The chloroplast ATP synthase catalyzes the light-driven synthesis of ATP and acts as a key feedback regulatory component of photosynthesis. *Arabidopsis* possesses two homologues of the regulatory γ subunit of the ATP synthase, encoded by the *ATPC1* and *ATPC2* genes. Using a series of mutants, we show that mutations within these subunits can support photosynthetic ATP synthase in vivo with similar specific activities, but that in wild-type plants, only γ1 is involved in ATP synthase in photosynthesis. The γ1-containing ATP synthase shows classical light-induced redox regulation, whereas the mutant expressing only γ2-ATP synthase (gamera) shows equally high ATP synthase activity in the light and dark. In situ redox titrations demonstrate that the regulatory thiol groups on γ1 ATP synthase remain reduced under physiological conditions but can be oxidized by the strong oxidant diamide, implying that the redox potential for the thiol/disulfide transition in γ2 is substantially higher than that for γ1. This regulatory difference may be attributed to alterations in the residues near the redox-active thiols. We propose that γ1-ATP synthase functions to catalyze ATP hydrolysis-driven proton translocation in nonphotosynthetic plastids, maintaining a sufficient transthylakoidal proton-gradient to drive protein translocation or other processes. Consistent with this interpretation, *ATPC2* is predominantly expressed in the root, whereas modifying its expression results in alteration of root hair development. Phylogenetic analysis suggests that γ2 originated from an ancient gene duplication, resulting in divergent evolution of functionally distinct ATP synthase complexes in dicots and mosses.

**Light-driven linear electron flow (LEF) in photosynthesis stores energy in reduced NADPH. It is also coupled to the translocation of protons into the thylakoid lumen, generating an electrochemical gradient of protons, the proton motive force (pmf), across the thylakoid.** The pmf in turn drives the synthesis of ATP from ATP and F1 via the CF0-CF1 ATP synthase (ATP synthase), storing energy in the form of the photophosphorylation potential. The plastid ATP synthase complex consists of nine different subunits. Four of these subunits (I, II, IIIγ, and IV, also called βc, b′c, a, and a) form the integral membrane CF0 subcomplex and the remaining five subunits make up the extrinsic CF1 subcomplex (αs, βs, γ, δ, and ε) that contains the catalytic sites of ATP synthase (1, 2). The CF0 subcomplex drives the synthesis of ATP at the CF1 subcomplex via the rotational-catalysis/binding-change mechanism (3–5).

The ATP synthase is regulated at several levels (6). It has been known for some time that the ATP synthase requires a substantial pmf to become activated (7–9). This pmf-activation is thought to involve interaction between the FO and F1 subcomplexes because detachment of FO activates ATP hydrolysis (10). A second level of regulation occurs in plants and green algae via thioredoxin-mediated thiol modulation of the γ subunit, altering the extent of pmf required to activate the complex (4). The structural basis of the regulation is assigned to a sequence of nine amino acid residues containing two Cys residues (Cys199–Cys205, in *Arabidopsis thaliana* numbering) able to form an intrapeptide disulfide bond upon oxidation (11). In the light, electron flow from photosystem I (PSI) reduces thioredoxin-f via ferredoxin/thioredoxin oxidoreductase (12), which in turn reduces the γ subunit, forming an ATP synthase that is activated with a relatively low pmf of about 50 mV (13). After dark adaptation for tens of minutes, the γ subunit cysteine residues become oxidized, imposing a higher pmf requirement for activation, of about 100 mV, thus resulting in ATP synthase inactivation, preventing rapid hydrolysis of ATP. Redox-regulation via thioredoxin is highly sensitive to light, and acts as a “switch,” rapidly transitioning between inhibition in the dark and full activity at even very low light intensities (14).

The ATP synthase is also fine-tuned in the light, during steady-state photosynthesis in response to changes in metabolism and/or environmental conditions, e.g., endogenous CO2 levels or drought stress (15–17). The resulting changes in proton efflux adjust the steady-state pmf and subsequently affect the regulation of light capture and electron transfer. The mechanism of this fine tuning is not yet clear, though a number of mechanisms have been proposed, including drawing down of inorganic phosphate and phosphorylation (18).

*Arabidopsis thaliana* possesses two genes that appear to code ATP synthase γ subunits, *ATPC1* and *ATPC2* (At4G04640 and At1G15700), located on chromosomes 4 and 1, respectively (19, 20), and showing 73% sequence similarity (19). Both *Arabidopsis* γ homologues contain the domain responsible for the redox regulation of the enzyme (19), (Fig. S6). The current study aims to answer the intriguing questions: In which tissues are γ1 and γ2 localized? Can both ATPC homologues function in ATP synthase? Are their regulatory and functional properties different? What functions might they serve in the plant?

**Results and Discussion**

As described in detail below, this study used a series of *Arabidopsis* plant lines, wild type (Columbia), two mutant lines lacking *ATPC2* (*atpc2* (T-DNA inserted in chl1_5402949), *atpc2_2* (T-DNA inserted in chl1_5404314), a mutant lacking *ATPC1* (dpa1) (21) complemented with 35S::*ATPC1*, and a mutant line lacking *ATPC1* but expressing *ATPC2* behind a 35S promoter (gamera).


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The γ2 Subunit Is Chloroplast Localized and Expressed at Very Low Levels. The primary sequences of γ1 (coded by ATPC1) and γ2 (coded by ATPC2) contain apparent N-terminal transit peptides of 50 and 60 amino acids, respectively, suggesting that γ2, like γ1, is targeted to the chloroplast. The subcellular localization of γ2 in chloroplast was confirmed by fluorescence microscopy on transiently transformed Arabidopsis protoplasts expressing γ2-GFP fusion protein (Fig. S1).

The ATPC1 gene was found to be highly expressed in photosynthetic tissues, and disrupting this gene abolished photosynthetic growth implying that it is essential for photosynthesis (21, 22). In contrast, ATPC2 was expressed at low levels in photosynthetic tissues, presumably insufficient to complement the ATPC1 knockout (21) (See also Fig. S3). Previous mass spectroscopy-based proteome analysis (23) was able to detect the γ2 subunit in wild-type chloroplasts, but we were unable to detect it using immunoblot analysis (Fig. S2B and C), likely indicating very low expression (Fig. S2). Knocking out this gene did not markedly affect growth (Fig. S2A) or photosynthesis (Fig. 1), implying that it plays little or no role in photosynthesis.

γ2 Can Form an Active ATP Synthase, Suppressing the Loss of γ1. To determine whether γ2 could, if expressed, support ATP synthase activity, we generated plants containing exclusively γ2—rather than γ1-ATP synthase by expressing ATPC2 under control of the constitutive 35S promoter in the ATPC1 null mutant background (see Supporting Information) (21). The resulting mutant was termed gamera (gamma exchange-revised ATP synthase). Using Western blot analysis with antibodies specific to γ1 and γ2 (Fig. S2B and C), we confirmed that gamera lacked detectable levels of the γ1 subunit, but gained γ2 subunit. On the other hand, leaves from wild type and the atpc2 mutant contained γ1, but no detectable γ2. Both γ1 in wild type and γ2 in gamera were found exclusively in the insoluble (membrane) fractions, indicating that these were likely bound into the ATP synthase complexes. All independent gamera transformants showed a lower than wild type accumulation of ATP synthase protein subunits, despite high levels of ATPC2 mRNA expression, suggesting that the γ2-ATP synthase may be less stable than γ1-ATP synthase (Fig. S2B and C).

The gamera mutant was able to grow photoautotrophically (Fig. S2A), indicating that higher (than wild type) expression levels of γ2 were able to functionally replace γ1 in the ATP synthase. However, the growth rates were somewhat slower in gamera than in wild type, resulting in rosette diameter 30% smaller in 4 wk old plants (Fig. S2A).

Photosynthetic Properties of Gamera. Fig. 1A shows the dependence of LEP, measured by saturation-pulse chlorophyll fluorescence yield analysis (24) on photosynthetically active radiation (PAR) for wild type, atpc2 and gamera. The initial slope of LEP against PAR was only slightly (approximately 10%) reduced in gamera compared to wild type or atpc2, indicating that the maximal quantum efficiency of photosynthesis was similar for all three genotypes. This result is also consistent with our observation that atpc2 and gamera had minimal effects on maximal photochemical quantum yield changes in dark-adapted leaves (0.83 ± 0.0019, 0.83 ± 0.0012, and 0.80 ± 0.0013 for wild type, atpc2, and gamera, respectively). However, LEP saturated at about 100 μmol photons m⁻² s⁻¹ in gamera compared to about 200 μmol photons m⁻² s⁻¹ for wild type and atpc2, whereas gamera reached a maximum LEP of approximately 33% (approximately 20 μmol electrons m⁻² s⁻¹) compared to wild type and atpc2, indicating that gamera had a rate-limitation at steps beyond PSII, most likely at the ATP synthase.

We confirmed a lower in vivo ATP synthase activity for gamera by probing proton flux and light-induced pmf by measuring the decay of the electrochemical shift (ECS) (Fig. 1) as described previously (15, 16, 25). The extent of the rapid decay of ECS upon switching off actinic light, termed ECSd, which reflects the light-driven thylakoid pmf was three- to fourfold larger in gamera than in wild type or atpc2 (Fig. 1C) suggesting that gamera generated a larger light-induced pmf than wild type or atpc2, consistent with a slow rate of proton efflux through the ATP synthase. Supporting this conclusion, gamera produced a substantially higher extent of energy-dependent exciton quenching (qE) than wild type or atpc2 (Fig. 1B), implying a more acidic lumen pH as a result of increased pmf.

The increased pmf in gamera could be attributed to a decrease in efflux of protons from the lumen. Fig. 1D plots the relative conductivity of the thylakoid membrane to protons (qH⁺), which predominantly reflects ATP synthase activity and can be estimated by the decay kinetics of the ECS (15, 24). The qH⁺ in atpc2 was similar to that in wild type, but remained consistently lower in gamera, indicating slower proton efflux. Western blot analysis
showed that ATP synthase β-subunit (Fig. S3B) decreased substantially in gamera compared to wild type and atpc2, implying that the total ATP synthase content, rather than decreased specific activity accounted for the lower $g_{H^+}$ in gamera.

Analysis of atpc1-complemented with ATPC1 (dps1 3SS::ATPC1) showed no differences from wild type in growth, photosynthetic performance, or in vivo ATP synthase activity (Fig. 1) indicating that the lowering of ATP synthase content or activity in gamera was due to substitution of ATPC1 by ATPC2, rather than secondary effects of altering ATPC promoter regions.

γ2-ATP Synthase Is Equally Active in Light- and Dark-Adapted Leaves. We analyzed ATP synthase activity in attached leaves by exciting PSI and PSII with short (100 μs), nonsaturating LED pulses to rapidly generate pmf, and probing the resulting proton flux through the ATP complex via the decay of the ECS absorbance change at 520 nm (13, 14) (Fig. 2 A–C). A few seconds after preillumination, ATP synthase activity of wild type remained active even at low pmf, as indicated by rapid nearly monophasic flash-induced ECS decay—with a fast phase of approximately 25 ms constituting approximately 80% of the decay, and a slower phase with lifetime longer than 250 ms, showing that the γ subunit was predominantly reduced. Upon extensive (>20 min), dark adaptation, the extent of the slow ECS phase markedly increased, reflecting oxidation of the γ subunit, which imposes an increased pmf requirement for activation of the ATP synthase. Owing to the slower decay of ECS in dark-adapted leaves, the contribution of the cytochrome $b_{6f}$ complex turnover to the electric field can be seen as a rise in ECS on the approximately 10 ms time scale. In light-adapted material, this phase is masked by the rapid decay of ECS. The extent of slow ECS decay phase showed a sigmoidal dependence on dark adaptation (Fig. 2D), with a pronounced lag phase from 0 to 10 min after preillumination, followed by an approximately 10 min half time decrease in activity. The sigmoidal oxidation kinetics were previously interpreted as reflecting the equilibration of γ subunit thiols redox state with that of a larger pool of redox carriers (14). Almost identical results were obtained for atpc2, indicating that γ2 is not required for regulation of ATP synthase. In contrast, wild type and atpc2, the ECS decay kinetics in gamera remained constant with a nearly monophasic decay showing halftime of about 21 ms, even after extensive (>60 min) dark-adaptation (Fig. 2B), indicating that γ2-ATP synthase is not down-regulated by thiol oxidation in the dark.

Equilibrium Redox Titrations of γ1 and γ2-ATP Synthase. In situ equilibrium redox titrations (22), were used to assess the redox properties of γ1- and γ2-ATP synthase in wild type and gamera, respectively (Fig. 3A). In wild type, the ECS decay was rapid at low redox potential and slow at high potential, where γ1 was expected to be reduced and oxidized, respectively. Assuming a stromal pH of 7.5, we estimate a redox midpoint potential for the γ1 subunit of approximately −356 mV (versus standard hydrogen electrode) with an $n=2$ redox behavior for the thiol/disulfide interconversion, similar to that reported previously (22). By contrast, ECS decay in gamera remained constant with changing the redox potential (Fig. 3A) over the entire range accessible by varying reduced and oxidized DTT (approximately −300 to −420 mV versus standard hydrogen electrode). The maximal activity of the ATP synthase under reducing conditions, as judged by the ECS decay, was about twofold higher in the wild type than in gamera, presumably reflecting the overall differences in ATP synthase content (Figs. S2B and C).

To determine whether the lack of redox regulation of γ2-ATP synthase was caused by a shift in the redox potential of the thiol/disulfide transition or by a change in the effects of such transition on the activity of the enzyme, we assayed the redox state of the γ1 and γ2 subunits directly, based on modification of free sulfhydryl groups with 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonate (AMS) followed by differential separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (26) (Fig. 3B). Wild-type leaves infiltrated with water showed classical light-induced changes in redox state of γ1, with the higher molecular weight reduced form, in light-adapted leaves. In contrast, we saw no light-induced changes in the apparent molecular weight of γ2 in gamera leaves infiltrated with water. To ensure maximal physiological differences in redox states, we repeated these experiments using two conditions designed to reflect the extremes of in vivo redox states: 1) oxidizing conditions in dark-adapted leaves in the presence of methyl viologen (MV) to prevent accumulation of electrons on thioredoxin (13); 2) reducing conditions in leaves illuminated with 300 μmol photons m$^{-2}$ s$^{-1}$ actinic light and infil
Evidence That  \( \gamma_2 \)-ATP Synthases Have a moonlighting Function in Root Morphogenesis. The gene expression profiles of \( \text{ATPC1} \) and \( \text{ATPC2} \) in different tissues were investigated using the publicly available software Genevestigator (27) (Fig. S4). In leaves, \( \text{ATPC1} \) expression was about 100-fold higher than that of \( \text{ATPC2} \) consistent with the failure to observe accumulation of \( \gamma_2 \) protein in wild-type leaves using \( \gamma_2 \)-specific antibodies (Fig. S2B). However, in radicle or mature root tissue, \( \text{ATPC2} \) expression was approximately equal to or above that of \( \text{ATPC1} \), suggesting a function of \( \gamma_2 \) in roots. To confirm the expression pattern of \( \text{ATPC2} \), we constructed plants with \( \beta \)-glucuronidase (GUS) driven by \( \text{ATPC1} \) (\( \text{ATPC1-GUS} \)) and \( \text{ATPC2} \) promoters (Supporting Information). In contrast to \( \text{ATPC1-GUS} \), which showed a strong staining over the whole seedling (Fig. S5), \( \text{ATPC2-GUS} \) expression was absent in the green tissues and appeared to be localized staining over the whole seedling (Fig. S5).

Equilibrium redox titration (22) of thiol/disulfide regulatory groups in the \( \gamma \) subunit of the chloroplast ATP synthase in wild type (WT) (closed squares) and gamera (opened triangles) (A). In situ fractional ATP synthase activity was estimated by probing ECS decay kinetics (\( \Delta \text{A}_{260} \)) as described in the text. (\( n = 4–6 \)). Detection of reduced (red) and oxidized (ox) \( \gamma \) subunit using gel shift assay, described in text (B). The redox states of \( \gamma_2 \) and \( \gamma_2 \) subunit thiols in wild type and gamma were visualized by AMS-labeling followed by SDS-PAGE and immnoblottting after treatment of leaf discs with water as control, MV, and reduced DTT under light (approximately 50 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\)) or dark as described in Materials and Methods.

Fig. 3. Equilibrium redox titration (22) of thiol/disulfide regulatory groups in the \( \gamma \) subunit of the chloroplast ATP synthase in wild type (WT) (closed squares) and gamera (opened triangles) (A). In situ fractional ATP synthase activity was estimated by probing ECS decay kinetics (\( \Delta \text{A}_{260} \)) as described in the text. (\( n = 4–6 \)). Detection of reduced (red) and oxidized (ox) \( \gamma \) subunit using gel shift assay, described in text (B). The redox states of \( \gamma_2 \) and \( \gamma_2 \) subunit thiols in wild type and gamma were visualized by AMS-labeling followed by SDS-PAGE and immnoblottting after treatment of leaf discs with water as control, MV, and reduced DTT under light (approximately 50 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\)) or dark as described in Materials and Methods.

Fig. 4. Evidence for a function of \( \text{ATPC2} \) in development. \( \text{ATPC2-GUS} \) staining in 10 d old seedlings in root system (A), hypocotyl-root junction (B), primary root (C), young leaves (D), and cotyledons (E). Representative photographs of root hairs of wild type (WT), two separate \( \text{atpc2} \) lines, and gamera (F). Scale bars 1.00 mm. Root hair lengths measured in wild type, two \( \text{atpc2} \) lines, and gamera (G) (\( n = 32–274 \)).

Species Distribution, Phylogeny, and Structural Aspects of \( \text{ATPC} \) Genes. Comparison of sequenced genomes showed that dicots contain two or more homologous duplicates (or multiplicates) of the \( \gamma \) subunit (Fig. S6). The monocots Zea mays and Oryza sativa, the green algae Ostreococcus lucimarinus and Chlamydomonas reinhardtii, or cyanobacteria (see also below) showed only a single copy, suggesting a possible role of the “secondary-duplicate” \( \gamma_2 \)-like subunit specifically in dicots. In all dicots, one homologue is clearly \( \gamma_1 \)-like, possessing a regulatory loop region with two cysteine residues. In many cases, though, one or more of the \( \gamma_2 \)-like subunit duplicates have clear differences in the redox regulatory loop, probably rendering it redox-insensitive. For examples, grape (Vitis vinifera), poplar (Populus trichocarpa), and lotus (Lotus japonicus, Nelumbo nucifera) are missing one of the two regulatory cysteine residues, whereas tobacco (Nicotiana tabacum), barrel clover (Medicago truncatula), felon herb (Artemisia indica), apple (Malus pumila var. domestica), and tomato (Solanum lycopersicum) are missing both. Soybean (Glycine max), castor bean (Ricinus communis), and selaginella (Selaginella tamariscina) \( \gamma \)-homologues retain both cysteine residues, but also contains the same threonine to glutamate substitution in the regulatory loop region as in Arabidopsis \( \gamma_2 \) (Fig. S6).

We investigated the possible evolutionary origins of the diversity of primary and secondary \( \gamma \) subunit homologues. The occurrence of duplicate (or multiplicate) genes coding for the \( \gamma \) subunit of the plastidic ATP synthase was screened in available genomes of uniferne multicellular representatives of viridiplanths and of the evolutionary ancestors of their plastids, i.e., the cyanobacteria. Multiple copies of \( \text{ATPC2} \) were only detected in multicellular spe-
cies whereas both cyanobacteria and unicellular green algae contain only one ATPC gene strongly corroborating the hypothesis put forward above that the duplicate genes are expressed differentially to adapt different types of tissues to their specific requirements. Consequently, duplicate versions of the gene are found in many subgroups of multicellular viridiplantae down to the very early branching mosses. An evolutionary analysis shows a far-going congruence between the phylogeny of these γ subunit genes and that of their parent species (Fig. S7).

The tree furthermore demonstrates that duplication of the ATPC gene had occurred frequently and independently during the evolution of multicellular plants. The two species of mosses, for example, as well as apple trees and glycines feature relatively recent duplication events within each of the mentioned lineages (Fig. S7). By contrast, an early duplication event apparently has given rise to two distinct ATPC genes in the ancestor of angiosperms with subsequent vertical inheritance of both copies towards most of the extant angiosperms. The resulting two clusters contain the above-mentioned ATPC1 and ATPC2 genes from Arabidopsis, respectively. The cluster encompassing the ATPC1 gene is substantially more compact (i.e., characterized by shorter branch lengths) than that containing the ATPC2 gene. This is in line with the ATPC1 gene operating under “standard” conditions in redox regulated ATP synthases, i.e., subjected to strong and unifunctional evolutionary constraints, whereas the secondary gene has evolved to fulfill different and possibly diverse functions in the various plants examined.

Conclusions: What Is the Function of γ-ATP Synthase in Arabidopsis?

We found that the regulatory behavior of the chloroplast ATP synthase is markedly altered by substitution of γ1 by γ2. Wild type and atpc2 plants, which express only γ1-ATP synthase in leaves, show classical light-dark regulation of ATP synthase activity via the TAT pathway (Fig. 2). In contrast, gamera, which expresses exclusively γ2-ATP synthase, shows light-dark-insensitive ATP synthase activity, due to a more positive redox potential for the regulatory thiol groups (Figs. 3 A and S4).

Because γ2-ATP synthase is equally active in the light and dark, we propose that it may function to catalyze low levels of ATP-driven proton translocation in nonphotosynthetic tissues or in the dark, i.e., proton pumping into the lumen driven by ATP hydrolysis) to maintain sufficient transthylakoid proton gradient when γ1-ATP synthase is inactivated. The proton gradient across the thylakoid membrane is required for nonphotosynthesis processes, for example, ion transport or the TAT pathway for targeting plastid proteins into the thylakoid lumen or membrane (28-30) especially in nonphotosynthetic organelles such as etioplasts that develop without photosynthetic electron flow to reduce thioredoxin. It is also possible that γ2-ATP synthase allows for ATP synthesis in nonphotosynthetic plastids, via a chlororespiratory pathway (31). In accord with these views, we showed that γ2-ATP synthase is expressed in the root-hypocotyl junction (Fig. 4B), and altering its expression level had striking effects on root hair morphology (Figs. 4F and G). This is consistent with recent proteomic analysis that shows that, unlike most photosynthetic proteins and complexes, the ATP synthase is already present in dark-adapted etioplasts (32). Although a full investigation of the function of γ2-ATP synthase in roots is beyond the scope of this paper, these observations support a role of γ2 not in photosynthesis but in development or maintenance/function of roots or other nonphotosynthetic tissues. Phylogenetic analyses (Fig. S7) likewise suggest the evolution of (possibly multiple) new “moonlight” functions for ATP synthase through divergent evolution of the γ subunits.

Materials and Methods

Plant Materials and Growth Conditions. Wild-type Arabidopsis thaliana (ecotype Columbia) and ATPC2 T-DNA knockout lines, atpc2 (T-DNA inserted in chl1_5402949) and gamera in which ATPC2 overexpressed in the ATPC1 T-DNA knock out line (dpa1) (21) (see SI Materials and Methods) were grown on soil under continuous light period at 20-30 μmol photons m⁻² s⁻¹ at 22 °C for 4 wks, as described previously (21).

In Vivo Spectroscopic Assays. Maximal PSII quantum efficiency, LEF, and energy-dependent excitation quenching (qE) were estimated from saturation-pulse chlorophyll a fluorescence yield measurements using the instrumenta-

Equilibrium Redox Titrations. Redox titrations were performed in situ in fully expanded detached leaves vacuum-infiltrated with varying ratios of oxidized and reduced 20 mM DTT solutions for 30 min incubation in the dark, as de-

Determination of Redox States of γ Subunits. The redox state of γ subunit of chloroplast ATP synthase was probed using the binding of AMS followed by

GUS Assay. Seedlings were washed in water after soaking in 3.7% formalde-

Observation of Root Phenotype. Wild type, two homozygous ATPC2 T-DNA knockout lines, atpc2 (T-DNA inserted in chl1_5402949), atpc2_2 (T-DNA inserted in chl1_540314A), and gamera were surface sterilized and germinated on Murashige and Skoog plates without sucrose in vertical transparent culture chambers as in (21). Plants were examined using light microscopy (Leica M165FC) after 5 d of growth. Tissue lengths were measured from recorded images using Image J (http://rsbweb.nih.gov/ij/). The lengths of root hairs were measured on the lower 5 mm of roots on four to five plants of each plant type (n = 32-274).

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