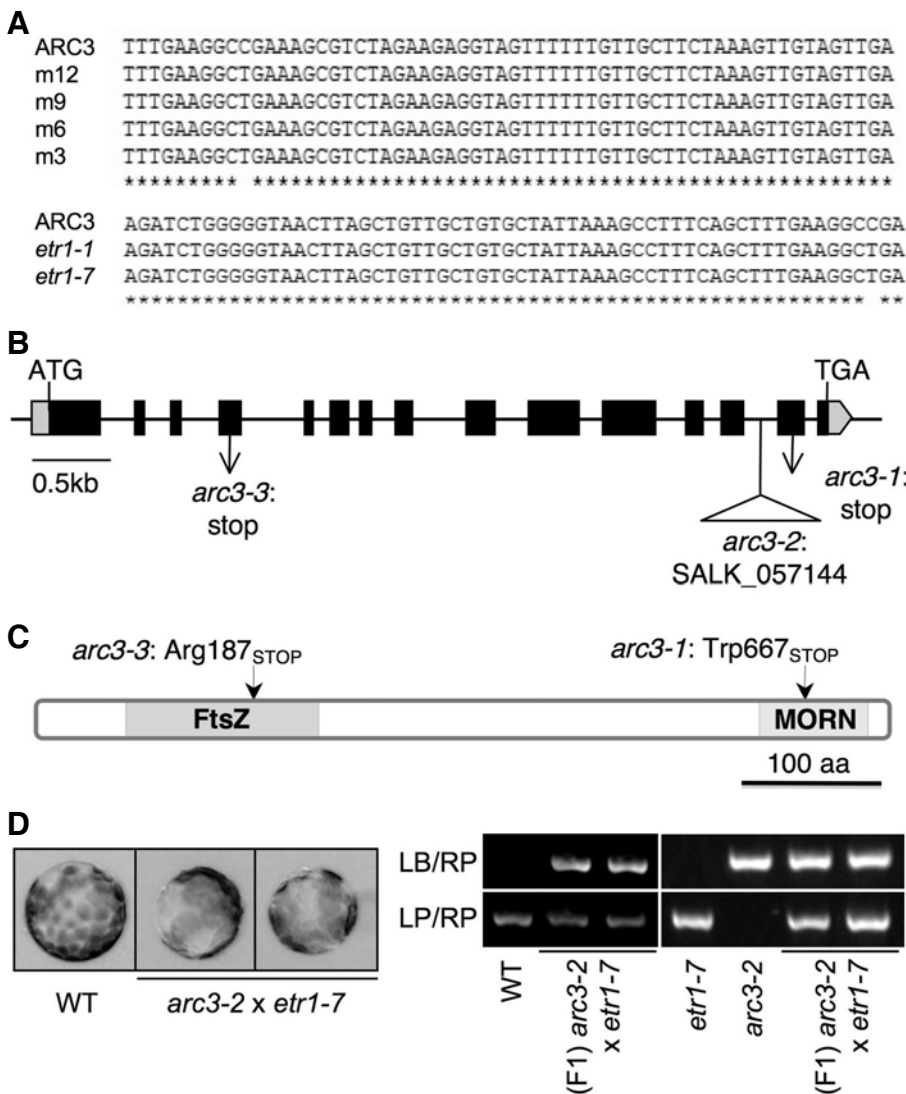
A

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ARC3   TTTGAAGGCCGAAAGCGTCTAGAAGAGGTAGTTTTTTGTTGCTTCTAAAGTTGTAGTTGA
m12    TTTGAAGGCTGAAAGCGTCTAGAAGAGGTAGTTTTTTGTTGCTTCTAAAGTTGTAGTTGA
m9     TTTGAAGGCTGAAAGCGTCTAGAAGAGGTAGTTTTTTGTTGCTTCTAAAGTTGTAGTTGA
m6     TTTGAAGGCTGAAAGCGTCTAGAAGAGGTAGTTTTTTGTTGCTTCTAAAGTTGTAGTTGA
m3     TTTGAAGGCTGAAAGCGTCTAGAAGAGGTAGTTTTTTGTTGCTTCTAAAGTTGTAGTTGA
       ********* **************************************************

ARC3   AGATCTGGGGGTAACTTAGCTGTTGCTGTGCTATTAAAGCCTTTCAGCTTTGAAGGCCGA
etr1-1 AGATCTGGGGGTAACTTAGCTGTTGCTGTGCTATTAAAGCCTTTCAGCTTTGAAGGCTGA
etr1-7 AGATCTGGGGGTAACTTAGCTGTTGCTGTGCTATTAAAGCCTTTCAGCTTTGAAGGCTGA
       ************************************************************ **
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B

C

D

Fig. 2. *ARC3* mutation cosegregation with *etr1-1*. (A) Nucleotide sequences of *arc3* isolated from four independent mapping lines (m12, m9, m6, and m3), as well as *etr1-1* and *etr1-7*, were aligned with WT sequence. (B) *arc3-3* was identified from the mapping population of *etr1-1* × L*er*. The genome structure of *ARC3* was shown with previously (*arc3-1* and *arc3-2*) and newly (*arc3-3*) identified alleles. (C) Schematic diagram of ARC3 protein was shown with *arc3-1* and *arc3-3* mutations. (D) Chloroplast phenotypes in leaf mesophyll protoplasts isolated from WT and two F1 lines of *arc3-2* × *etr1-7*. Molecular marker analysis for *arc3-2* was shown.

errant chloroplast phenotype in the dominant ethylene-insensitive mutant *etr1-1* (Fig. 1A). This mutant had enlarged chloroplasts that were much reduced in number compared to WT.

To investigate whether ETR1 function in ethylene signaling is involved in chloroplast phenotype, we observed the chloroplast morphology of another dominant allele of *etr1*, *etr1-2*, together with other ethylene-response mutants, including the ethylene constitutively responsive mutant, *ctr1-1* and ethylene-insensitive mutants, *ein2-1* and *ein3-1*. The abnormal phenotype was not observed in any of these mutants (Fig. 1A), indicating that ethylene signaling is irrelevant to the chloroplast phenotype of *etr1-1*. We next generated F1 plants by crossing *etr1-1* with WT in a reciprocal manner; F1 plants were ethylene insensitive due to the dominant nature of the *etr1-1* allele in ethylene signaling (Fig. 1B). However, the leaf cells showed a normal chloroplast shape (Fig. 1A), providing another evidence that the ethylene signaling function of ETR1 is irrelevant to the abnormal chloroplast phenotype in *etr1-1*.

We noted that the chloroplast abnormality in *etr1-1* resembled the *arc* phenotype resulting from defects in organelle division as in *arc3-2* (Fig. 1A; Marrison et al., 1999; Pyke and Leech, 1994). To further examine whether the ethylene signal-

ing-independent *arc* phenotype was specific to the *etr1-1* allele, we observed the chloroplast phenotype of *etr1-7*, an intragenic suppressor of *etr1-1* that has no obvious developmental defect compared to WT and *etr1-1* (Fig. 1C) (Hua and Meyerowitz, 1998). Unexpectedly, the *arc* phenotype was also observed in *etr1-7* (Fig. 1D), even though ETR1 expression was greatly diminished in the mutant (Cho and Yoo, 2007). A peculiarity of chloroplast division is that both loss- and gain-of-function alleles involved in chloroplast division often result in the same *arc* phenotype. This indicates that the molecular ratio among the machinery proteins is important for the precise control of plastid division (Maple et al., 2007). To test if this was the case in chloroplast aberrations in gain-of-function *etr1-1* and loss-of-function *etr1-7* mutants, we isolated and observed leaf mesophyll protoplasts from transgenic *etr1-7* lines that expressed *ETR1* cDNA, genomic DNA, or cDNA with a histidine kinase mutation (*H353Q*) in *ETR1* under the control of the *ETR1* promoter (Cho and Yoo, 2007). The *arc* phenotype in *etr1-7* was again present in all of the transgenic *etr1-7* lines (Fig. 1D). These results indicated that *ETR1* itself was not the genetic factor underlying the chloroplast phenotype; instead, there had to be another mutation that was cosegregating with *ETR1* and
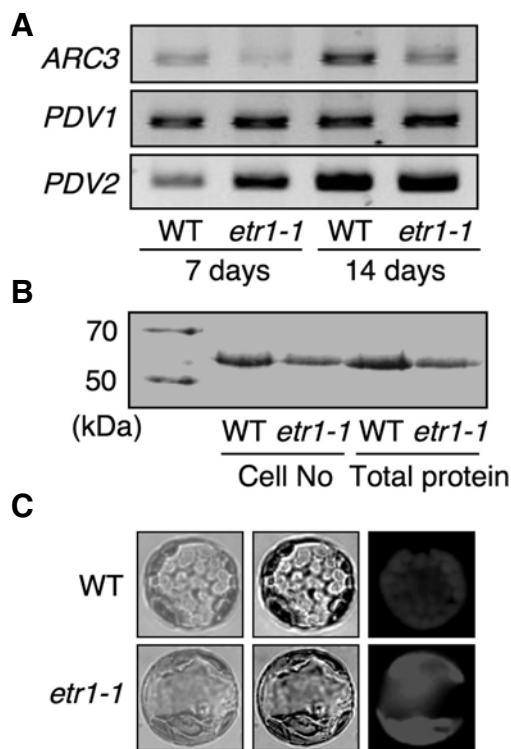
A

B

C

Fig. 3. *arc3-3* related molecular and chloroplast phenotypes in *etr1-1*. (A) *ARC3*, but not *PDV1* and *PDV2*, transcript level was reduced in *etr1-1*. Transcript levels were monitored using semi-quantitative PCR with cDNA templates generated from whole seedlings. (B) Rubisco protein was visualized by Coommassie Blue staining. (C) Chloroplast phenotypes in leaf mesophyll protoplasts. Image was taken under a fluorescence microscope (200×).

causing the *arc* phenotype in *etr1-1*.

A second mutation in *ARC3* of *etr1* mutants

To isolate and characterize the genetic factor causing the *arc* phenotype in *etr1-1* (Fig. 1A), we crossed *etr1-1* with Landsberg *erectra* (L*er*) and mapped a second mutation by marker-assisted allele positioning. The mutation was placed near *ETR1* after the first round of marker analysis. In all of the mapping lines with *arc* phenotype (m12, m9, m6, and m3), the second mutation was identified as a single nucleotide change in *ARC3* (AT1G75010) that caused a premature translation stop at Arg187 (Fig. 2A). We named this allele *arc3-3,* complying with previously described alleles (Fig. 2B). As expected, the same mutation was found on *ARC3* in *etr1-7*, because *etr1-1* and *etr1-7* share genetic backgrounds (Fig. 2A) and showed the same *arc* phenotype (Fig. 1D).

ARC3 is located on the stromal side of chloroplasts and is involved in FtsZ-ring positioning in the middle of the organelle (Supplementary Fig. S1; Maple et al., 2007; Shimada et al., 2004). *arc3-1* has a point mutation that leads to a premature translation stop at Trp667, and *arc3-2* has T-DNA insertion at the 13th intron (Fig. 2B). Both alleles cause defects in the MORN domain at the C-terminal end of ARC3 (Fig. 2C), and mutants carrying either allele have average 13 chloroplasts (Fig. 1A; Maple et al., 2007; Shimada et al., 2004). The new allele *arc3-3* had a defect in the FtsZ domain at the N-terminal of the protein, which caused much shorter ARC3 peptide synthesis, if any, compared to those in *arc3-1* and *arc3-2* (Fig. 2C).

To further verify our discovery, *etr1-7* was crossed with *arc3-2* and F1 plants were used for genetic complementation test. As shown in Fig. 2D, F1 plants were heterozygous for *arc3-2*, but kept *arc* phenotype due to *arc3-3* in *etr1-7* (Fig. 2D). This result conclusively demonstrated that the loss of ARC3 in *etr1-7* caused the giant chloroplast phenotype.

At the plastid division site of chloroplasts, PDV1 and PARC6 interact in the inner membrane space and PARC6 may associate with ARC3 in the stroma (Supplementary Fig. S1; Okazaki et al., 2009; Osteryoung and McAndrew, 2001). Likewise, PDV2 and ARC6 interact each other and ARC6 also associates with FtsZ1 and FtsZ2 (Glynn et al., 2008; Osteryoung and McAndrew, 2001). All of these protein-protein interactions collectively support the precise localization of the plastid division ring to the outer membrane and the Z-ring to the inner membrane at the chloroplast division site.

To examine if *arc3-3* affects other plastid division machineries in *etr1-1*, we measured the transcript levels of *PDV1* and *PDV2* together with *ARC3*. When we quantified the transcript levels with cDNA generated from seedlings grown on full-strength B5 agar media containing 1% sucrose, relatively lower levels of *ARC3* were detected in *etr1-1* compared to WT (Fig. 3A). Perhaps because of the premature stop codon, *ARC3* transcript was relatively unstable in *etr1-1* (Fig. 2C). In both young and mature seedlings, *PDV1* transcript levels were more or less similar in WT and *etr1-1*. *PDV2* accumulation was only slightly higher in young *etr1-1* seedlings compared to WT. The higher accumulation of *PDV2* may reflect its early compensatory response with respect to the defect in the PDV1 complex caused by the *arc3-3* allele in *etr1-1* mutants during the early stage of plastid division (see the topological model of the chloroplast division site in Supplementary Fig. S1).

We then measured the protein levels of rubisco (RBC) in WT and *etr1-1* and monitored the effects of chloroplast structure on photosynthetic machinery. In the same number (4×10^4 cells) of leaf mesophyll cells, RBC proteins in *etr1-1* were relatively less abundant than in WT (Fig. 3B). In the same amount of total protein (5 μg), RBC protein accumulation was also reduced in *etr1-1* compared to WT (Fig. 3B). These results indicated that the smaller number of chloroplasts was not fully compensated for by their enlarged size in *etr1-1 arc3-3* mutants.

Chlorophyll fluorescence from leaf protoplasts delineated chloroplasts as many small structures in WT and a few larger structures in *etr1-1* (Fig. 3C). The quantitative analysis of chlorophyll fluorescence has been utilized to acquire information about the photosynthetic yields of photosystem II, the relative level of the reduced quinone pool, and the extent of non-photochemical quenching during photosynthetic light reactions (Akimoto and Mimuro, 2007; Karukstis and Sauer, 1983). It will be interesting to further examine if the altered structure of plastids can cause changes in chlorophyll fluorescence and photosynthesis activity.

In summary, we have unravelled that an unexpected chloroplast phenotype in *etr1-1* is caused not by *etr1-1*, but by a second mutation, *arc3-3*, that cosegregates with *etr1-1*. It appears that the increased size of the abnormal chloroplasts may not be able to fully compensate for the loss of a greater array of small chloroplasts.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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