

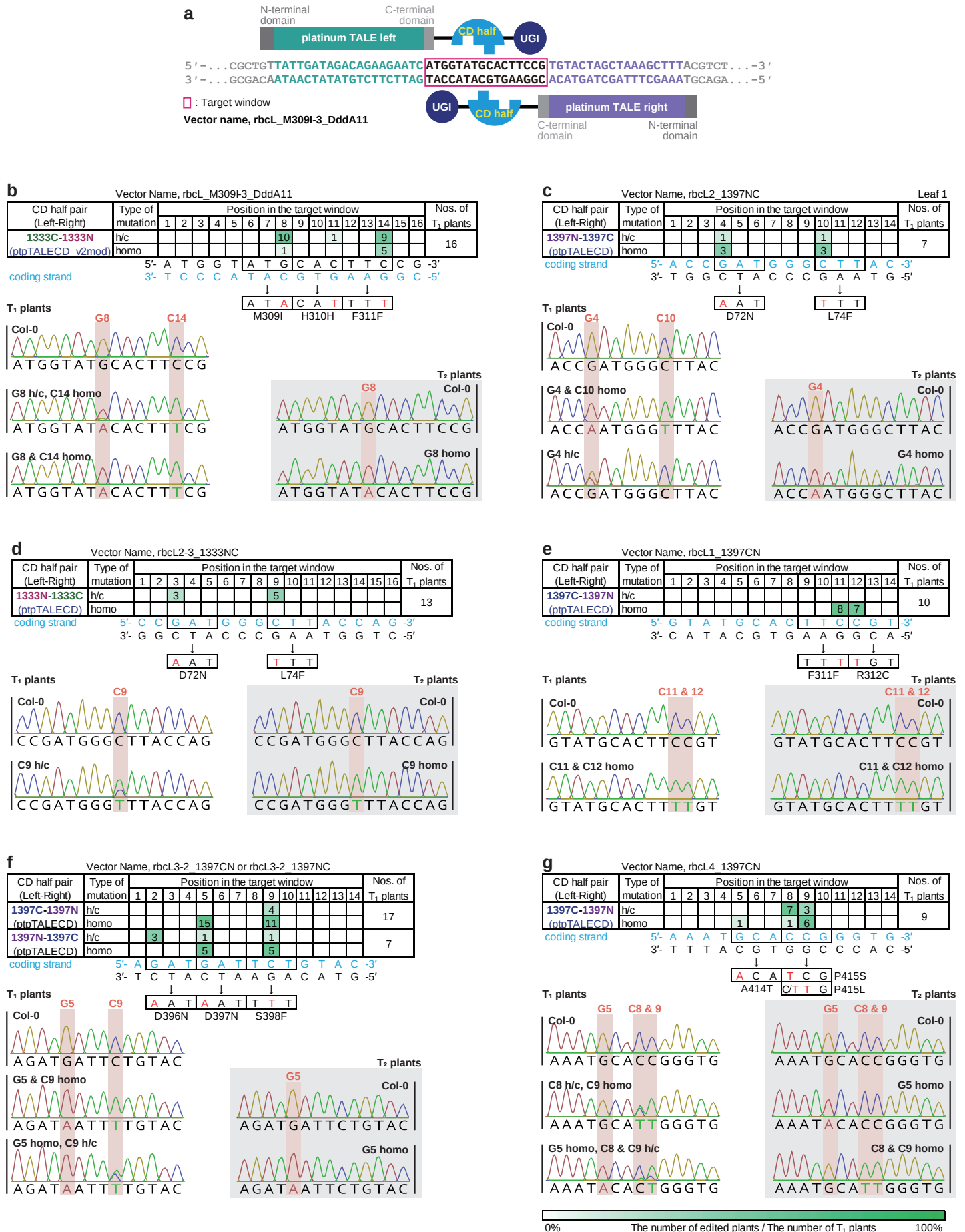


Fig. S1 Base editing in the T₁ generation. **a**, A schematic image of interaction between the base editor and their target sequence. platinum TALE, the DNA binding domain of platinum TALEN (Sakuma *et al.*, 2013); CD, cytidine deaminase; UGI, uracil glycosylase inhibitor. modified from Nakazato *et al.*, 2021. **b-g**, Base editing efficiency in the T₁ generation and Sanger traces of T₁ and T₂ plants. The tables in the upper panel show which bases in the target window were edited, how frequently bases were edited, and how many plants underwent base editing. Genotypes were determined by Sanger sequencing, and the representative Sanger traces of T₁ and T₂ plants are shown in the lower left and right panels, respectively. The Col-0 trace is representative of three individuals. The number of individuals represented by the T₁ and T₂ traces varies depending on the genotype; allele pattern information is provided in Tables S2 and S3. The constructs were rbcL_M309I-3_DddA11 (b), rbcL2_1397NC (c), rbcL2-3_1333NC (d), rbcL1_1397CN (e), rbcL3-2_1397CN (or NC) (f), and rbcL4_1397CN (g). homo, homoplasmically edited; h/c, heteroplasmically or chimerically (not homoplasmically) edited.

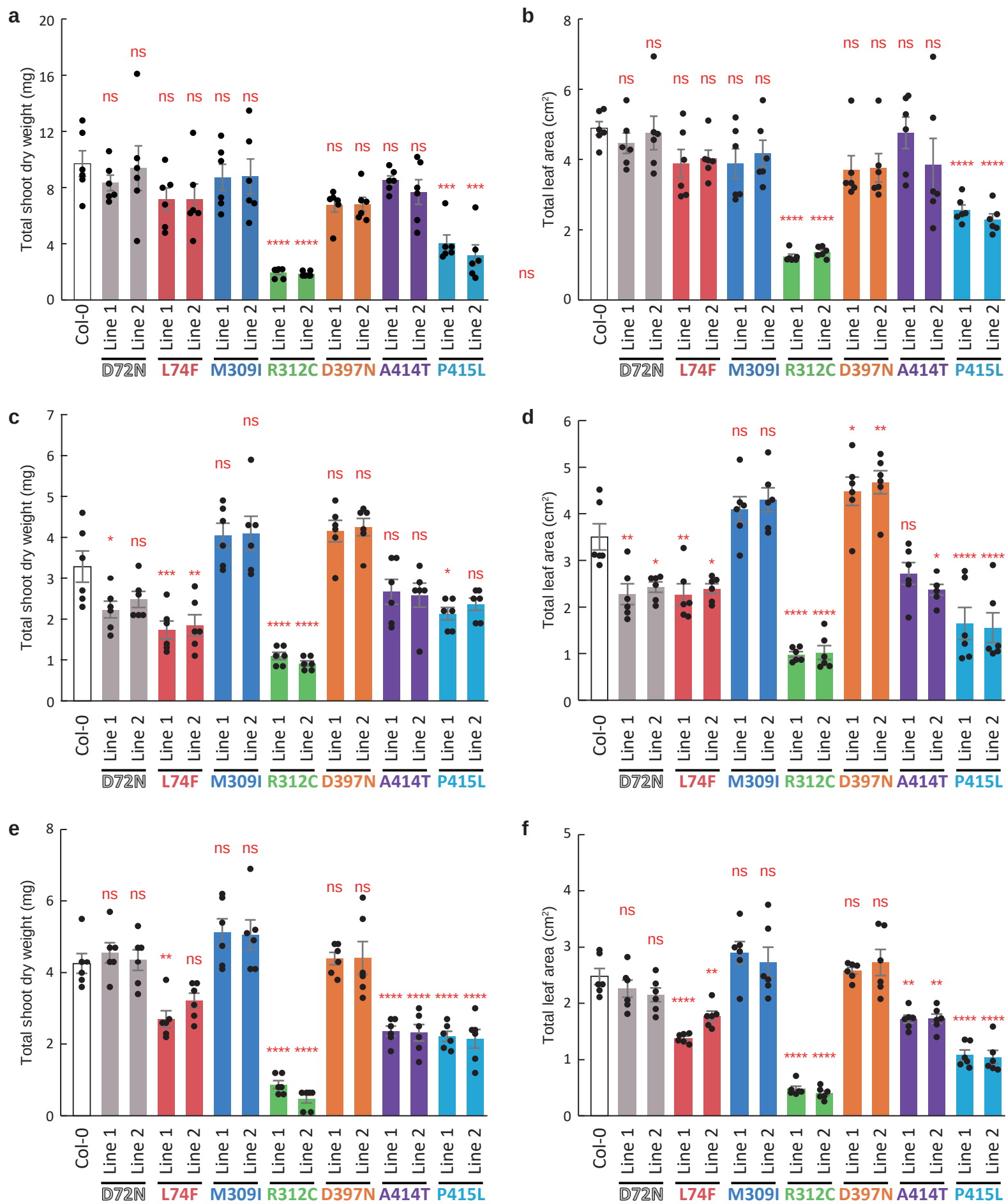


Fig. S2 Plant growth under (286 $\mu\text{mol mol}^{-1} \pm 14$), ambient (381 $\mu\text{mol mol}^{-1} \pm 14$), and high (549 $\mu\text{mol mol}^{-1} \pm 23$) CO₂ concentration. a, c, e, Total shoot dry weight of plants cultivated under low CO₂ concentration at 32 days after sowing (a), ambient (c), and high (e) CO₂ concentration at 25 days after sowing. b, d, f, Total leaf area of plants cultivated under low CO₂ concentration at 32 days after sowing (b), ambient (d), and high (f) CO₂ concentration at 25 days after sowing. Data is mean $\pm$ standard error. Individual data points were displayed as black dots. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$, **** $p < 0.0001$, $n = 6$ plants. Dunnett's two-tailed test for multiple comparisons.**

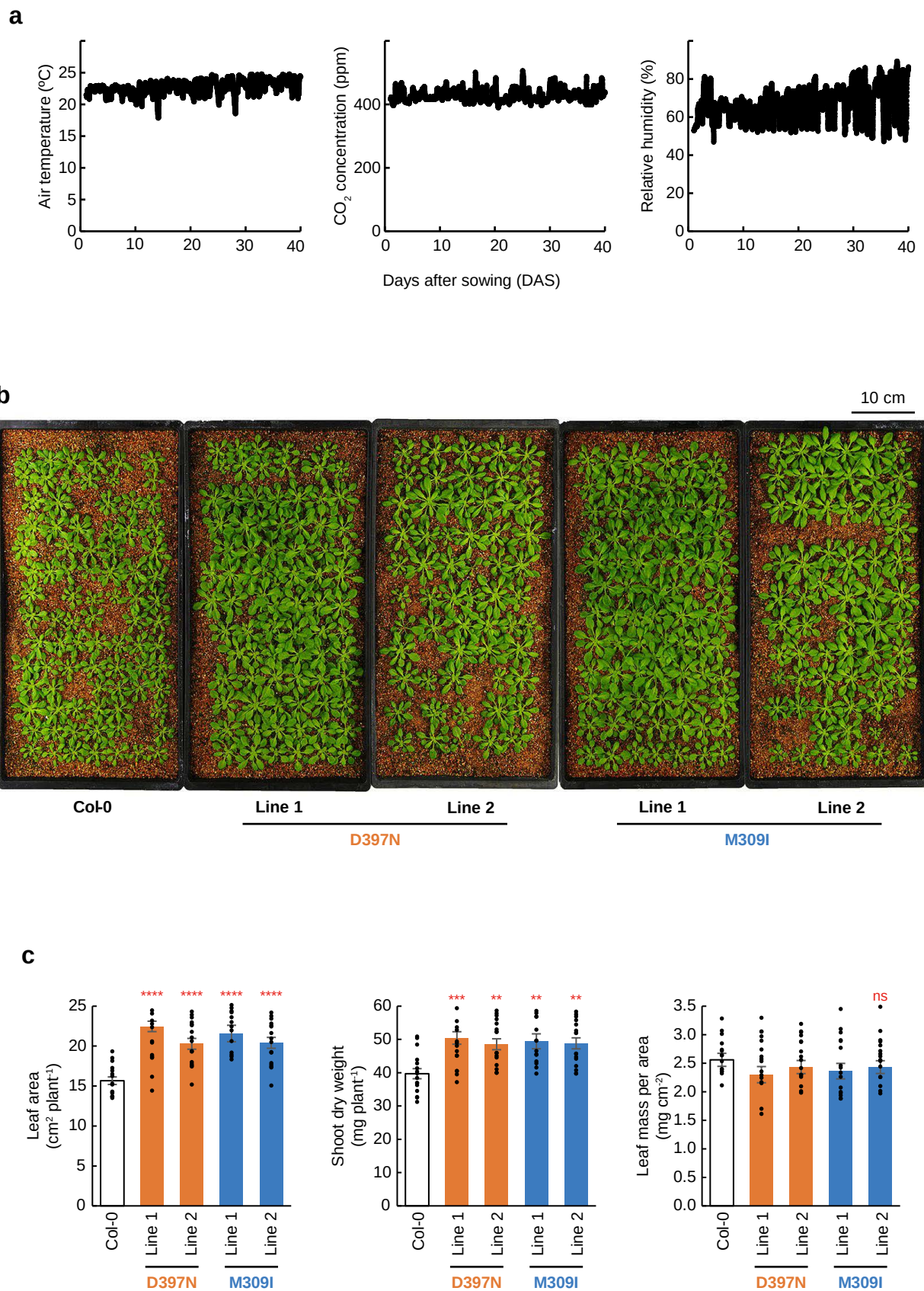


Fig. S3 Plant growth of different lines cultivated in an environment-controlled condition for 36 days.

a, Cultivation condition. Plants were grown in a growth room with a 10 h daylight and 14 h dark period, a photosynthetic photon flux density of 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, an average air temperature of 22.7°C, a relative humidity of 66.9%, and a CO₂ concentration of 428.8 $\mu\text{mol mol}^{-1}$. **b**, Photograph of plants right before harvest. **c**, Leaf area, shoot dry weight, and leaf mass/ leaf area ratio of plants by 28 days. Data is mean $\pm$ SE. ns $p > 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 16$ plants.

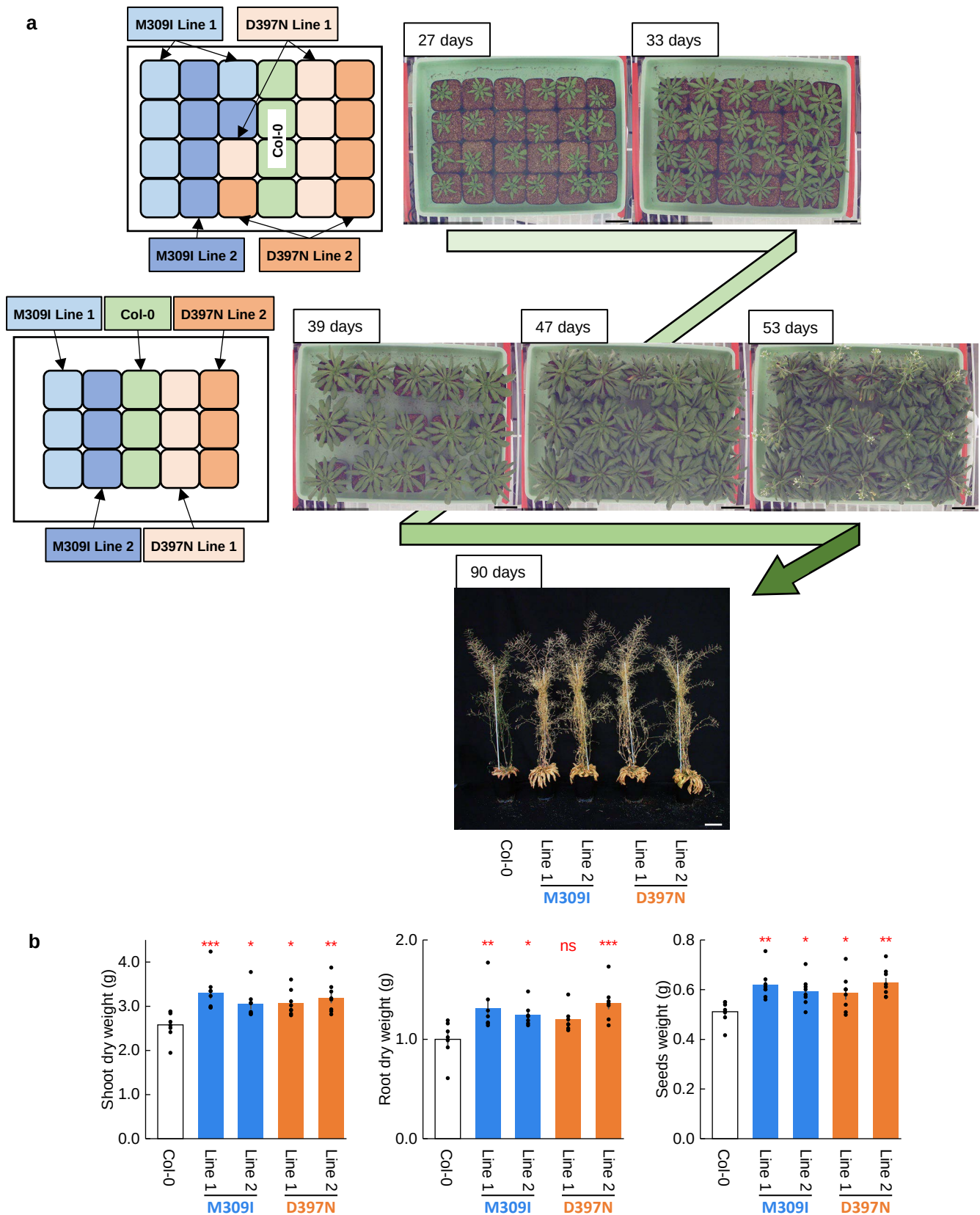


Fig. S4 Plant growth in ambient CO₂ concentration (381 $\mu\text{mol mol}^{-1}\pm 14$). **a**, Plant growth over a 90-day period, from seedling to harvest. No significant differences in bolting time were observed across all plants. Plants were grown with a daylight/dark period of 10/14 h, and photosynthetic photon flux density (PPFD) in the daylight period was set to 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Air temperature and relative humidity were maintained at 22°C and 60%, respectively. Bars = 5 cm. **b**, Weights of dried shoots and roots and seeds harvested at day 90. Data are means $\pm$ SE, n = 5 plants. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, indicating significant differences of each column compared with Col-0. Dunnett's two-tailed test for multiple comparisons.

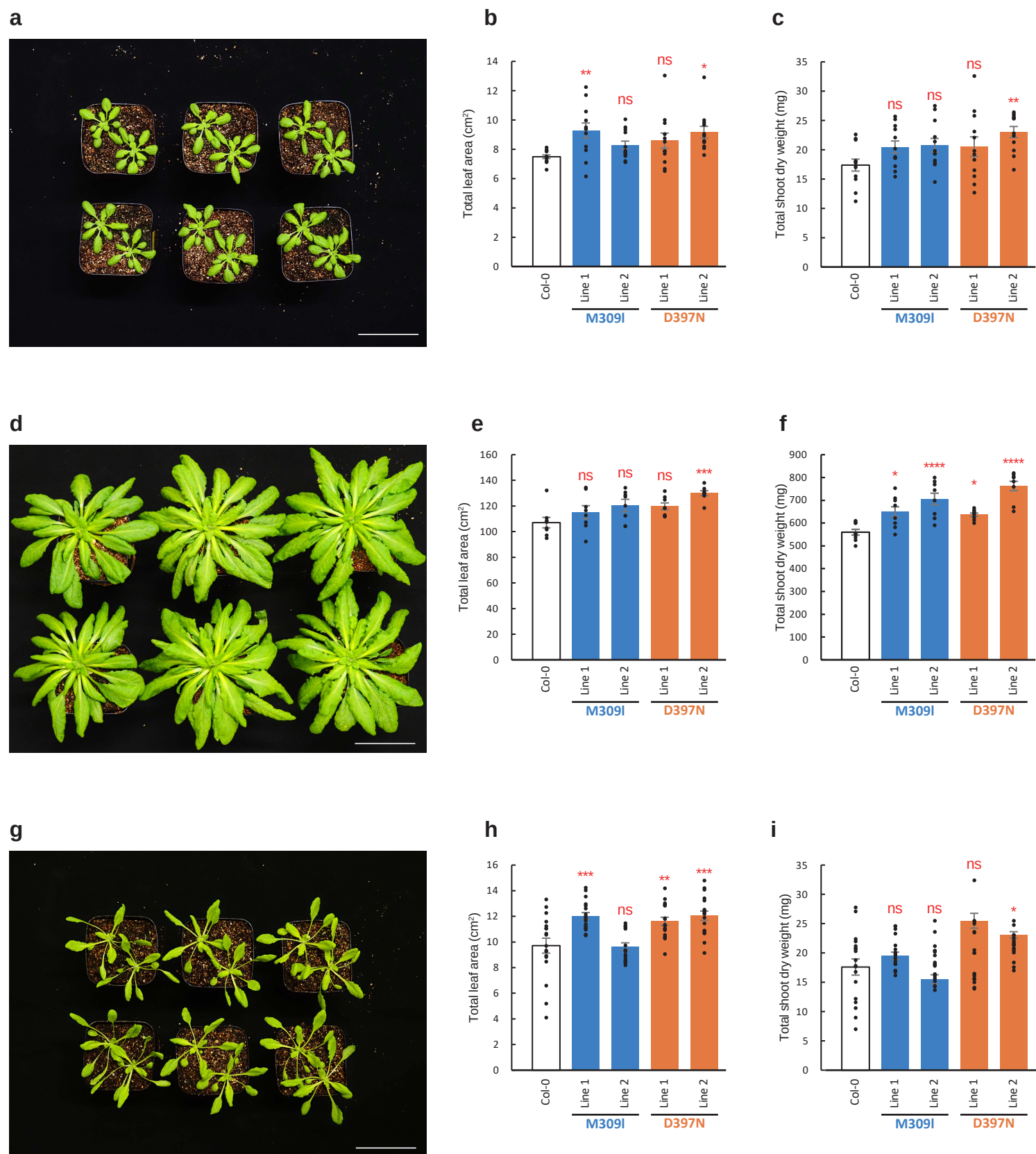


Fig. S5 Plant growth under CO_2 concentration at 25°C and 30°C. **a, d, g,** The visual images of plants cultivated under high (549 $\mu\text{mol mol}^{-1} \pm 23$) CO_2 concentration at 25°C for 25 days (a) and for 42 days (d), and at 30°C for 25 days (g). Bars = 5 cm. **b, e, h,** Total leaf area of plants cultivated under high (549 $\mu\text{mol mol}^{-1} \pm 23$) CO_2 concentration at 25°C for 25 days (b) and for 42 days (e), and at 30°C for 25 days (h). **c, f, i,** Total shoot dry weight of plants cultivated under high (549 $\mu\text{mol mol}^{-1} \pm 23$) CO_2 concentration at 25°C for 25 days (c) and for 42 days (f), and at 30°C for 25 days (i). Data is mean $\pm$ SE. Individual data points were displayed as black dots. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n = 6 plants. Dunnett's two-tailed test for multiple comparisons.

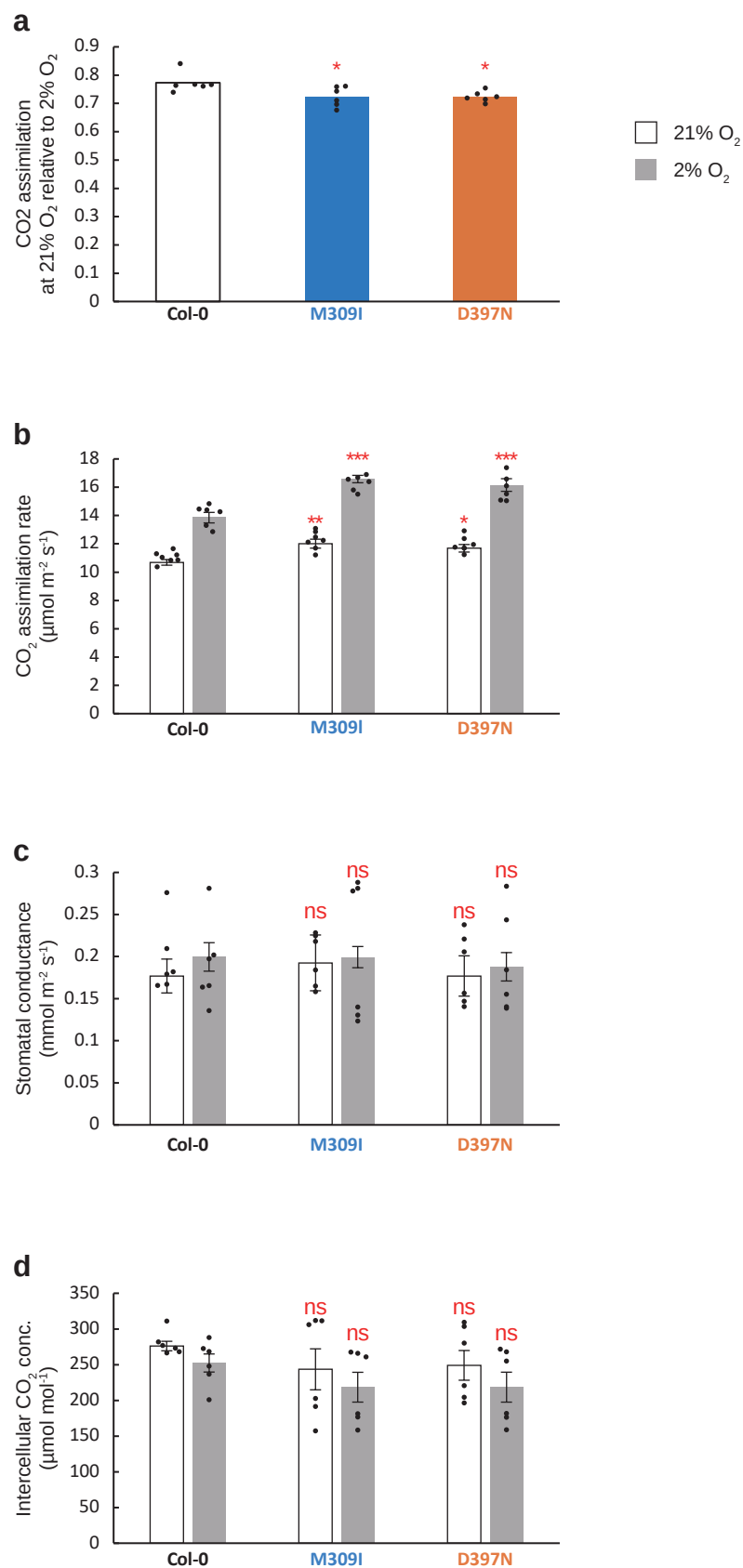


Fig. S6 Photosynthesis parameters at 2% and 21% O₂. **a**, CO₂ assimilation at 21% O₂ relative to 2% O₂. **b**, CO₂ assimilation rate at 2% and 21% O₂. **c**, Stomatal conductance and **d**, Intercellular CO₂ concentration at 2% and 21% O₂. Data is mean ± SE. Individual data points were displayed as black dots. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n = 6 plants. Dunnett's two-tailed test for multiple comparisons.

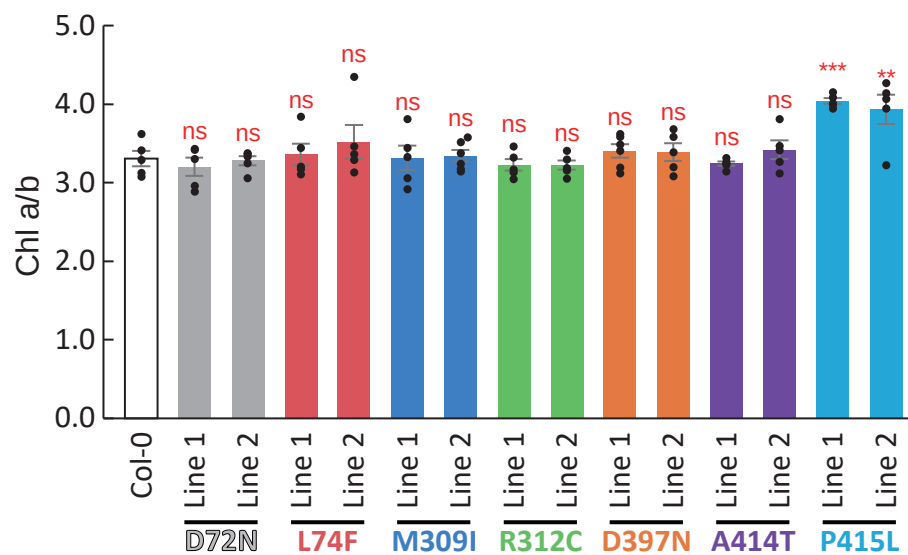


Fig. S7 Chlorophyll a/b ratio in different lines. Data is mean $\pm$ SE. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n = 6 plants. Dunnett's two-tailed test for multiple comparisons.

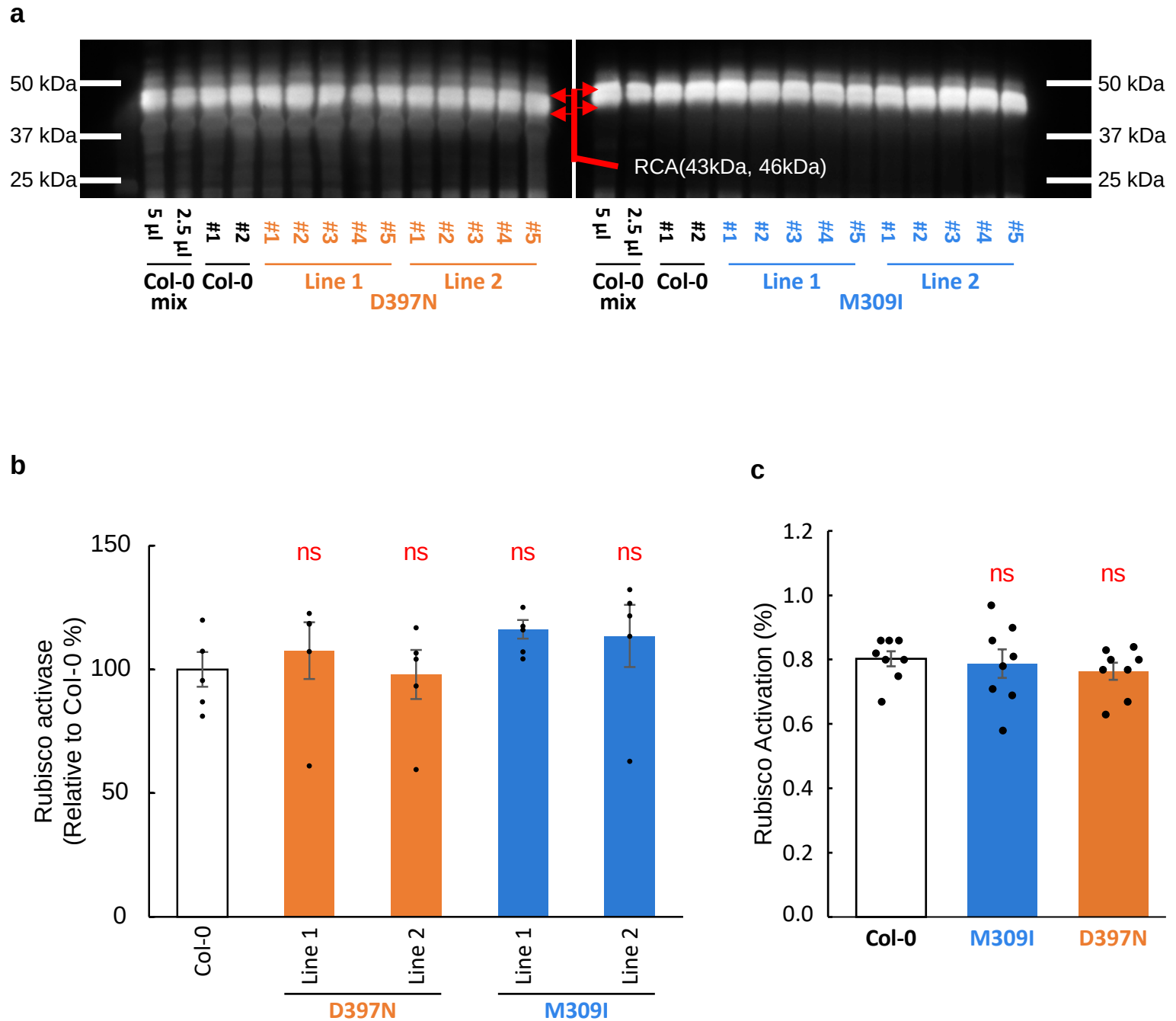


Fig. S8 Analysis of Rubisco activase expression in M309I and D397N single mutants. **a**, Western blots targeting Rubisco activase (RCA). Western blots were probed with polyclonal antibodies raised against rice Rubisco activase, and visualized using alkaline phosphatase conjugated to a secondary antibody. Two RCA isoforms, with molecular weights of 43 kDa and 46 kDa, were detected. Except for the lane labeled “Col-0 mix 2.5 μ L”, 5 μ L of protein solution was loaded into each well. **b**, Relative amount of Rubisco activase against Col-0 (normalized to 100%) in different lines. Data are means $\pm$ SE, $n = 4$ (for Col-0) or 5 (for each different line) biological replicates. NS, not significant by Dunnett's two-tailed test for multiple comparisons. **c**, Rubisco activation state of Col-0, M309I, and D397N. Data are means $\pm$ SE, $n = 4$ (for Col-0) or 5 (for each different line) biological replicates. NS, not significant by Dunnett's two-tailed test for multiple comparisons.

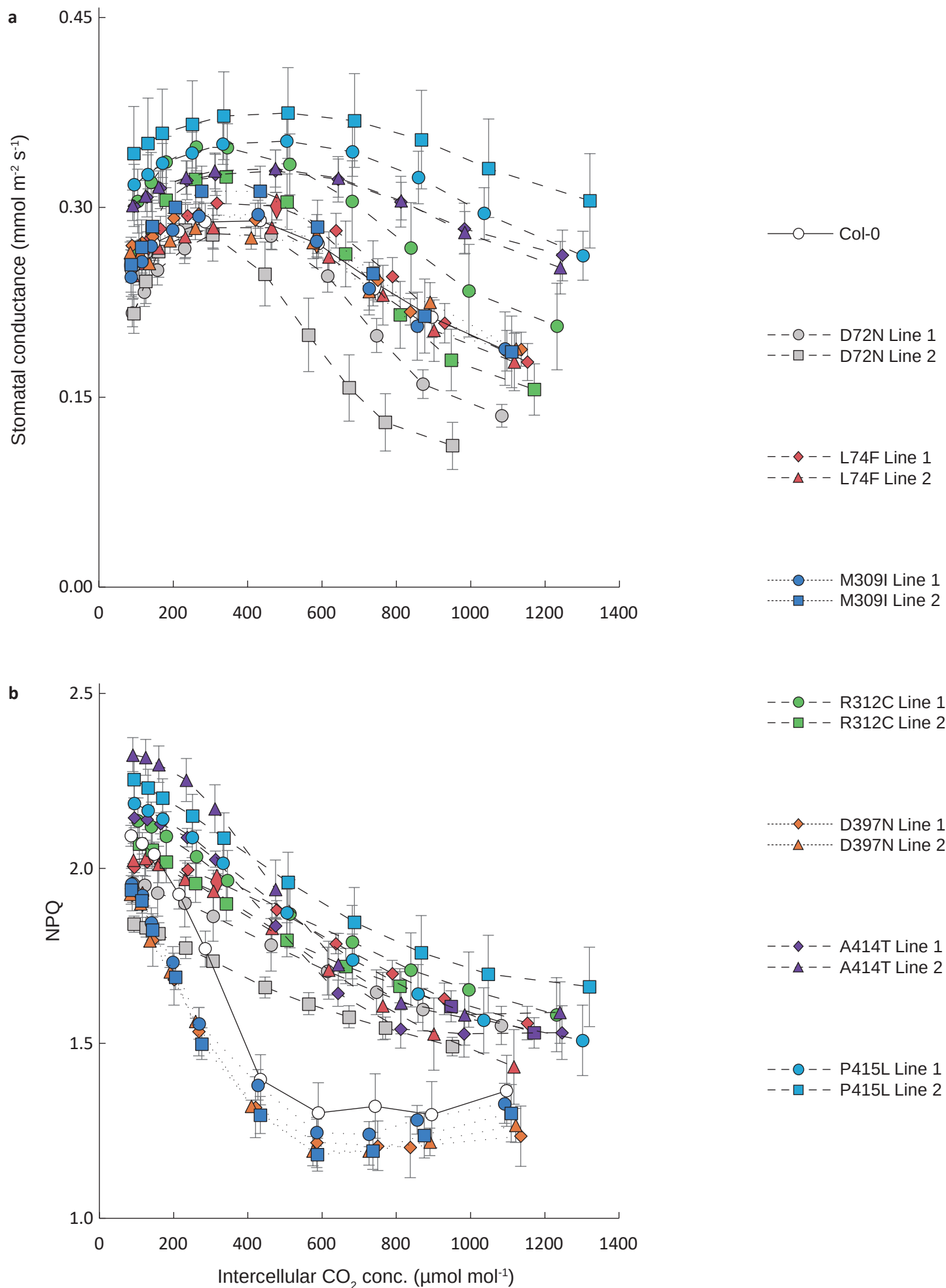


Fig. S9 Photosynthesis parameters in various intercellular CO_2 concentrations of different lines.

a, Stomatal conductance of different lines in various intercellular CO_2 concentrations. **b**, NPQ of different lines in various intercellular CO_2 concentrations. Data is mean $\pm$ SE. Individual data points were displayed as black dots. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 6$ plants. Dunnett's two-tailed test for multiple comparisons.

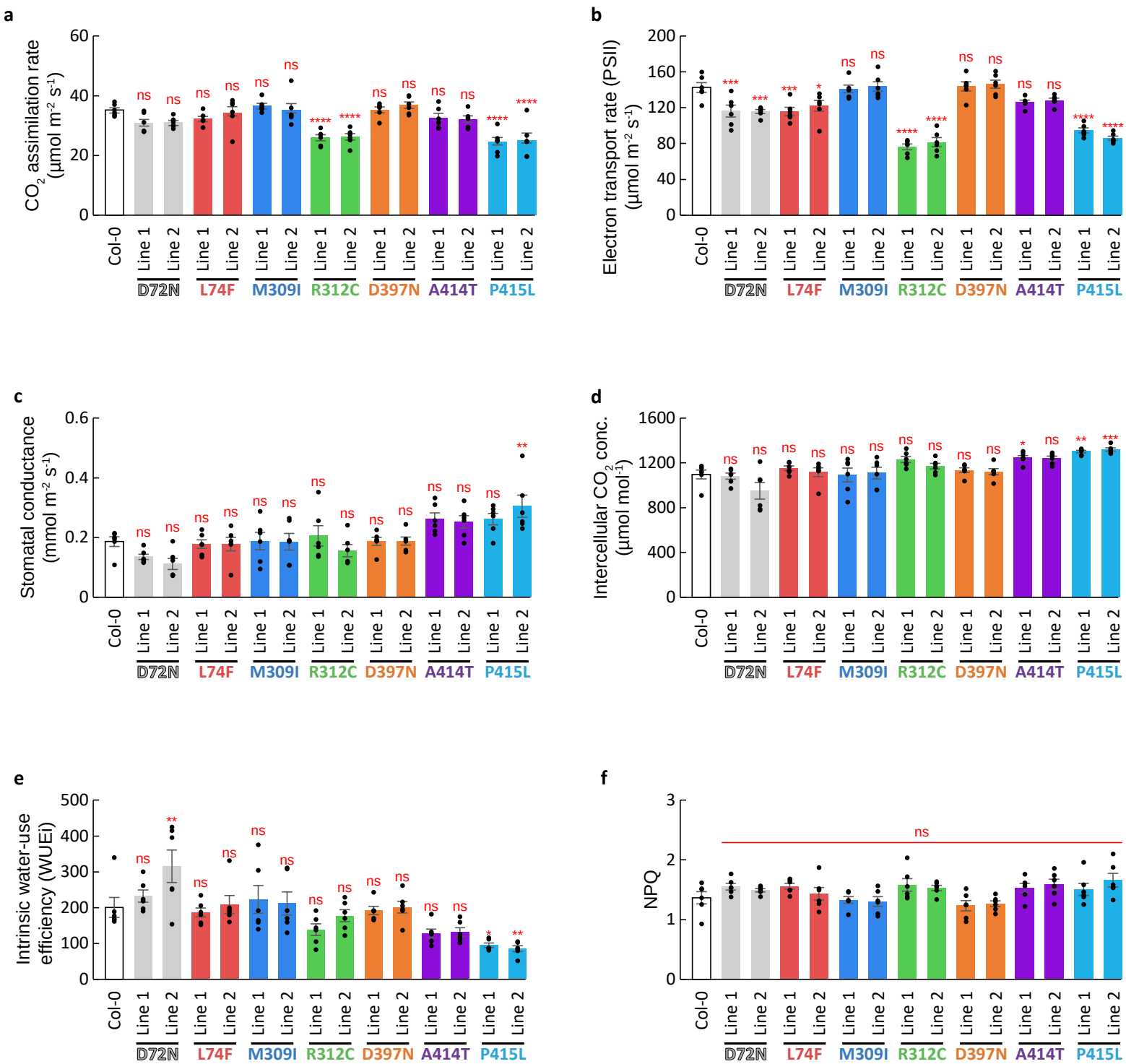
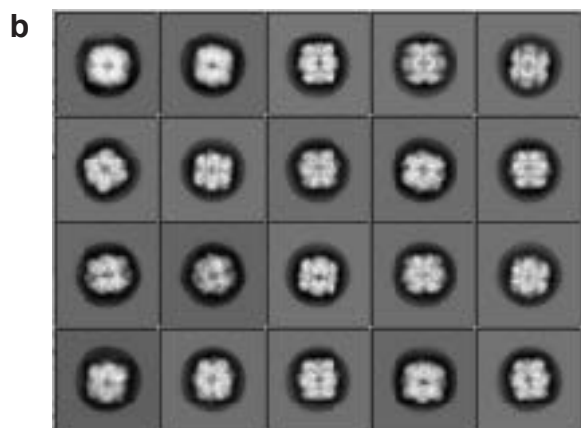
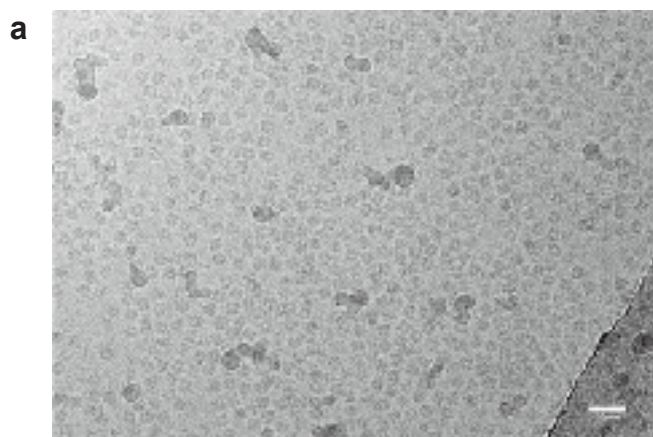


Fig. S10 Photosynthesis parameters in 1200 μmol mol⁻¹ CO₂ of different lines. **a**, CO₂ assimilation rate. **b**, Electron transport rate of PSII. **c**, Stomatal conductance. **d**, Intercellular CO₂ concentration. **e**, Intrinsic water-use efficiency (WUEi). **f**, NPQ. Data is mean ± SE. Individual data points were displayed as black dots. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n = 6 plants. Dunnett's two-tailed test for multiple comparisons.



c 2,732 movies

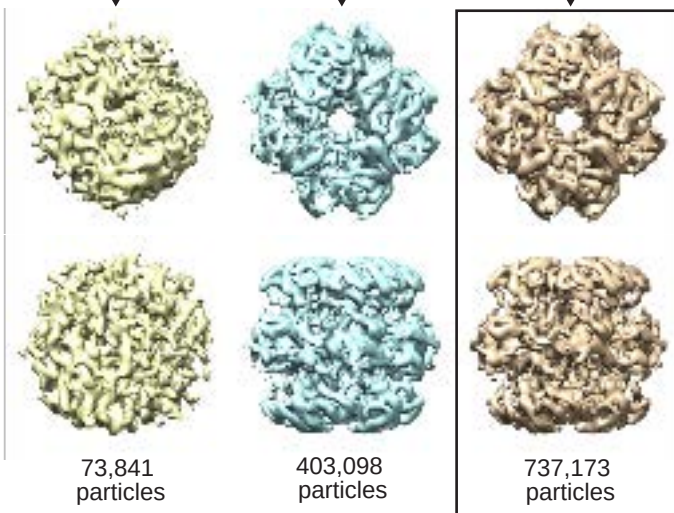
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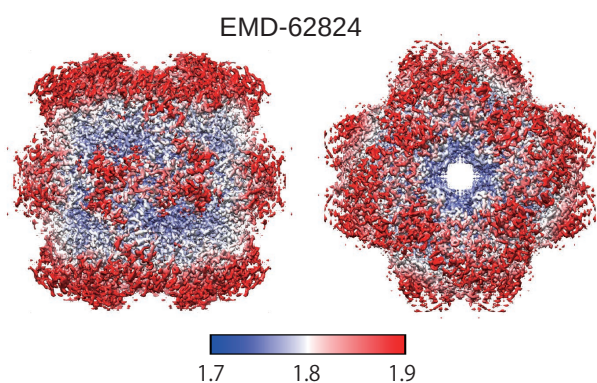
Extract with 4x binning
2D classification (by Relion)

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Ab-initio reconstruction



Re-extract without binning
Refine3D with C1 symmetry
CTF Refinement, Polish



Refine3D with
D4 symmetry

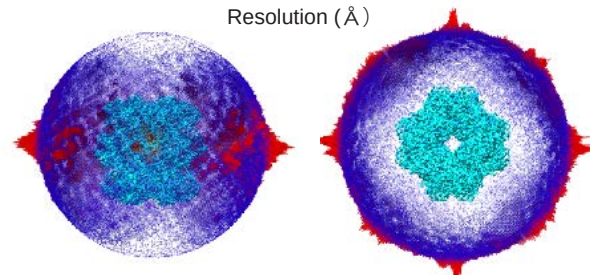
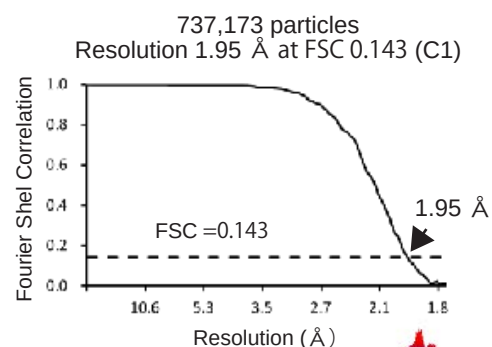
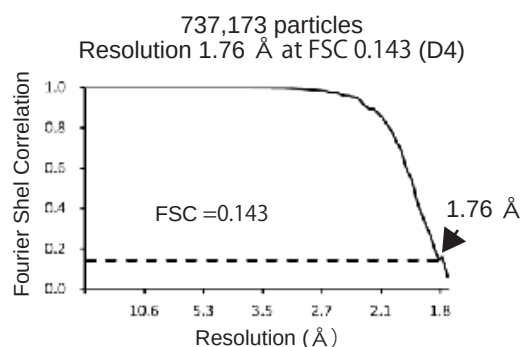
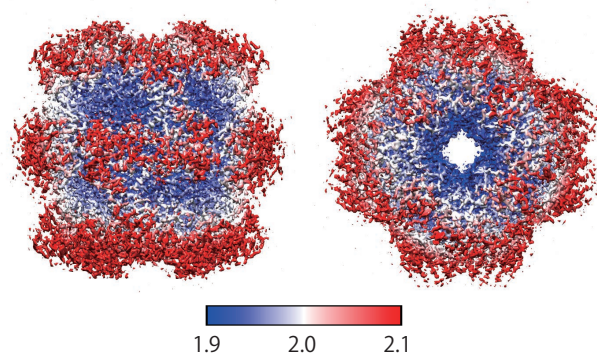


Fig. S11 Cryo-EM single-particle 3D image analysis of the Col-0 Rubisco. **a**, Representative cryo-EM image of Col-0 Rubisco. Bar = 50 nm. **b**, Reference-free two-dimensional averages of Col-0 Rubisco. **c**, Work process of Cryo-EM single-particle 3D reconstruction of Col-0 Rubisco and Fourier Shell Correlation (FSC) curves of the final density maps of Col-0 Rubisco reconstructed with C1 symmetry. The local resolution of the maps is indicated by a color gradient. Euler angular distribution of particles contributing to the final reconstruction is shown at the bottom. The number of views at each angular orientation is represented by length and color of cylinders where red indicates a greater number of views.

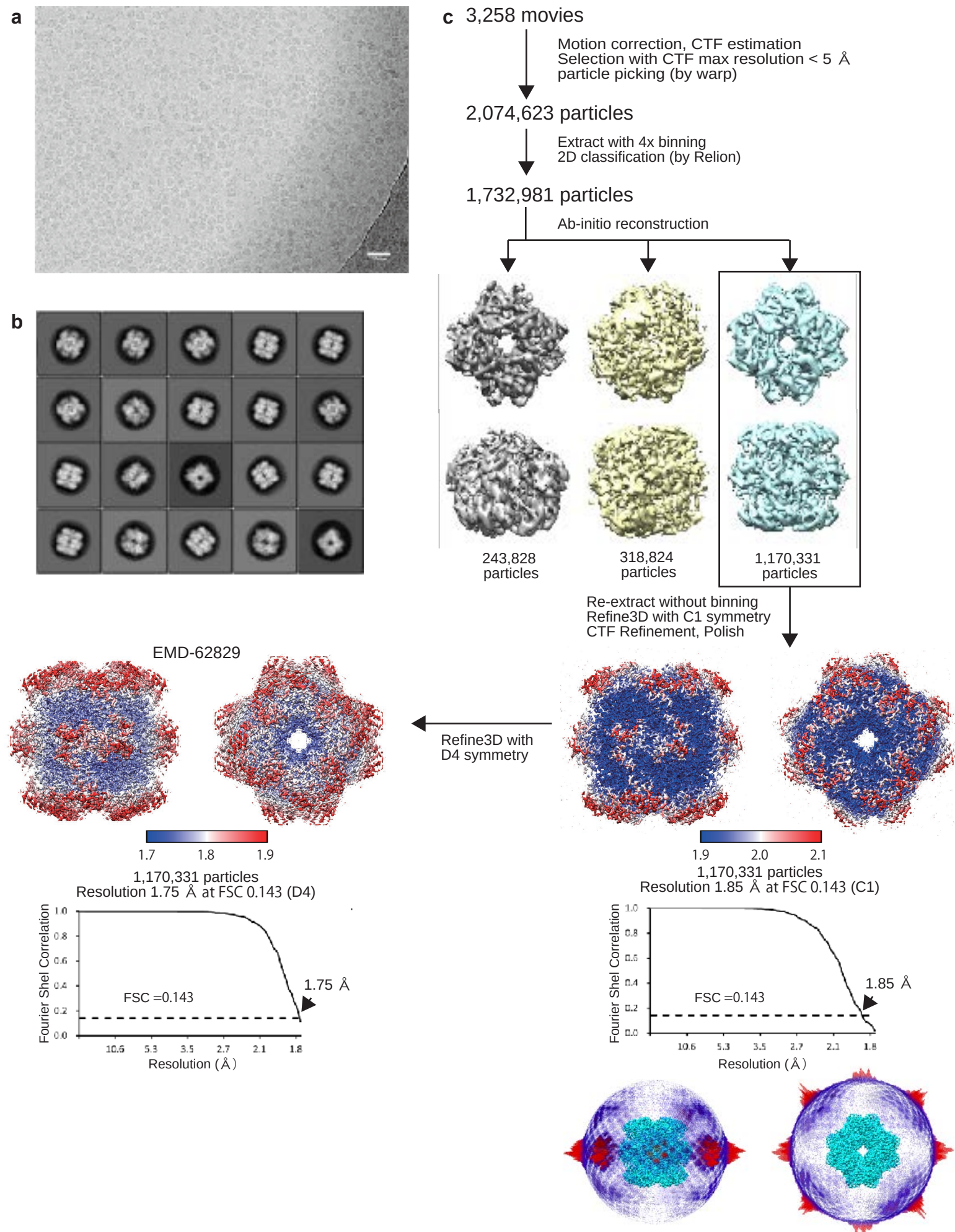


Fig. S12 Cryo-EM single-particle 3D image analysis of the M309I Rubisco. **a**, Representative cryo-EM image of M309I Rubisco. Bar = 50 nm. **b**, Reference-free two-dimensional averages of M309I Rubisco. **c**, Work process of Cryo-EM single-particle 3D reconstruction of M309I Rubisco and Fourier Shell Correlation (FSC) curves of the final density maps of M309I Rubisco reconstructed with C1 symmetry. The local resolution of the maps is indicated by a color gradient. Euler angular distribution of particles contributing to the final reconstruction is shown at the bottom. The number of views at each angular orientation is represented by length and color of cylinders where red indicates a greater number of views.

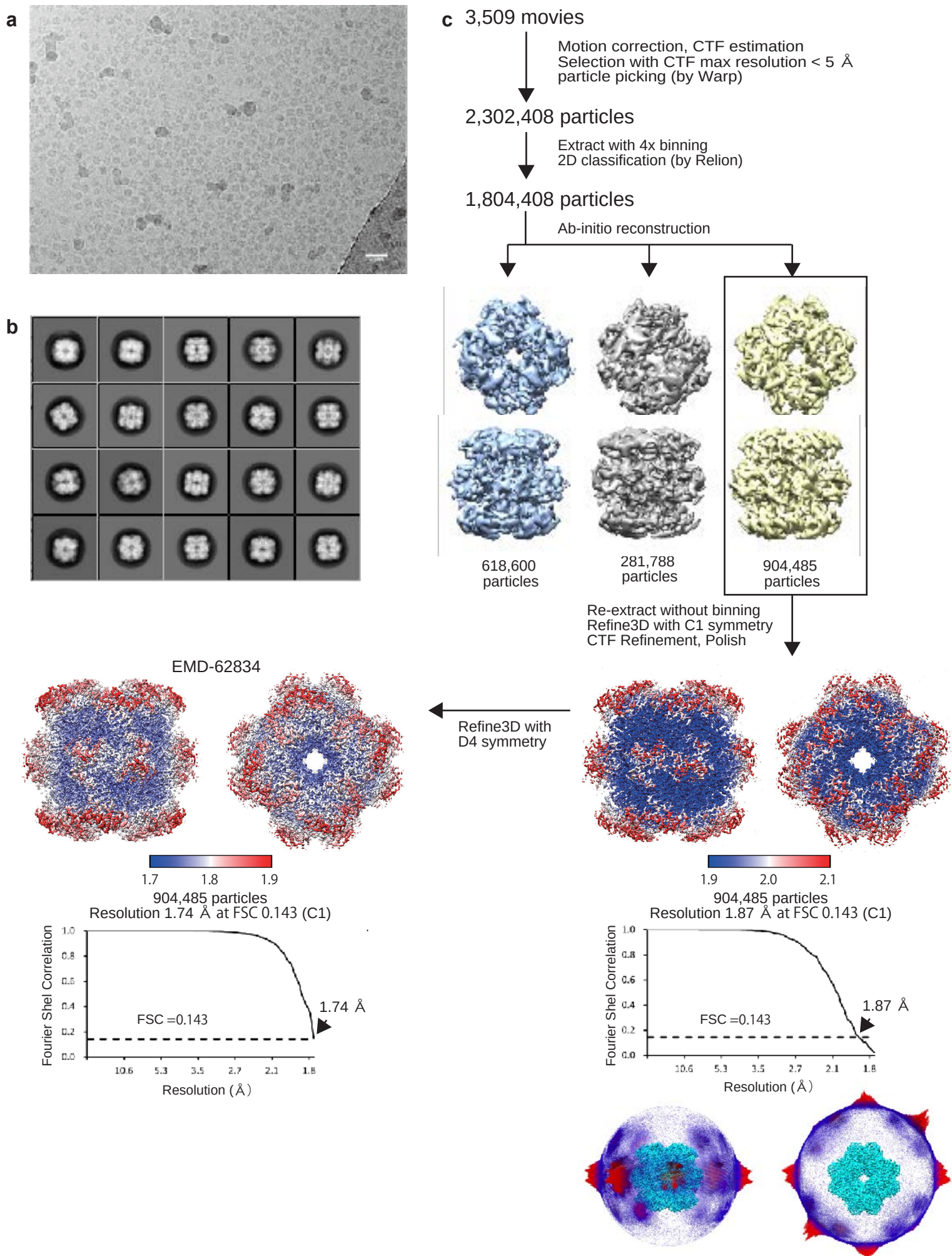


Fig. S13 Cryo-EM single-particle 3D image analysis of the D397N Rubisco. **a**, Representative cryo-EM image of D397N Rubisco. Bar = 50 nm. **b**, Reference-free two-dimensional averages of D397N Rubisco. **c**, Work process of Cryo-EM single-particle 3D reconstruction of D397N Rubisco and Fourier Shell Correlation (FSC) curves of the final density maps of D397N Rubisco reconstructed with C1 symmetry. The local resolution of the maps is indicated by a color gradient. Euler angular distribution of particles contributing to the final reconstruction is shown at the bottom. The number of views at each angular orientation is represented by length and color of cylinders where red indicates a greater number of views.

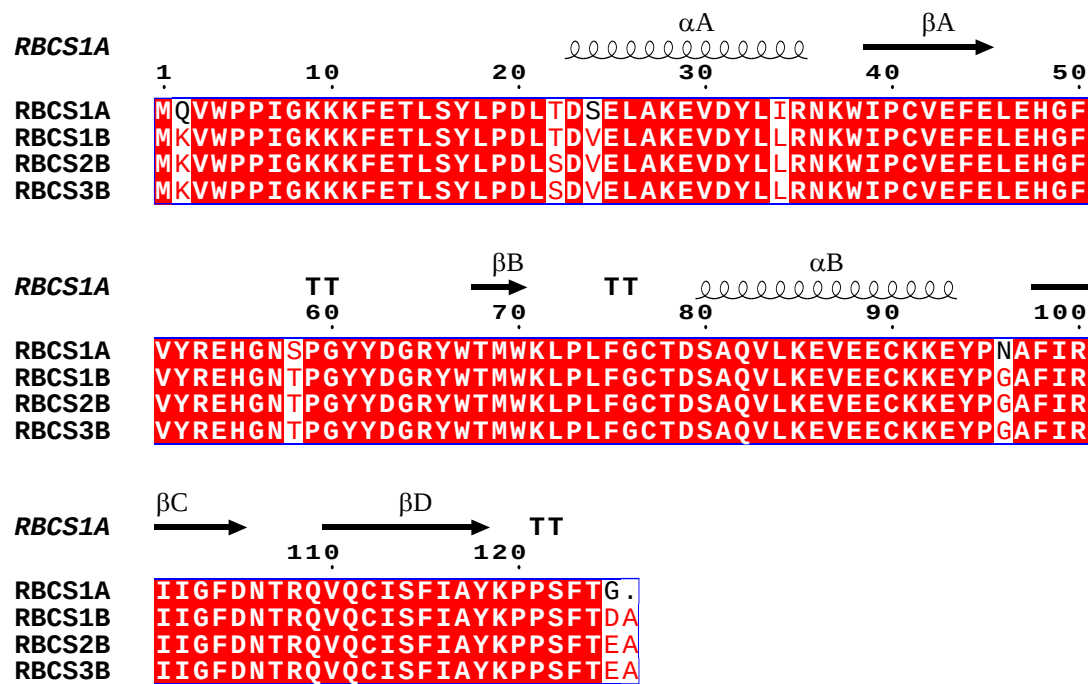


Fig. S14 Sequence alignment of RbcS isoforms in *A. thaliana*. The amino acid sequences of RBCS1A, RBCS1B, RBCS2B, and RBCS3B are provided in GenBank accession number AEE34594.1, BAB09355.1, AAO29974.1, and AAL47390.1, respectively. Secondary structural elements are indicated above the amino acid sequence by arrows and coils for β -strands and α -helices, respectively. In addition, amino acids conserved across all four isoforms are highlighted with a red background.

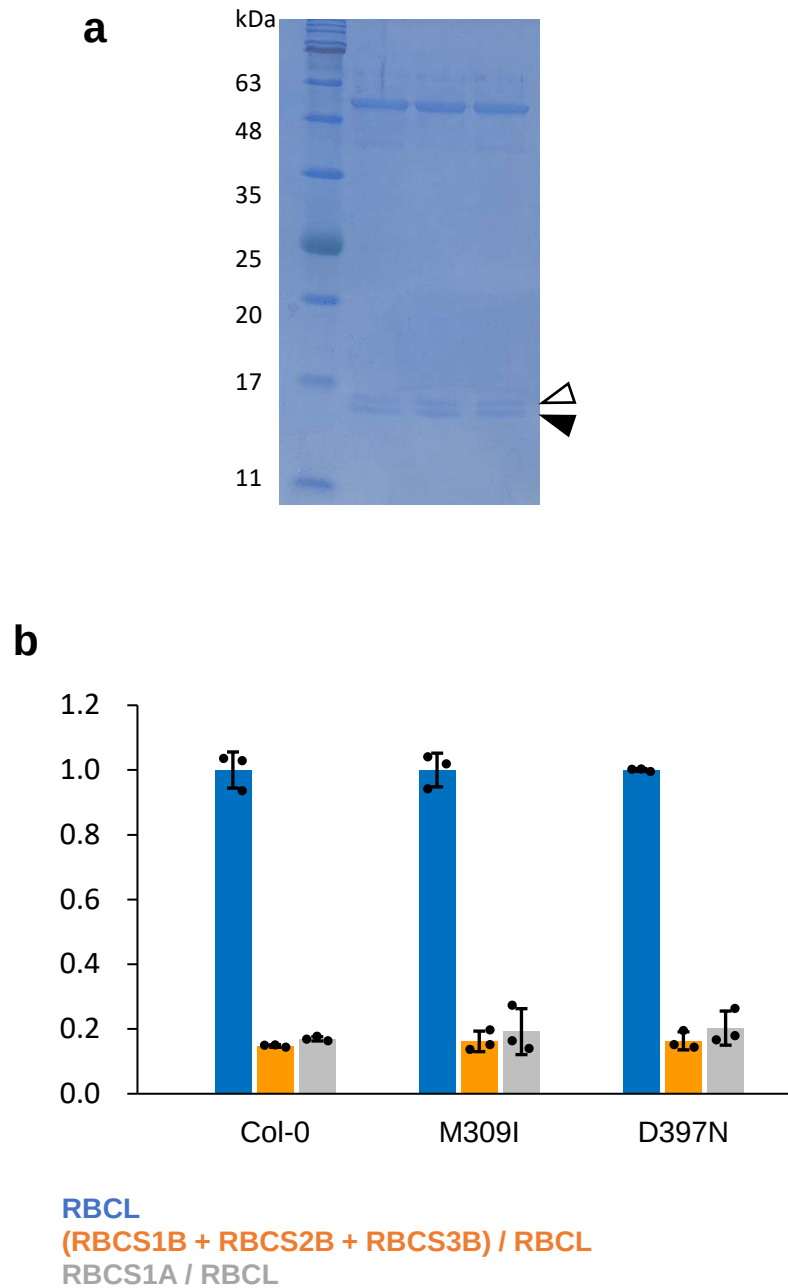


Fig. S15 SDS-PAGE analysis of *A. thaliana* Rubisco purified from Col-0, M309I, and D397N leaves. **a**, A representative SDS-PAGE image of Col-0, M309I, and D397N Rubisco. The white arrowhead indicates the RBCS1B, RBCS2B, and RBCS3B proteins, while the black arrowhead indicates RBCS1A, as reported by Izumi *et al* 2012 (Ref. 23). **b**, Ratios of RBCS protein band intensities to RBCL proteins. The blue bars represent the relative amount of RBCL (normalized to 1.0). The ratios of RBCS1B, RBCS2B, and RBCS3B proteins to RBCL are shown in orange, while the ratios of RBCS1A proteins to RBCL are shown in grey. Band intensities were quantified using ImageJ version 1.53q. Data represent the average of three independent experiments, with error bars indicating standard deviation.

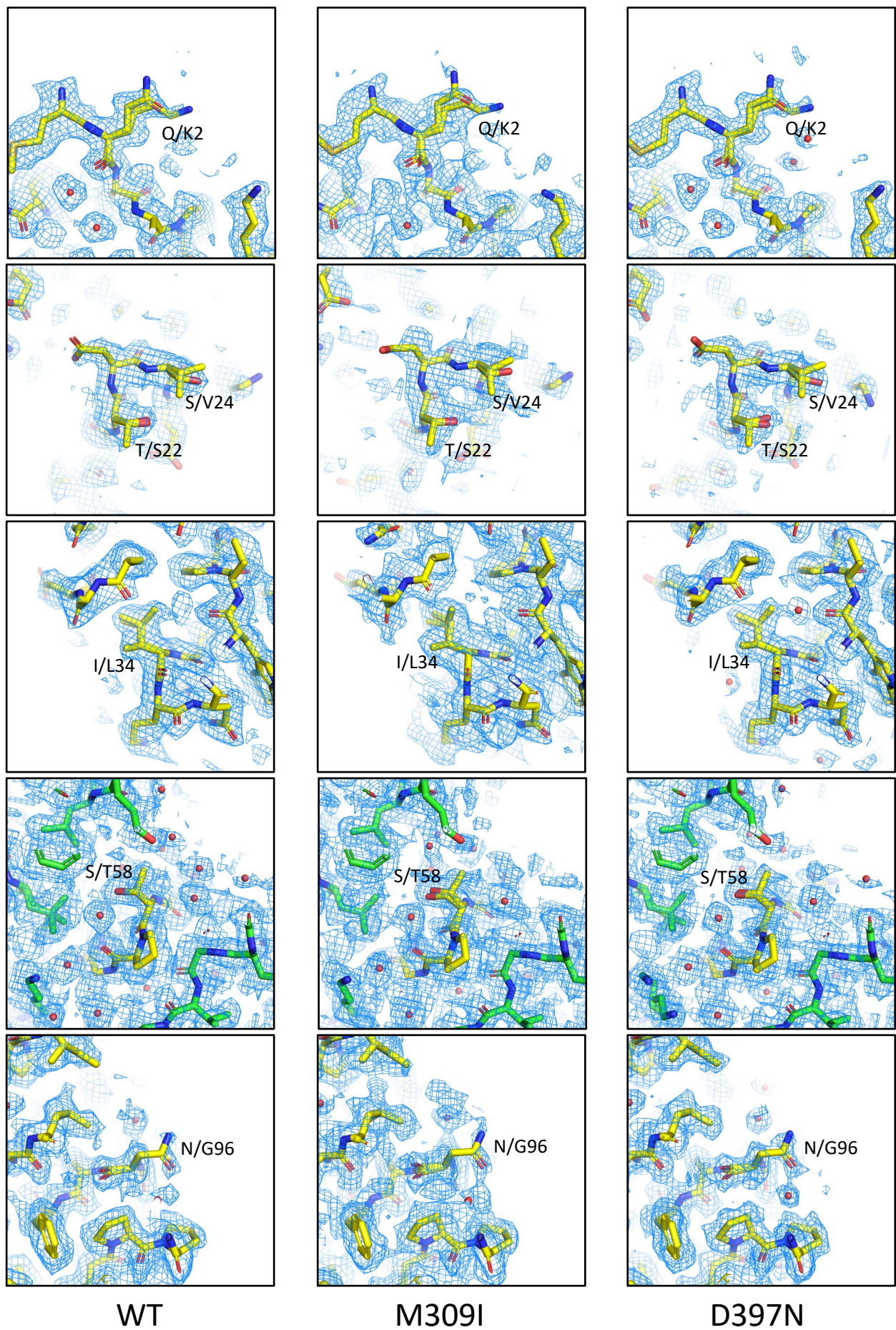
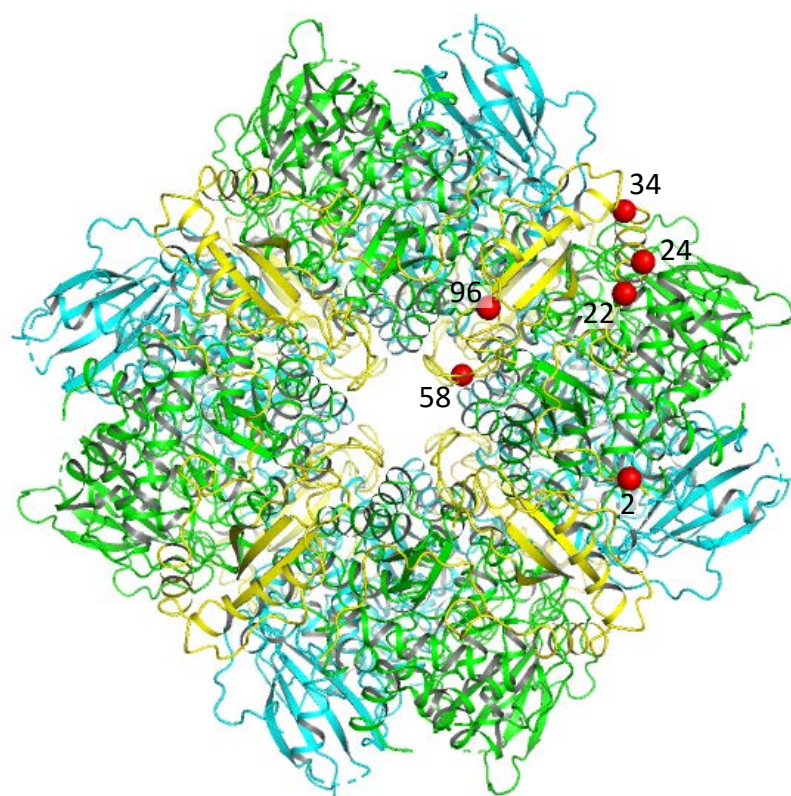


Fig. S16 Cryo-EM density maps of *A. thaliana* RbcS1A/RbcS2B (RbcS3B). Cryo-EM density maps contoured at 1.5 σ around the amino acid residues that differ among RbcS isoforms. Maps of Col-0, M309I, and D397N are shown in the left, middle, and right panels, respectively. The amino acid residues are annotated based on the RbcS1A/RbcS2B (RbcS3B) sequence (Fig. S14).



↻ -90°

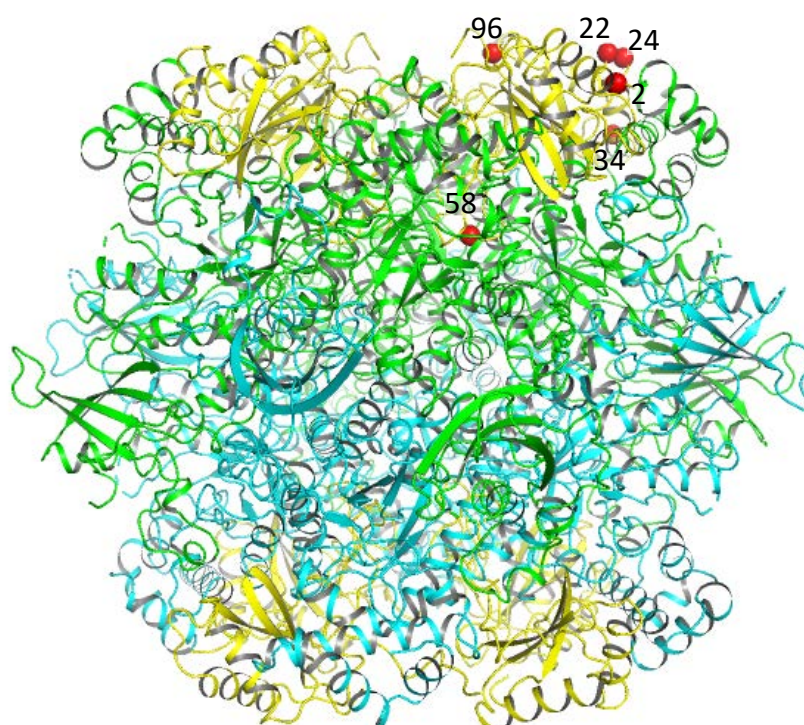


Fig. S17 Locations of the residues 2, 22, 24, 34, 58, and 96. The relative locations of the Ca atoms of the amino acids are shown as red spheres.

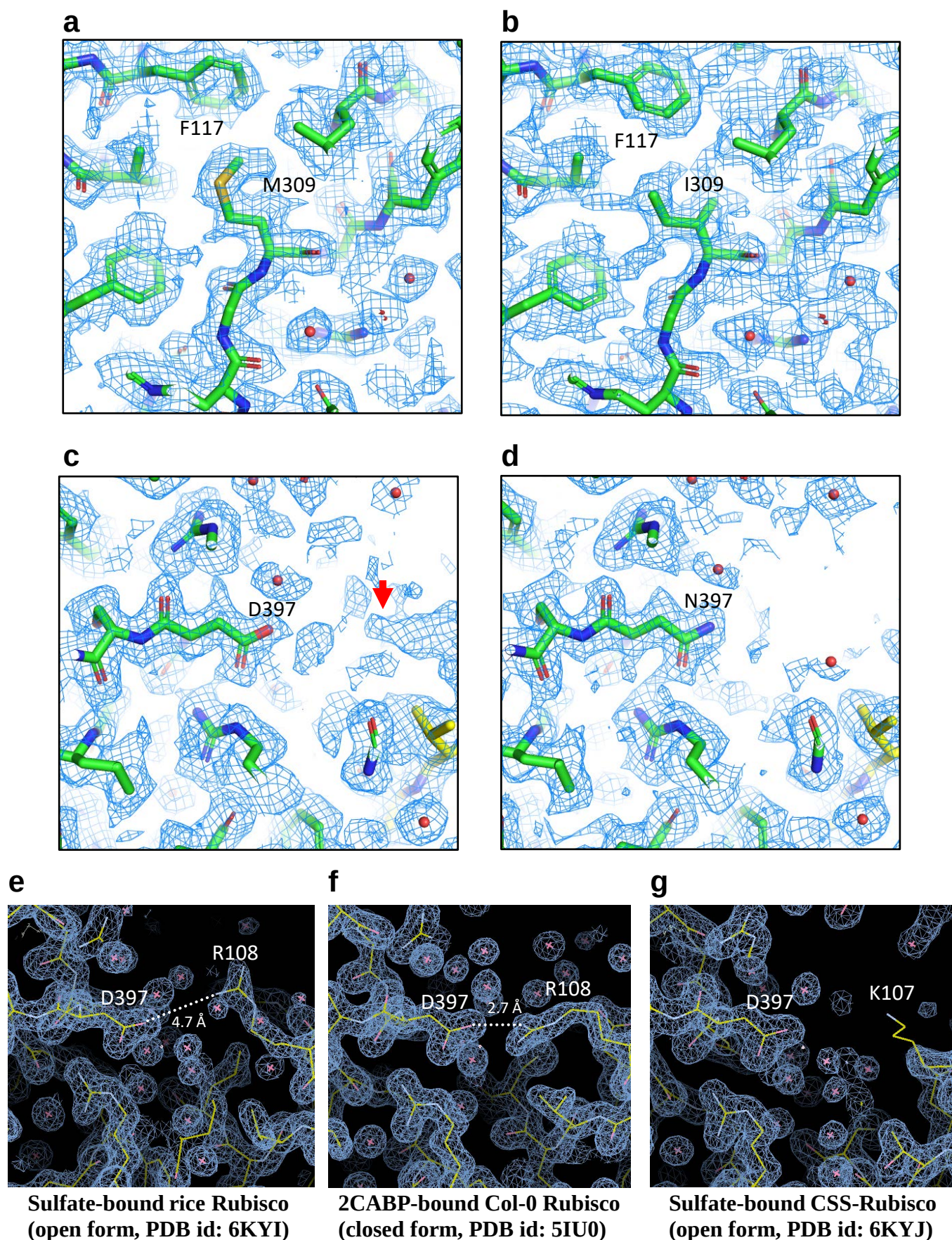


Fig. S19 Cryo-EM density maps around M309I and D397N. **a, b**, The cryo-EM density maps around M309/I309 contoured at 1.5 σ for Col-0 (a) and M309I (b). The orientation of the side chains of F117 is different between (a) and (b). **c, d**, The cryo-EM density maps around D397/N397 contoured at 1.5 σ for Col-0 (c) and D397N (d). **e**, The crystal structure and the 1.5 σ electron density map of sulfate-bound rice Rubisco (open form, PDB ID: 6KYI) around D397. **f**, The crystal structure and the 1.5 σ electron density map of 2CABP-bound Col-0 Rubisco (closed form, PDB ID: 5IUO) around D397. **g**, The crystal structure and the 1.5 σ electron density map of sulfate-bound CSS-Rubisco composed of sorghum RbcS and rice RbcL (open form, PDB ID: 6KYJ) around D397.

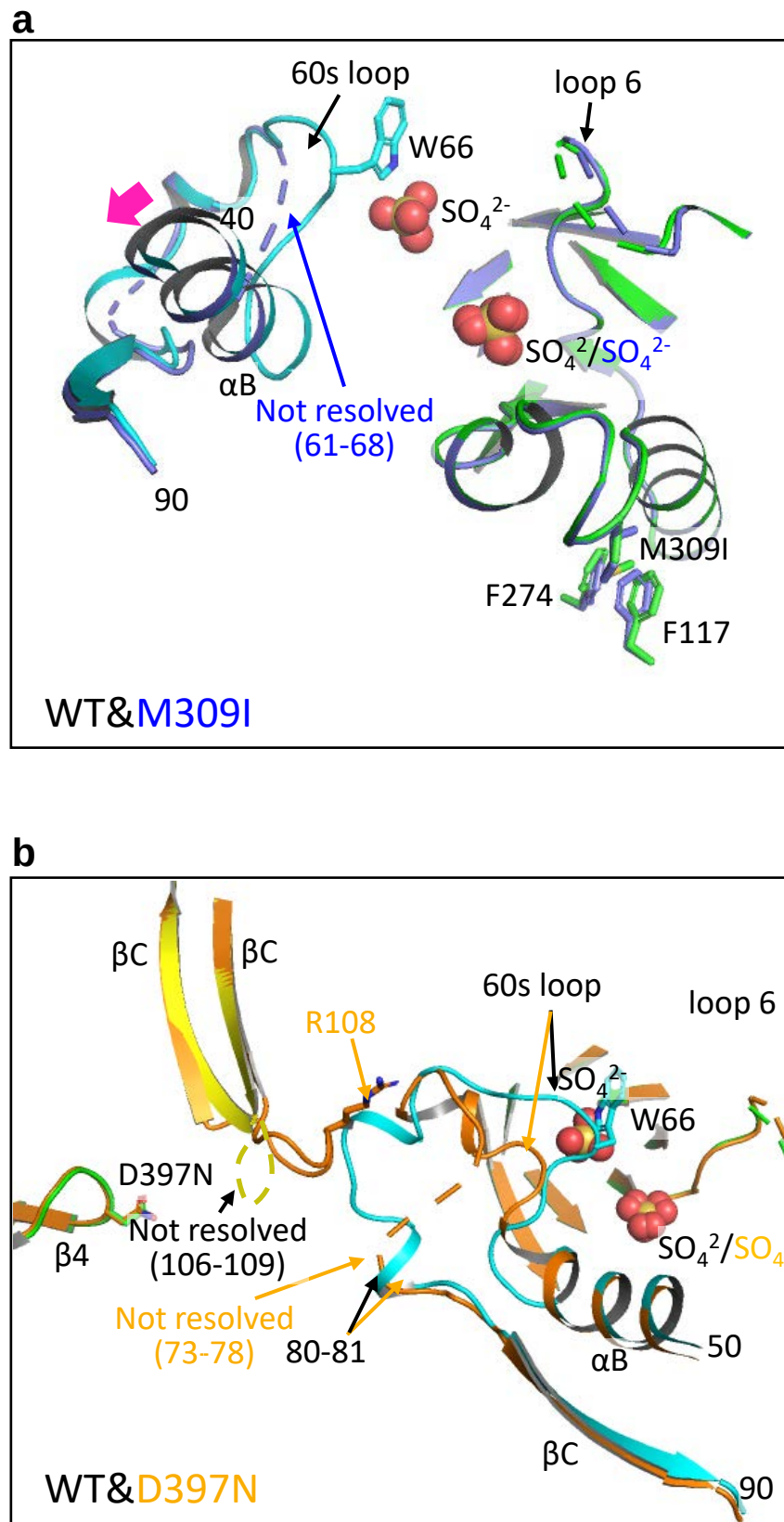


Fig. S20 Superimposition of the active site structures of the *A. thaliana* Rubisco in complex with sulfate ions.

a, Rubisco structure around the 60s loop and loop 6 in Col-0 (green and cyan) and M309I (blue). The magenta arrow indicates a shift of approximately 0.5 Å in the αB helix. Key interacting residues and regions are labeled. **b**, Rubisco structure around the 60s loop and loop 6 in Col-0 (green, cyan, and yellow) and D397N (orange). Black and orange arrows indicate the partial structures of Col-0 and D397N Rubisco, respectively.

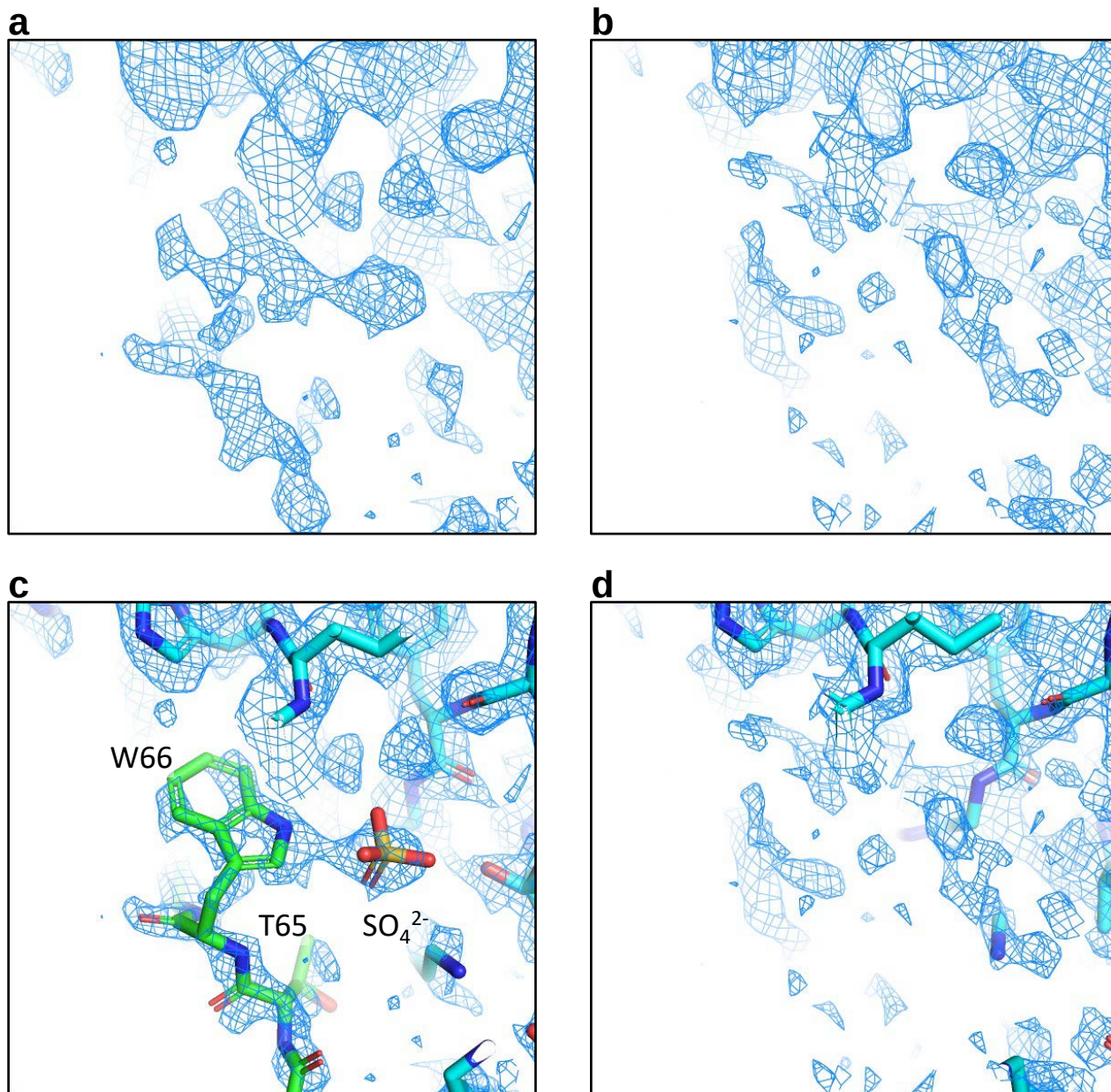


Fig. S21 Differences in cryo-EM density maps around the 60s loop between Col-0 and M309I Rubisco. a, b, The cryo-EM density maps contoured at 2.0 σ for Col-0 (a) and M309I (b). **c, d,** Fitness of residues in the 60s loop in Col-0 (c) and M309I (d). The cryo-EM density maps of Col-0 allowed fitting all portions of the 60s loop, while the region corresponding to residues 61–68 in this loop could not be fitted due to lower cryo-EM density.

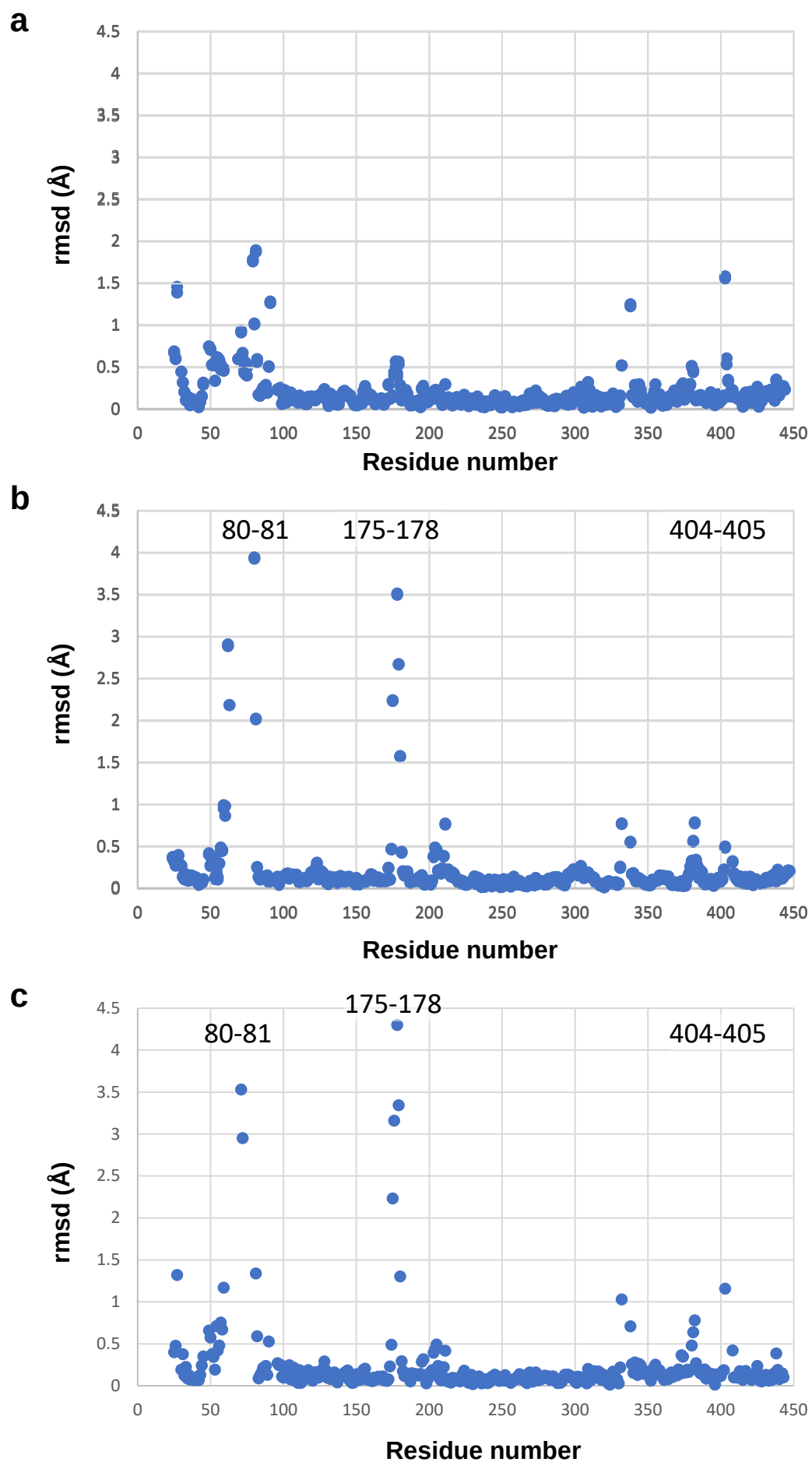


Fig. S22 Backbone C α atoms rmsd of RbcL. a-c, Rmsd of RbcL between Col-0 and M309I (a), Col-0 and D397N (b), and M309I and D397N (c)

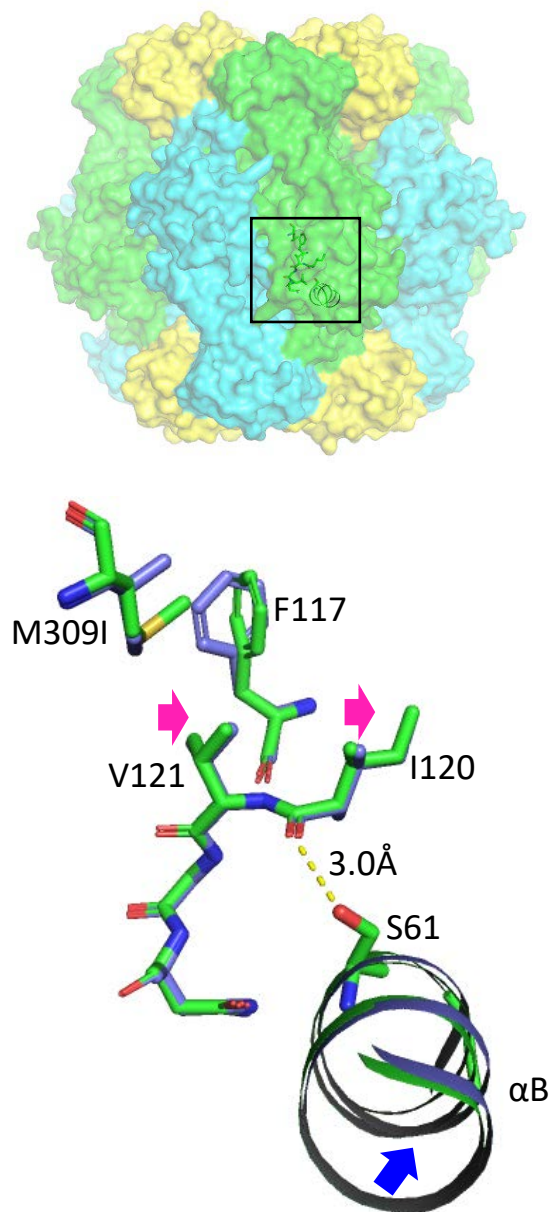


Fig. S23 Hypothetical mechanism of structural changes induced by the M309I substitution.

Superimposed structures of Col-0 (green) and M309I (blue) Rubisco are depicted. The overall structure of Rubisco is shown at the top, while the magnified view of the region surrounding the α B helix is presented at the bottom. The magenta arrows indicate potential structural shifts caused by the substitution of M309 to I.

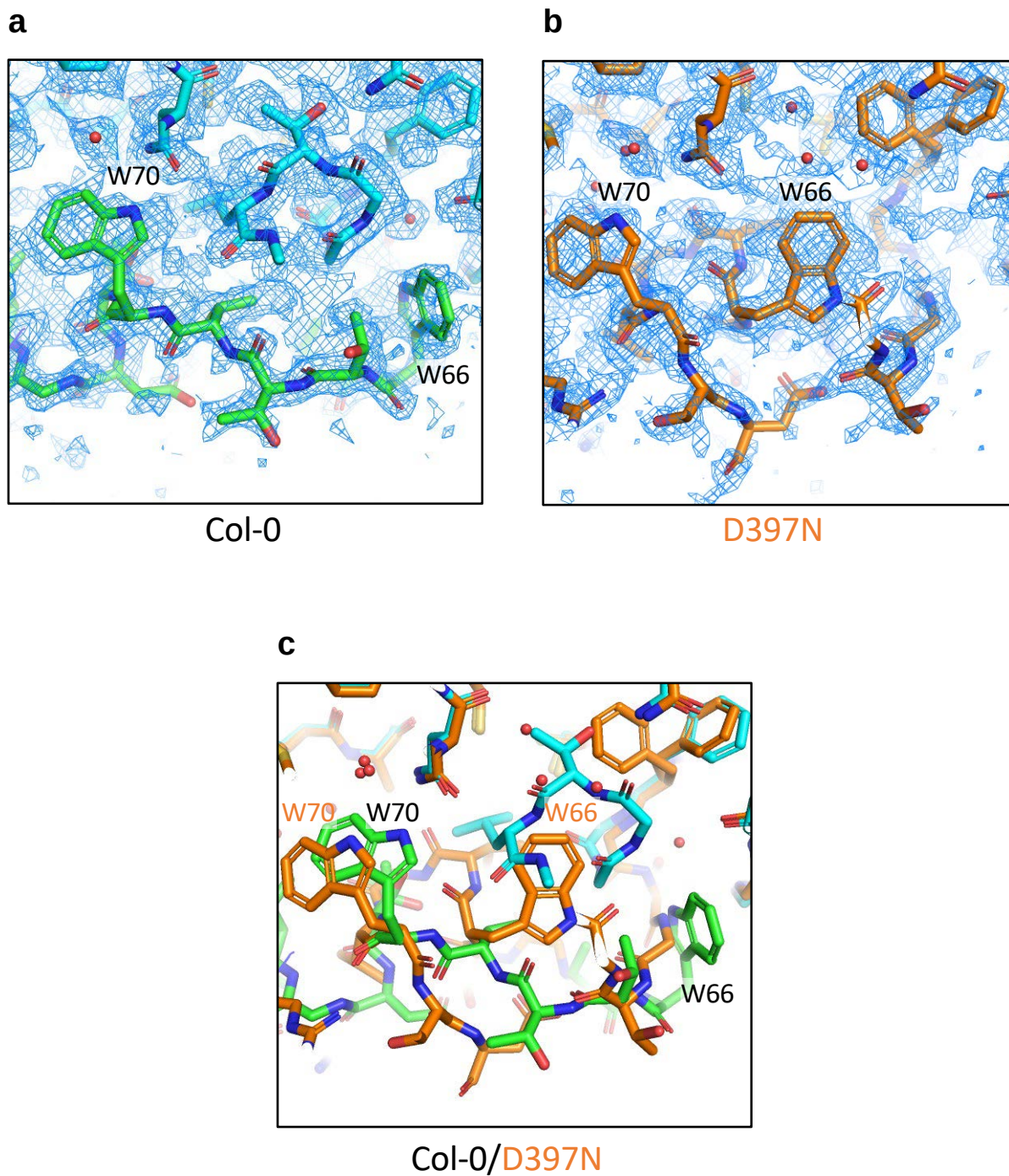


Fig. S24 Differences in cryo-EM density maps around the 60s loop between Col-0 and D397N Rubisco. **a, b,** The cryo-EM density maps contoured at 1.5 σ for Col-0 (**a**, green) and D397 (**b**, orange). **c**, Superimposition of the Col-0 and D397N Rubisco structures around the 60s loop.

Supplementary Text

Chloroplast genome editing of Rubisco boosts photosynthesis and plant growth

Authors

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† These authors contributed equally.

Supplementary Text

The underlying reason for structural changes observed near the 60s loop in the M309I mutant remains uncertain. Superimposition of Col-0 and M309I RbcLs gave relatively small root mean square deviations (rmsd) (0.302 Å for 414 C α atoms, Fig. S22). M309 is located at the dimer interface, forming a hydrophobic cluster with residues F117 and F274. Compared to the Col-0 structure, M309I structure showed that the substitution of M309 to I induces rotations in side chains of F117, along with a shift of approximately 0.5 Å in the α B helix (blue arrow in Fig. S23). The shift in the α B helix likely triggers a transition from ordered to disordered structure in the 60s loop of M309I (Fig. S21). The following discussion is necessarily speculative, as it concerns subtle structural differences. Considering the structural differences between M309I and Col-0, several local structural features can be noted around F117 and the adjacent regions, including the “not resolved” 60–68 region. In both Col 0 and M309I, M309 or I309 makes van der Waals contact with the side chain of F117. Consequently, the M309I substitution is associated with a change in the rotameric orientation of the side chain of F117, and the shortest carbon–carbon distance within the hydrocarbon atoms of the F117 and V121 side chains is approximately 3.9 Å, which is just barely sufficient to maintain van der Waals contact between these residues. In addition, although small (approximately 0.1–0.3 Å), a shift is observed in the 120–121 region (indicated by magenta arrows in Fig. S23). In Col-0, the backbone carbonyl of I120 forms a hydrogen bond with the S61 side chain, and S61 is located at the C-terminus of the α B helix, while the 60–68 region of M309I is “not resolved”. Taken together, these findings define a structural context that may be consistent with altered conformational behavior toward the 60–68 region, although the present data do not allow definitive conclusions regarding the functional or mechanistic significance of these features.

The rmsds of RbcL between Col-0 and D397N are relatively large (0.425 Å for 405 C α atoms, Fig. S22). We carefully fitted the D397N model into the cryo-EM density map, as the region around the 60s loop notably differed from the Col-0 structure (Fig. S24). The question arises as to how the D397N substitution induces structural changes

in the 60s loop. In the sulfate-bound rice Rubisco (open form, PDB id: 6KYI), which represents a structural state similar to that observed in our current study, the distance between D397 of RbcL and R108 of RbcS is 4.7 Å (Fig. S19e). In the current structures, R108 of WT could not be modeled because the density is insufficiently resolved to interpret it; however, some density is present in the region where R108 may be partially located (red arrow in Fig. S19c). In contrast, no such density is observed at this position in the D397N mutant (Fig. S19d). These observations imply that the D397N substitution weakens the interaction with R108, which, in turn, may trigger structural changes in the 60s loop. In the 2CABP-bound Col-0 Rubisco (closed form, PDB id: 5IU0), D397 forms a salt bridge with Arg108 at a distance of 2.7 Å (Fig. S19f). Again, in the sulfate-bound rice Rubisco (open form, PDB id: 6KYI), the distance between D397 and R108 is 4.7 Å (Fig. S19e). These findings suggest that the salt bridge between D397 and R108 is weakened during the structural transition from the closed to the open state. Notably, in the sulfate-bound hybrid Rubisco (CSS-Rubisco) composed of sorghum RbcS and rice RbcL (open form, PDB id: 6KYJ), which exhibits an increased k_{cat} , the residue corresponding to R108 is a lysine (K107 in sorghum numbering, Fig. S19g); therefore, the interaction between residues 397 of RbcL and 108 of RbcS is inherently weaker. This tentatively suggests a potential correlation between a weaker salt bridge and a higher k_{cat} , though further careful evaluation is needed.

Supplementary References

61. Knight, S., Andersson, I. & Brändén, C.-I. Crystallographic analysis of ribulose 1, 5-bisphosphate carboxylase from spinach at 2· 4 Å resolution: Subunit interactions and active site. *Journal of molecular biology* **215**, 113–160 (1990).