nature portfolio

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Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐	☒	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	No custom software was used. Peptides were analyzed by nano-LC-MS/MS using a Waters CSH C18 column online to a Thermo Orbitrap Eclipse mass spectrometer. Spectra were searched using Mascot Server (v2.7.0, www.matrixscience.com) with a fragment ion mass tolerance of 0.060 Da and a parent ion tolerance of 15.0 ppm. Searches were performed against the Pisum sativum v1a annotated protein database (57,835 entries) and a common contaminants database cRAP_20150130.fasta (www.thegpm.org/crap/). Peptide and protein identifications were validated using Scaffold (v5.3.3, www.proteomesoftware.com).
Data analysis	No custom software was used. FIJI 2.16.0, CellProfiler (v4.2.7), CellProfiler Analyst (v3.0.4), Python (v3.12.6) using seaborn (v0.13.2), pandas, and matplotlib (v 3.8.4), Perseus v2.1.3.0, Metascape v3.5, MCSdatabase v 1.7, InteredgeDistance macro (v1.3), AlphaFold v3 with PPM 3.0, GraphPad Prism v10.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The proteomics data generated in this study have been deposited in the ProteomeXchange Consortium database via the PRIDE partner repository under accession code 10.6019/PXD065686. The confocal and transmission electron microscopy data generated in this study have been deposited in the Figshare database under accession code https://doi.org/10.6084/m9.figshare.31290871. Plasmids generated in this study have been deposited in Addgene, under the accession codes 224830 to 224858. All other data supporting the findings of this study are available within the Article and its Supplementary Information files.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	This information has not been collected.
Reporting on race, ethnicity, or other socially relevant groupings	This information has not been collected.
Population characteristics	This information has not been collected.
Recruitment	This information has not been collected.
Ethics oversight	This information has not been collected.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	For microscopy, we ensured robust biological replication by sampling non-overlapping, high-quality images that captured all unique transformants within the optimal imaging window. To ensure statistical representation, we established a target of at least 100 chloroplasts per investigated protein, a threshold consistent with standard quantitative imaging practices. Ultimately, all samples exceeded this minimum count due to the efficiency of the imaging pipeline. For proteomic analysis, we initially targeted six independent biological replicates, each derived from separate growth and fractionation cycles. Following the exclusion of low-yield samples to maintain data stringency (see Methods), the final dataset comprised n=4 to n=6 replicates per fraction. These sample sizes provided sufficient statistical power to resolve distinct proteomic profiles and identify significant differences in protein abundance using permutation-based FDR control.
Data exclusions	For the proteomics analysis, to ensure comparability across samples, the two lowest-yielding heavyIE and lightThy samples (based on total spectral counts) were excluded from downstream analysis. The final dataset consisted of 19 samples: four inner envelope (IE), five heavyIE, four thylakoid (Thy), and six light thylakoid (lightThy) replicates.
Replication	All experiments were performed using multiple biological replicates across at least two independent growth cycles. For transient expression assays, localization was assessed at various time points post-transformation; observations were confirmed in at least three independent transformation experiments. Early time points were selected specifically to mitigate potential artifacts or mislocalization often associated with prolonged protein overexpression.
Randomization	Experimental groups were determined by biological similarity (same genotype, biological fraction, etc.) No population studies are included. Similarly, we controlled key covariates experimentally and remaining sources of variation were handled through standardized workflows and replication rather than post-hoc statistical adjustment.
Blinding	Fluorescence microscopy data was coded by the original experimenter numerically, and analyzed by a second experimenter using Cell Profiler (blinded). Electron microscopy was similarly coded by the original experimenter, then analyzed by a team of secondary experimenters (blinded). Final data for each experiment was graphed by the original experimenters (non-blinded).

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☐	☒ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Dual use research of concern

Policy information about dual use research of concern

Hazards

Could the accidental, deliberate or reckless misuse of agents or technologies generated in the work, or the application of information presented in the manuscript, pose a threat to:

No	Yes
☒	☐ Public health
☒	☐ National security
☒	☐ Crops and/or livestock
☒	☐ Ecosystems
☒	☐ Any other significant area

Experiments of concern

Does the work involve any of these experiments of concern:

No	Yes
☒	☐ Demonstrate how to render a vaccine ineffective
☒	☐ Confer resistance to therapeutically useful antibiotics or antiviral agents
☒	☐ Enhance the virulence of a pathogen or render a nonpathogen virulent
☒	☐ Increase transmissibility of a pathogen
☒	☐ Alter the host range of a pathogen
☒	☐ Enable evasion of diagnostic/detection modalities
☒	☐ Enable the weaponization of a biological agent or toxin
☒	☐ Any other potentially harmful combination of experiments and agents

Plants

Seed stocks	Homozygous T-DNA insertion lines tvpfp (SALK_095443.29.45.x) and pmfp (SALK_149886.49.50.x) were obtained from the Arabidopsis Biological Resource Center.
Novel plant genotypes	N/A
Authentication	PCR-based verification of T-DNA insertion sites (Supplementary Figure 5).