

PLANT SCIENCES

A multiomics atlas of the *Arabidopsis* nuclear envelopeHeejae Nam^{1†}, Doogie Kim^{1†}, Yu Tang^{1†}, Yiling Fang¹, Maryam Haghani², Krishna V. Dhulipalla², Marvin Chen¹, Song Li², Yangnan Gu^{1*}

The nuclear envelope (NE) is a critical regulatory interface that preserves genome integrity and mediates nucleocytoplasmic transport, yet its compositional and functional landscape in plants remains poorly defined. Here, we present a multiomics atlas of the *Arabidopsis* NE. By integrating proximity-labeling proteomics with single-cell/single-nucleus transcriptomics, we uncover distinct NE coexpression networks that dynamically shape NE composition and function in cell type-specific manners, enabling adaptation to diverse physiological and environmental demands. Notably, this analysis revealed that nuclear pore complex (NPC) biogenesis is transcriptionally restricted primarily to proliferative cells, whereas the protein complex exhibits remarkable stability and persists throughout cellular differentiation despite the absence of sustained high-level transcription. This supports an “assembly-and-maintenance” model in which NPCs are predominantly assembled during cell division and subsequently maintained as exceptionally long-lived structures throughout developmental progression. Together, this atlas provides a high-resolution framework for plant nuclear organization and establishes the NE as a spatiotemporally regulated hub central to plant growth, development, and responses to stimuli.

INTRODUCTION

The nuclear envelope (NE) encases the cell nucleus as a double membrane structure comprising the inner nuclear membrane (INM) and outer nuclear membrane (ONM), separated by the perinuclear space that is continuous with the endoplasmic reticulum (ER) lumen (1). This architecture maintains nuclear shape through interactions with the underlying nuclear lamina, a meshwork of intermediate filament proteins. In addition, it organizes perinuclear chromatin organization via chromatin-NE attachments and mediates nucleocytoplasmic transport through both passive diffusion and active transport mechanisms (2–4).

The NE harbors diverse membrane-embedded proteins and protein assemblies, with the linker of nucleoskeleton and cytoskeleton (LINC) complex being a notable example that spans the perinuclear space through SAD1/UNC-84 DOMAIN (SUN) proteins in the INM and Klarsicht, ANC-1, Syne Homology (KASH) domain proteins in the ONM. LINC complexes form structural bridges connecting the nucleoskeleton (and chromatin) to the cytoskeleton, thereby enabling force transmission across the nuclear boundary and ensuring proper nuclear positioning, mechanotransduction, and mechanical stability during cellular movements and development (5, 6). At hundreds of distinct sites, the INM and ONM converge to form nuclear pores through local fusion events, where the massive nuclear pore complex (NPC) assembles as a ~110- to 125-MDa supramolecular structure. Each NPC contains multiple copies of ~30 to 40 different nucleoporins (Nups) organized into distinct subcomplexes—the inner ring, the outer ring, FG-repeat Nups forming the permeability barrier, cytoplasmic filaments, and the nuclear basket—arranged in an eightfold radial symmetry around a central transport channel of ~40 to 60 nm in diameter (7–12). This intricate macromolecular assembly, among the largest protein complexes in eukaryotic cells, regulates the selective bidirectional trafficking of RNAs, proteins, and other macromolecules between the nucleus and cytoplasm (13–15). Beyond LINC and NPC,

the NE harbors numerous additional proteins and protein complexes that together establish an intricate molecular network, positioning the nuclear membrane as a central regulatory hub that integrates structural stability, chromatin organization and modification, signal transduction, and nucleocytoplasmic transport (16–26).

In *Arabidopsis*, nucleoskeleton proteins, including CROWDED NUCLEI (CRWNs), function as master regulators of nuclear architecture (27–31). These proteins, together with INM proteins such as PLANT NUCLEAR ENVELOPE TRANSMEMBRANE 2 (PNET2), orchestrate the genome organization by establishing plant lamina-associated domains (LADs) and tethering specific chromatin domains to the nuclear periphery, thus modulating transcriptional accessibility through chromatin compaction and epigenetic modifications (32–37). Meanwhile, the plant NPC is emerging as a central hub integrating diverse signaling pathways. Nearly one-third of known nucleoporins have been reported to play essential roles in plant responses to specific environmental stimuli, including photoperiod, temperature fluctuations, drought, salinity, pathogen attack, and wound-induced tissue regeneration (19, 20, 31, 36, 38–44). Recent evidence demonstrates that the plant NPC functions as an integrated transcriptional factory by spatially concentrating chromatin remodeling complexes, transcriptional activators, RNA polymerase II machinery, and mRNA processing factors all at the nuclear basket, creating microenvironments that facilitate cotranscriptional mRNA processing and quality control to enable rapid transcriptional reprogramming (45).

Despite these recent advances in plants, our comprehensive understanding of the NE protein composition and function remains largely confined to humans and yeasts, where ~300 to 400 NE-localized proteins have been identified, establishing extensive functional networks and regulatory pathways (46–51). In contrast, the characterized NE proteome in other eukaryotic lineages, including plants, remains considerably smaller and less well-defined, limiting our ability to understand how different organisms have adapted NE composition and functions to meet specific physiological and environmental demands (52, 53). Many known plant NE proteins exhibit profound divergence from their functional counterparts in mammals and fungi, making traditional comparative genomics approaches unsuitable for systematic identification of plant NE proteins (16, 54–57). This necessitates alternative methodologies such as proteomics, which

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themselves face substantial technical challenges: The intrinsic association of the NE with the ER, combined with the abundance of plant-specific membrane organelles such as plastids, complicates the extraction of pure plant NE for proteomic profiling, even with modified biochemical fractionation approaches (52, 58). Consequently, efforts to assemble an accurate plant NE proteome have been historically impeded by nonspecific protein contamination and complicated data interpretation.

Mutations in *Arabidopsis* NE-associated genes frequently result in tissue-specific developmental defects (40, 59–61), suggesting that many NE components are tissue specific and likely form distinct protein interaction networks and functional modules tailored to meet functional demands of different plant organs and cell types. Therefore, the systematic exploration of tissue-specific NE composition, particularly through advanced proteomics and tissue/cell type-resolved analyses, represents a critical and largely unexplored frontier in plant NE biology research.

In this study, we used an optimized proximity-labeling proteomics approach using a broad set of NE-resident proteins as baits, generating a comprehensive map of the NE-associated proteome in *Arabidopsis* seedlings. This approach identified ~700 distinct proteins, including a rich array of plant lineage-specific NE integral and associated proteins. By integrating proteomic data with recently published single-nucleus (snRNA-seq) and single-cell RNA sequencing (scRNA-seq) datasets, we revealed spatiotemporal expression patterns of NE components and their assembly into functional modules in a tissue- and cell type-specific manner, underscoring their specialized roles in developmental plasticity and environmental responsiveness. Most notably, we found that nucleoporin genes exhibit predominant and clustered expression in proliferative tissues and cells, followed by sustained protein persistence during cell differentiation with remarkably long half-lives, suggesting a distinctive assembly-and-maintenance homeostasis model for the NPC. This work provides a foundational resource for future investigations of NE function in plants.

RESULTS

Profiling NE-associated proteins using proximity labeling

In our previous work, we conducted proximity-labeling proteomics using several well-characterized PNET proteins as baits (52). However, the overlap among candidates identified by these PNETs is limited, suggesting that they localize to distinct NE subdomains and could not fully capture the NE proteome. To achieve extensive coverage of NE-associated proteins, particularly at the INM side that contains NE-specific components, we strategically selected CRWN proteins as alternative baits for proximity labeling because they are functional analogs of the human lamins and are uniformly distributed beneath the INM, where they maintain close associations with INM components (30, 62). These properties make CRWN proteins ideal for comprehensive profiling of the plant NE-associated proteome (Fig. 1A). In addition, we used TurboID as the labeling enzyme due to its enhanced catalytic efficiency and faster kinetics relative to earlier versions such as BioID.

In our exploration to determine the optimal TurboID orientation for proximity labeling, we initially fused it to either the N- or C-terminus of CRWN1 (fig. S1A). Unexpectedly, when expressed under the native promoter, neither of these fusions could complement the altered nuclear morphology phenotype characteristic of *crwn1* mutants, a critical indicator of CRWN1 functionality (fig. S1B). To overcome

this, we engineered an alternative fusion construct by inserting TurboID into a middle region of CRWN1 (after G899), following its tandem coiled-coil domain (fig. S1A). With comparable expression level to the N- and C-terminal fusion in transgenic plants (fig. S1C), this middle-insertion construct (*pCRWN1::CRWN1-M-TurboID*) successfully complemented the small nucleus phenotype in the *crwn1* mutant (fig. S1B). This indicates that TurboID insertion into an internal permissive region enabled both proximity labeling and tagging without compromising CRWN1 function. We subsequently confirmed that this middle insertion strategy was equally effective for CRWN2 and CRWN3 proteins (fig. S1B), establishing a consistent approach for maintaining CRWN protein function while enabling proximity labeling. However, we were unable to obtain a CRWN4 line with detectable expression or inducible biotinylation; therefore, CRWN4 was excluded from the analysis.

Next, we used 10-day-old *pCRWN::CRWN-M-TurboID* transgenic *Arabidopsis* seedlings to perform proximity labeling (fig. S1C), followed by total protein extraction, affinity purification, and label-free quantitative mass spectrometry (MS) as previously described (52, 63). Biotin-treated wild-type plants were included as control for the selection of specifically probed protein candidates, and three biological replicates were used for each sample. A total of 54, 54, and 90 significantly enriched candidate proteins were identified by CRWN1, CRWN2, and CRWN3, respectively (Fig. 1A and table S1). These include many known INM-associated PNET proteins (e.g., SUN1 and SUN2), nucleoplasmic-facing nuclear pore components (e.g., basket nucleoporins Nup136, Nup82, and Nup50), and other nucleoskeleton proteins (e.g., KAKU4 and GBPL3).

In addition to CRWN1/2/3, we expanded our profiling by including two previously identified PNET proteins, PNET7 and PNET9 (52), as bait for proximity labeling (Fig. 1B). Unlike most other PNETs used in earlier studies, these two baits demonstrated exceptional versatility by effectively labeling proteins located in both the INM and ONM. Notably, PNET7 and PNET9 shared substantial overlap in their probed prey proteins, with 26% common hits (Fig. 1B and table S1), indicative of broad and extensive labeling capacity. Moreover, their proximity-labeling profiles successfully recovered nucleoskeleton proteins, NPC components, and many previously reported or characterized PNET proteins, including PNET8/10/11/13, WIP1/2/3, and WIT1/2. This broad coverage suggests that PNET7 and PNET9 exhibit distinctive mobility or access within the nuclear membrane system, enabling them to label a wide and diverse spectrum of NE-associated proteins.

Assembly of the *Arabidopsis* NE proteome

To generate a comprehensive map of the NE proteome, we integrated these five new proximity-labeling datasets with previously reported PNET proximiomes from distinct NE subdomains, including the INM (SUN1, NEAP1, MAN1, and PNET2_A), the ONM (WIP1, SINE1, and WIT1), and the NPC-nuclear lamina junction (GBPL3 and KAKU4), representing a systematic spatial sampling strategy that captures the architectural complexity of the NE (Fig. 1C). This integration of 14 proteomics datasets yielded an *Arabidopsis* NE proteome comprising 734 proteins (table S2), representing the most comprehensive survey of the plant NE proteome by proximity labeling to date.

Domain prediction revealed that these 734 candidates include 98 membrane proteins with predictable transmembrane (TM) domain(s) and 466 proteins with predicted intrinsically disordered region (IDR) (table S2). We categorized the protein population into

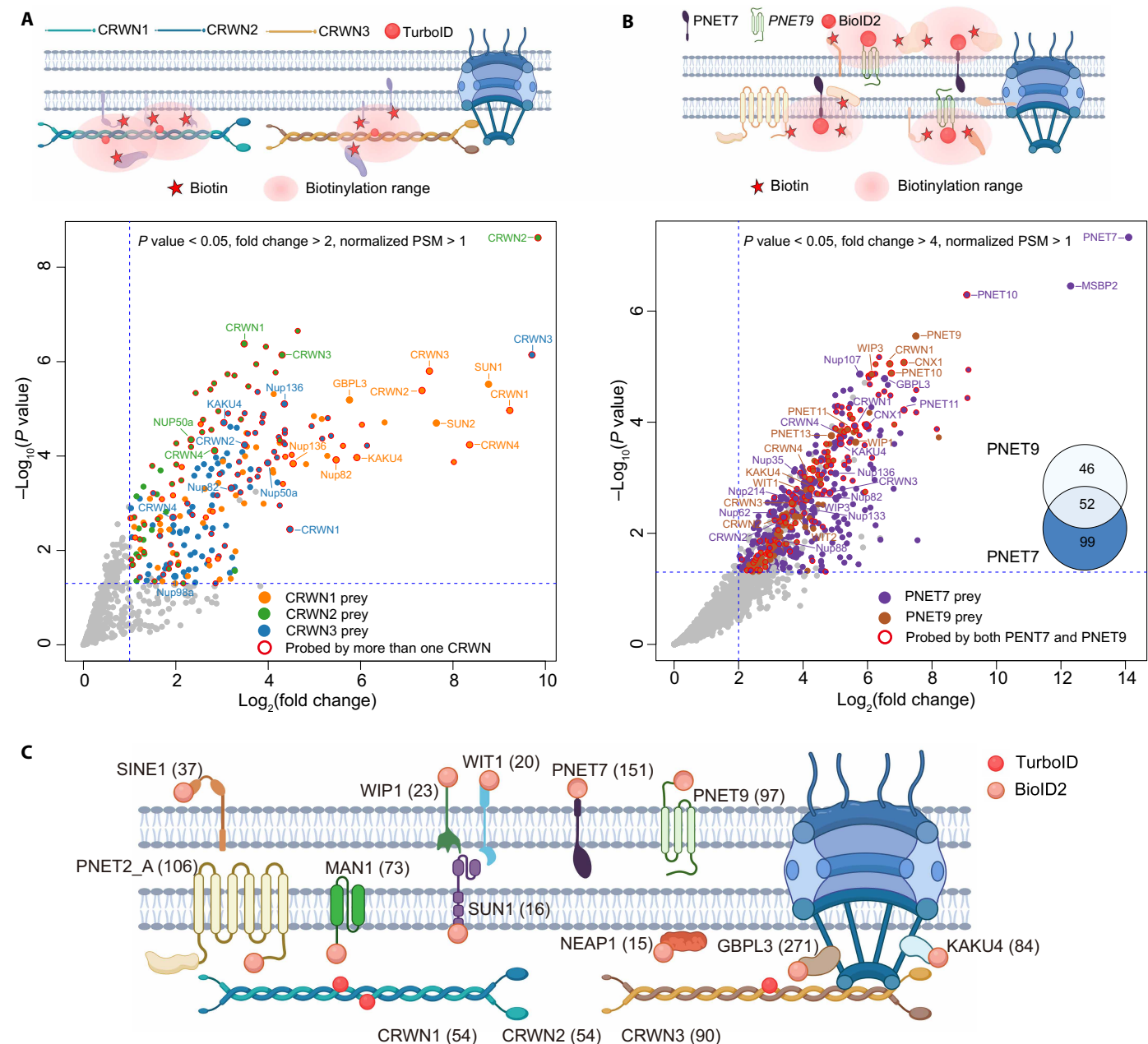


Fig. 1. Assembly of the *Arabidopsis* NE proteome using proximity-labeling proteomics. (A) Schematic illustration of the TurboID-based proximity-labeling strategy using the nuclear lamina protein CRWN1, CRWN2, and CRWN3 as baits to capture NE-associated proteins (top). TurboID tags were inserted into the middle regions of CRWNs. Biotinylated proteins were affinity-purified from 10-day-old *Arabidopsis* seedlings and analyzed by label-free quantitative mass spectrometry. Scatter plots (bottom) display significantly enriched proteins in each CRWN proximiome relative to biotin-treated wild-type controls ($P < 0.05$, fold change > 2 , normalized PSM > 1). Common preys probed by different CRWNs are highlighted by red circles, and known NE components are labeled. (B) Schematic diagram of the proximity labeling using PNET7 and PNET9 as baits (top). These integral NE proteins display broad labeling capacity spanning both the INM and ONM. Scatter plots show proteins significantly enriched in the PNET7 or PNET9 proximiomes ($P < 0.05$, fold change > 4 , normalized PSM > 1). Common preys probed by PNET7 and PNET9 are highlighted by red circles. Known NE components are labeled. (C) Summary of the integrated NE proteome. The five new proximiomes (CRWN1/2/3, PNET7, and PNET9) were combined with nine previously reported NE proximiomes generated using distinct NE proteins as baits (PNET2_A, MAN1, SUN1, NEAP1, WIP1, SINE1, WIT1, GBPL3, and KAKU4), yielding a total of 734 NE-associated proteins. The number of prey proteins identified by each bait is indicated in parentheses.

four groups based on the presence and absence of TM and IDR, both of which have been reported as direct mechanisms facilitating protein targeting and/or suborganellar organization (Fig. 2A). The group harboring both TM and IDR domain contains the highest proportion of previously validated NE integral proteins, suggesting that this domain combination represents a notable feature that may facilitate NE localization in plant cells. We subsequently validated the NE localization of at least six proteins from this category in both transient expression systems and stable transgenic lines and designated these PNET15-20 (Fig. 2B and fig. S2).

To assess the comprehensiveness of NE proteome coverage and to examine the interconnections between different NE subdomains and structures, we constructed a Circos plot integrating all 14 proximity-labeling datasets. In this visualization, shared proteins across proximiomes are linked by connecting lines, highlighting both protein overlap and NE subdomain cross-talk (Fig. 2C). This analysis revealed three key patterns: First, strong intragroup connectivity among structurally and functionally related NE proximiomes, e.g., extensive overlap among nucleoskeletal proteins (CRWNs and KAKU4), indicating near-saturation coverage of their respective protein neighborhoods within detection limits; second, substantial cross-subdomain connectivity, particularly between the NPC basket and nucleoskeletal components as well as between INM proximiomes and nucleoskeletal proteins, consistent with known physical connections among these NE structures; and third, broad connectivity profiles of PNET7 and PNET9 proximiomes, which not only overlapped strongly with each other but also showed extensive connections across multiple NE modules, reinforcing their utility as probes for mapping diverse NE-associated protein networks.

Functional annotation through Gene Ontology (GO) analysis of the NE proteome revealed that the plant NE functions as a multifaceted cellular platform orchestrating diverse essential processes, including but not limited to RNA processing and metabolism, transcriptional regulation and chromatin organization, lipid biosynthesis and membrane trafficking, and protein folding and targeted degradation (Fig. 2D). Each functional category is supported by contributions from multiple proximiomes rather than being driven by single bait proteins (Fig. 2E), eliminating concerns about experimental artifacts or bait-specific biases. Notably, proteins involved in RNA processing and transcription are highly enriched in the proximiomes of CRWNs and the NPC basket (Fig. 2E and table S2), underscoring the functional integration of these NE subdomains in gene expression regulation. This observation aligns with recent findings that CRWNs and KAKU4 contribute to histone modification (34, 64, 65) and that RNA processing factors are recruited to the nuclear periphery in both mammalian and plant cells, where they play crucial roles in RNA metabolism (44, 66).

Evolutionary analysis of the 734 NE-associated genes across prokaryotic and eukaryotic lineages revealed four distinct clusters that shed light on the stepwise evolutionary assembly of the NE proteome: prokaryote-eukaryote conserved (cluster 1; reflecting potential ancient cellular functions co-opted by the NE), eukaryote specific (cluster 2; representing innovations associated with nuclear compartmentalization), eukaryote specific but notably absent from the fungal lineage (cluster 3; indicating lineage-specific divergence or loss in fungi), and plant specific (cluster 4; indicating lineage-specific adaptations to plant species) (Fig. 3A and table S3). GO enrichment analysis of these evolutionary groups revealed distinct functional trajectories: Prokaryote-eukaryote conserved genes were predominantly involved

in protein folding, protein maturation, and metabolic pathways, indicating that the eukaryotic NE has co-opted these ancient roles in coordinating proteostasis and energy metabolism during nuclear evolution. In contrast, eukaryote-specific NE genes showed strong enrichment for RNA processing, splicing machinery, and nucleocytoplasmic transport functions, consistent with the integration of compartmentalization and gene expression regulation at the NE. Last, plant-specific NE genes were enriched for cellular organization, lipid biosynthesis, and abiotic stress responses, consistent with the specialized developmental programs and environmental adaptations required for sessile lifestyles (Fig. 3B).

Spatial analysis of the NE proteome links NPC biogenesis to cell proliferation in plants

Our proximity-labeling proteomics approach profiled NE-associated proteins from whole *Arabidopsis* seedlings, precluding distinction of protein profiles at the tissue or single-cell level. To address this limitation and investigate the spatiotemporal distribution of plant NE proteins, we analyzed the expression profiles of the 734 NE candidates together with the complete set of nucleoporin genes using a recently published, comprehensive *Arabidopsis* snRNA-seq atlas spanning multiple developmental stages and covering diverse tissues and cell types (CNP0002614) (67). Given the NE's multifaceted roles, we reasoned that different NE components might exhibit distinct expression patterns adapted to specific cellular contexts. The analysis revealed that the NE proteome encompasses proteins with both ubiquitous and spatially restricted expression patterns. This spatial specialization suggests that different cell types may assemble functionally distinct NE compositions tailored to their unique regulatory requirements (fig. S3A and table S4).

To further explore the functional significance of this spatial heterogeneity, we performed high-dimensional weighted gene coexpression network analysis [hdWGCNA; (68)] on the snRNA-seq data of NE genes across four major *Arabidopsis* organs. This analysis identified 10, 12, 7, and 12 spatially regulated gene coexpression clusters (modules) in root, shoot, silique, and leaf, respectively, revealing tissue-specific regulatory networks and functional associations among NE components at single-cell resolution (Fig. 4A and table S5). A notable and consistent feature across organs was that many core NPC genes formed distinct, tightly clustered coexpression modules, including module 6 (M6) in root, module 1 (M1) in shoot, and module 3 (M3) in silique (Fig. 4, B and C), suggesting a tightly coregulated gene set. Critically, mapping these NPC-enriched modules onto the snRNA-seq data showed that they are strongly expressed in proliferative cell types, including pluripotent initial cells surrounding the quiescent center in root meristems (Fig. 4D, M6), actively dividing cells in shoot apical meristems (Fig. 4E, M1), and developing embryonic tissues within siliques (Fig. 4F, M3). These data indicate a robust association between nuclear pore biogenesis and proliferative cells. Across organs, core NPC components also showed consistent coexpression with chromatin regulators and RNA splicing machinery (Fig. 4B). This is consistent with the recently reported physical interactions between the NPC and transcription/cotranscriptional RNA processing machinery (44). In addition, certain PNET proteins—including NEAP3, SINE1, SUN1, PNET3, PNET4, PNET10, CRWN2, and KAKU4—coexpressed with Nups (Fig. 4C and table S5), suggesting that the early cell division window likely represents a critical period not only for NPC assembly but also for broader NE remodeling and membrane protein integration.

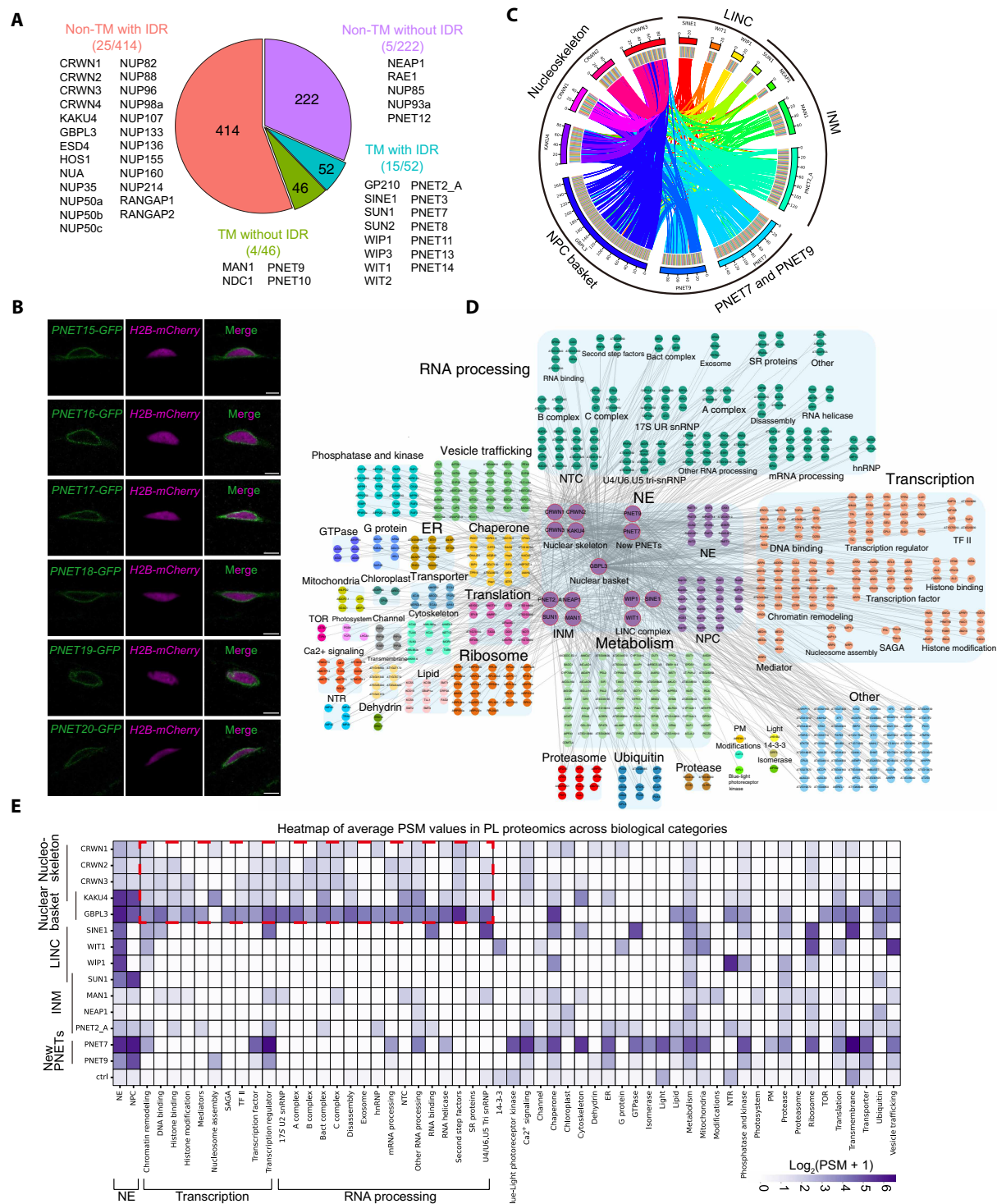


Fig. 2. Classification, validation, and functional annotation of *Arabidopsis* NE proteins. (A) Classification of 734 NE-associated proteins identified from the integrated NE proximate based on predicted transmembrane (TM) domains and intrinsically disordered regions (IDRs). Four structural categories are shown: TM with IDR ($n = 52$), TM without IDR ($n = 46$), non-TM with IDR ($n = 414$), and non-TM without IDR ($n = 222$). Representative known NE proteins from each category are listed. (B) Subcellular localization of six previously unidentified TM + IDR NE proteins (PNET15 to PNET20). Each candidate was fused to GFP and stably coexpressed with histone H2B-mCherry (nuclear marker) in double transgenic *Arabidopsis* plants. Nuclei from leaf epidermal cells were imaged. Scale bars, 5 μ m. (C) Circos plot showing the overlap of identified proteins across 14 NE proximates. Connecting lines indicate shared prey proteins. (D) Gene Ontology (GO) enrichment analysis of the integrated NE proteome. Bait proteins are highlighted by red circles. (E) Heatmap showing the distribution of average peptide-spectrum match (PSM) values for proteins within major NE functional categories across the 14 proximates. The dashed red box highlights transcription and RNA processing-related proteins probed by NE baits.

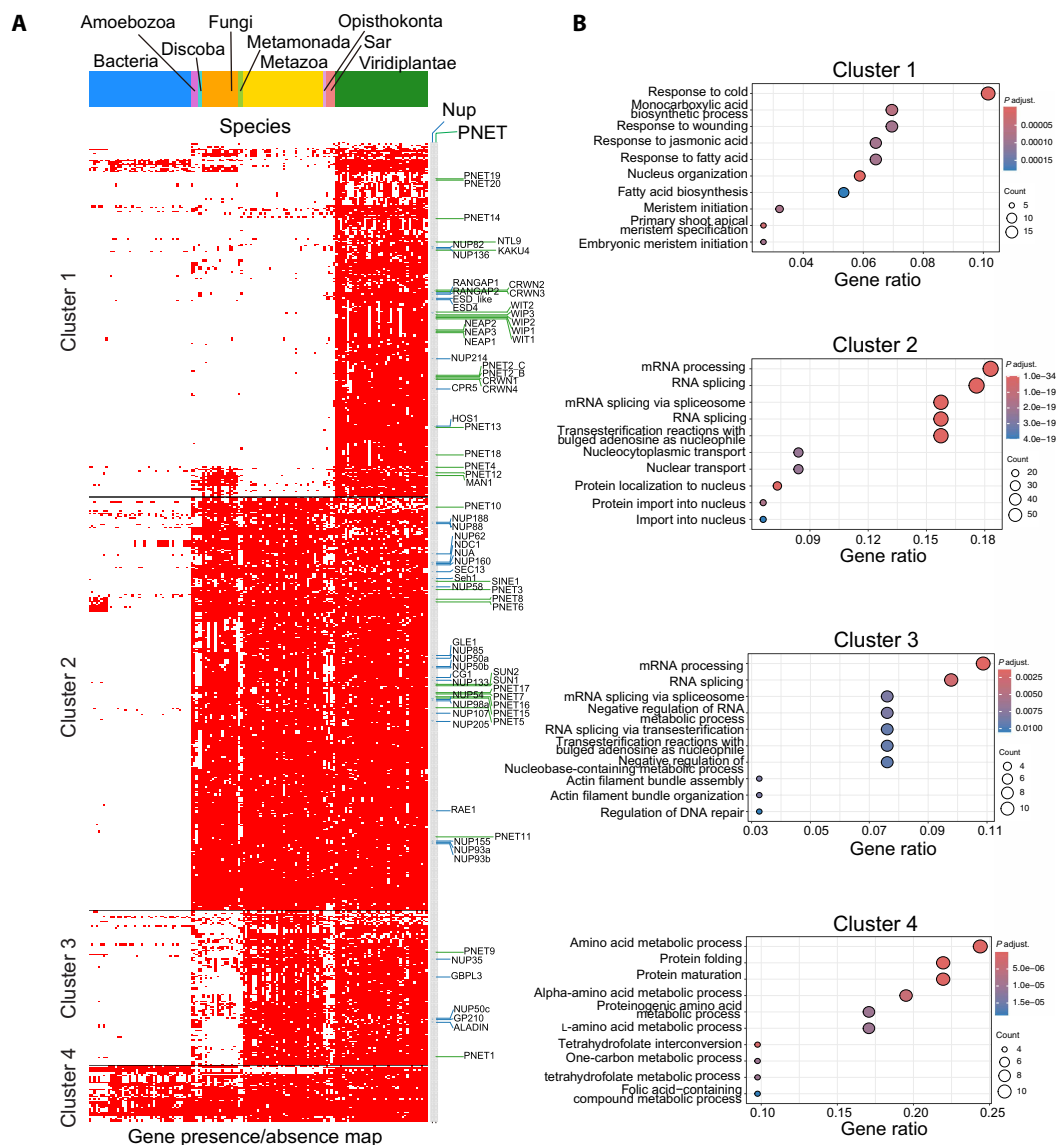


Fig. 3. Evolutionary analysis of NE proteome. (A) Evolutionary conservation of the 734 *Arabidopsis* NE-associated genes across major eukaryotic and prokaryotic lineages was assessed and visualized by heatmap based on the presence or absence of homologs. Species are grouped by phylogenetic relationships, with most genes classified into four conservation categories: cluster 1, prokaryote-eukaryote conserved (ancient core functions co-opted by the NE); cluster 2, eukaryote specific (eukaryote innovations); cluster 3, eukaryote specific but notably absent in fungi (eukaryote innovations absent in fungi); cluster 4, *Viridiplantae* lineage specific (plant-specific adaptations). It should be noted that gene assignment to these clusters is not absolute; minority constituents within each group may deviate from the primary category classification. Heatmap colors (red versus white/blank) represent the presence and absence of homologs, respectively. Known NE and NPC proteins are labeled with text on the right. The full ortholog edge list, species codes, taxonomy table, and species inventory are provided in table S3. (B) GO enrichment analysis of the four clusters of NE-associated genes using *Arabidopsis* databases.

Beyond the general enrichment of NE components in proliferative cells, we also identified compelling examples of tissue- and cell type-specific NE gene expression that reflect functional adaptations to local cellular environments and physiological demands. *Nup205*, a nucleoporin with a reported role in plant immunity regulation (69), exemplified this specialization. While *Nup205* coexpressed with other Nup genes in root and silique tissues, it shifted to a stress granule-enriched module (shoot M12) in shoot tissue and to defense- and immunity-related modules (leaf M10) in leaf tissue (Fig. 4H and table S5). Notably, leaf M10 genes are predominantly expressed in epidermal cells, the primary entry point for many pathogens (Fig. 4G). These findings support the idea that *Nup205* has evolved specialized

regulatory functions in coordinating biotic stress responses, in addition to its core structural role within the NPC. Similarly, *PNET7*, an integral NE protein containing steroid-binding domains, showed highly specific expression in the suberized endodermis layer of roots (Fig. 4D, M3), where it coexpressed with genes involved in fatty acid biosynthesis and lipid metabolism pathways (Fig. 4B), suggesting a specialized regulatory role in coordinating NE-associated lipid homeostasis with the biosynthesis of protective barrier compounds essential for root function.

We further applied Pearson/Spearman-based pseudo-bulk correlation analyses to validate hdWGCNA-derived NE coexpression patterns, obtaining results that were highly consistent with above

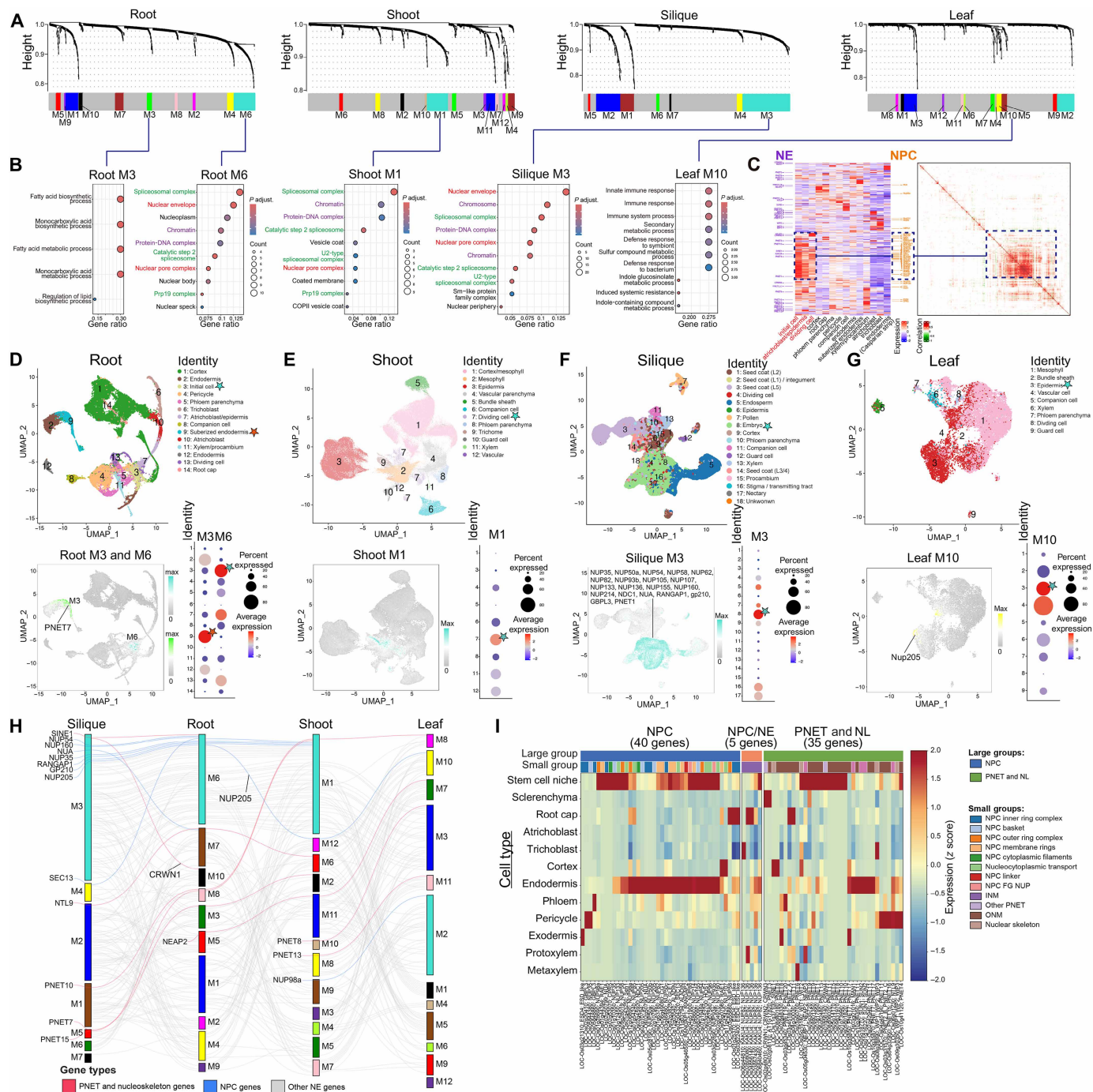


Fig. 4. Tissue- and cell type-specific coexpression network of NE-associated genes across *Arabidopsis* organs. (A) hdWGCNA of NE-associated genes across root, shoot, leaf, and silique using snRNA-seq datasets (CNP0002614). Identified coexpression modules (M) are color-coded and labeled in each tissue. See table S5 for module gene lists. (B) GO enrichment plots showing top functional categories in selected coexpression modules. (C) Heatmap and expression-correlation map of NE-associated genes across annotated root cell types based on snRNA-seq datasets. The position of NPC and NE genes are labeled on the right and left, respectively. Cell type annotations for each organ are shown at the bottom of heatmap. The coexpression pattern of NE-associated genes analyzed by weighted correlation are shown on the right. (D to G) UMAP visualization of single-nucleus transcriptomes from root (D), shoot (E), silique (F), and leaf (G), with cell type identities annotated (top panels). Module eigengene expression is overlaid in lower panels to reveal tissue- and cell type-specific enrichment of selected modules, including root M3 and M6, shoot M1, silique M3, and leaf M10. Accompanying bubble plots indicate expression frequency and levels of module genes within each cell type. (H) Sankey diagram across four *Arabidopsis* organs (silique, root, shoot, and leaf). Each line represents a gene and traces its assignment to coexpression modules in different tissues, thereby illustrating conserved or tissue-specific expression patterns. PNET genes (red lines), nucleoporin genes (blue lines), and other NE-associated genes (gray lines) are distinguished by color coding. (I) Expression heatmap of NE- and NPC-associated genes across rice tissues and cell types based on z score-normalized scRNA-seq expression values (GSE251706). Genes are subdivided into functional subcategories (NPC inner ring, basket, outer ring, membrane rings, cytoplasmic filaments, nucleocytoplasmic transport, linker Nups, FG Nups, INM proteins, ONM proteins, nucleoskeleton, and other PNETs).

findings (e.g., in root tissue; fig. S3B). To enable the community to explore NE gene coexpression relationships across *Arabidopsis* tissues in greater depth, we developed an interactive web platform (<https://kdhulipalla13-gene-co-expression-2-0.share.connect.posit.cloud/>) that supports customizable searches of tissue-specific NE coexpression patterns and facilitates hypothesis generation regarding NE functions in tissue- and cell type-specific contexts.

To examine whether the spatial transcriptional architecture of NE-associated genes observed in *Arabidopsis* represents a conserved principle across plant lineages or reflects species-specific adaptations, we extended our analysis to include a previously published, root scRNA-seq data (GSE251706) from rice (*Oryza sativa*), a monocot that diverged from the eudicot *Arabidopsis* by ~140 to 150 million years (70). The cross-species comparison of NPC and PNET gene expression profiles revealed both notable conservation and divergence in the spatial deployment of NE components. Consistent with *Arabidopsis*, most rice NPC genes exhibited high expression levels and coexpression patterns within the stem cell niche (Fig. 4I and fig. S3, C and D), confirming that the association between nuclear pore biogenesis and proliferative cell identity represents a conserved feature of plant development that may transcend major phylogenetic divisions. However, rice NPC genes also displayed robust expression in endodermal cells (Fig. 4I), a spatial pattern absent in *Arabidopsis* roots. This difference plausibly reflects species-specific anatomy and developmental programming in rice roots, where the endodermis contributes to lateral root and middle-cortex formation and therefore contains proportionally actively dividing cells (71); in this context, endodermal NPC expression may track local proliferative activity. Thus, while the fundamental relationship between NPC assembly and cell proliferation remains evolutionarily conserved, the spatial deployment of NE components in differentiated tissues has diversified to support lineage-specific physiological adaptations and developmental programming.

Temporal restriction of NPC gene expression to early developmental stages

Beyond spatial regulation across tissues and organs, we also investigated the temporal dynamics of NE-associated gene coexpression during cellular differentiation using root development as a model. We leveraged a previously published scRNA-seq dataset of the *Arabidopsis* root (GSE152766) (72), which provides high-resolution, transcriptomic profiles across well-defined cell states and developmental trajectories (Fig. 5A). We performed a pseudotime analysis on this dataset that enables the reconstruction of continuous cell differentiation (T0 to T9), providing temporal resolution of gene expression dynamics associated with progressive cell fate specification (Fig. 5A). This temporal analysis showed that most NPC genes display highly restricted expression dynamics, with predominant activation and peak coexpression at the earliest developmental stage (T0), corresponding to meristem cell states and newly divided cells, followed by a rapid transcriptional decline across subsequent pseudotime intervals as cells progress to later developmental stages (Fig. 5B). This observation reinforces the hypothesis that NPCs are assembled primarily during the initial phases of cellular differentiation and subsequently maintained as stable structures throughout differentiation without requiring continuous gene expression or active protein turnover. The hdWGCNA analysis confirmed this temporal specificity, identifying a coexpression module (M1) highly enriched in nucleoporin genes during early developmental stages (Fig. 5C),

defining a distinct temporal window for coordinated NPC biogenesis. In contrast, NE-associated non-NPC genes—including PNET, nucleoskeleton, and LINC proteins—displayed considerably more heterogeneous temporal expression profiles, with different gene subsets activated at distinct pseudotime intervals (Fig. 5B). Beyond those coexpressing with Nup genes in M1, a distinct subset of NE proteins clustered into M2, which corresponds to the cellular elongation stage. This module showed functional enrichment for vesicle-mediated transport, consistent with the progressive acquisition of specialized physiological roles during differentiation (Fig. 5C).

We further extended this analysis to leaf and silique tissues by including developmental stage-resolved snRNA-seq datasets (CNP0002614) (67). For each tissue, we constructed stage-specific coexpression modules and visualized their temporal transitions using Sankey diagrams (fig. S4, A and B, and table S6). In the silique, NPC genes exhibited minimal coexpression during early and mid stages (D0 to D4) but converged into a conserved coexpression module at the terminal stages (D4 and D5), suggesting a late wave of NPC assembly during silique development, likely a result of active cell division associated with embryo and endoderm formation at this stage (fig. S4B). In contrast, coexpression among NPC genes can be sparse and transient in the leaf, and coexpression modules may emerge and disappear between adjacent developmental stages, consistent with the generally low transcript abundance for NPC genes in leaves (fig. S4A).

Differential regulation of transcription and protein stability supports the NPC assembly-and-maintenance model in plants

The spatiotemporal transcriptomic analyses revealed an unexpectedly restricted expression pattern of NPC genes, with a large fraction of them predominantly expressed in actively dividing cell populations. This finding seems to contrast sharply with the essential function of NPC proteins in nucleocytoplasmic transport, which demands their presence in all cell types. However, this paradox may be reconciled by evidence from animal and fungal systems demonstrating extremely long protein half-lives for core scaffold Nups (73–75). This suggests a model where NPCs are predominantly assembled during the cell cycle in dividing cells and subsequently maintained as stable, long-lived structures throughout cellular differentiation and maturation. However, this assembly-and-maintenance model remains largely unexplored and entirely unvalidated in plants.

To test this model in *Arabidopsis*, we examined both the transcriptional activity and protein distribution of selective NPC components, including Nup35 (IRC Nup), Nup54 (FG Nup), Nup93a (Linker Nup), and Nup160 (ORC Nup). For each Nup analyzed, we generated two types of transgenic lines: promoter-reporter lines, in which nuclear localization signal (NLS)–green fluorescent protein (GFP) was driven by the native *Nup* promoter, to assess transcriptional activity, as well as translational GFP fusion lines, which include *Nup* promoter and their endogenous genomic sequence, to visualize protein distribution. Consistent with the above spatiotemporal transcriptomic analyses, confocal microscopy revealed that the promoter activity of all test *Nups* was largely restricted to initial cells and nearby actively dividing cells at the root tip (Fig. 5D). Conversely, the corresponding translational fusion lines showed a markedly different pattern: GFP-tagged Nup proteins were broadly detected across all root cells spanning diverse developmental stages, although fluorescence intensity was not uniform across different root zones. This disparity indicates that Nup protein distribution is far less spatially

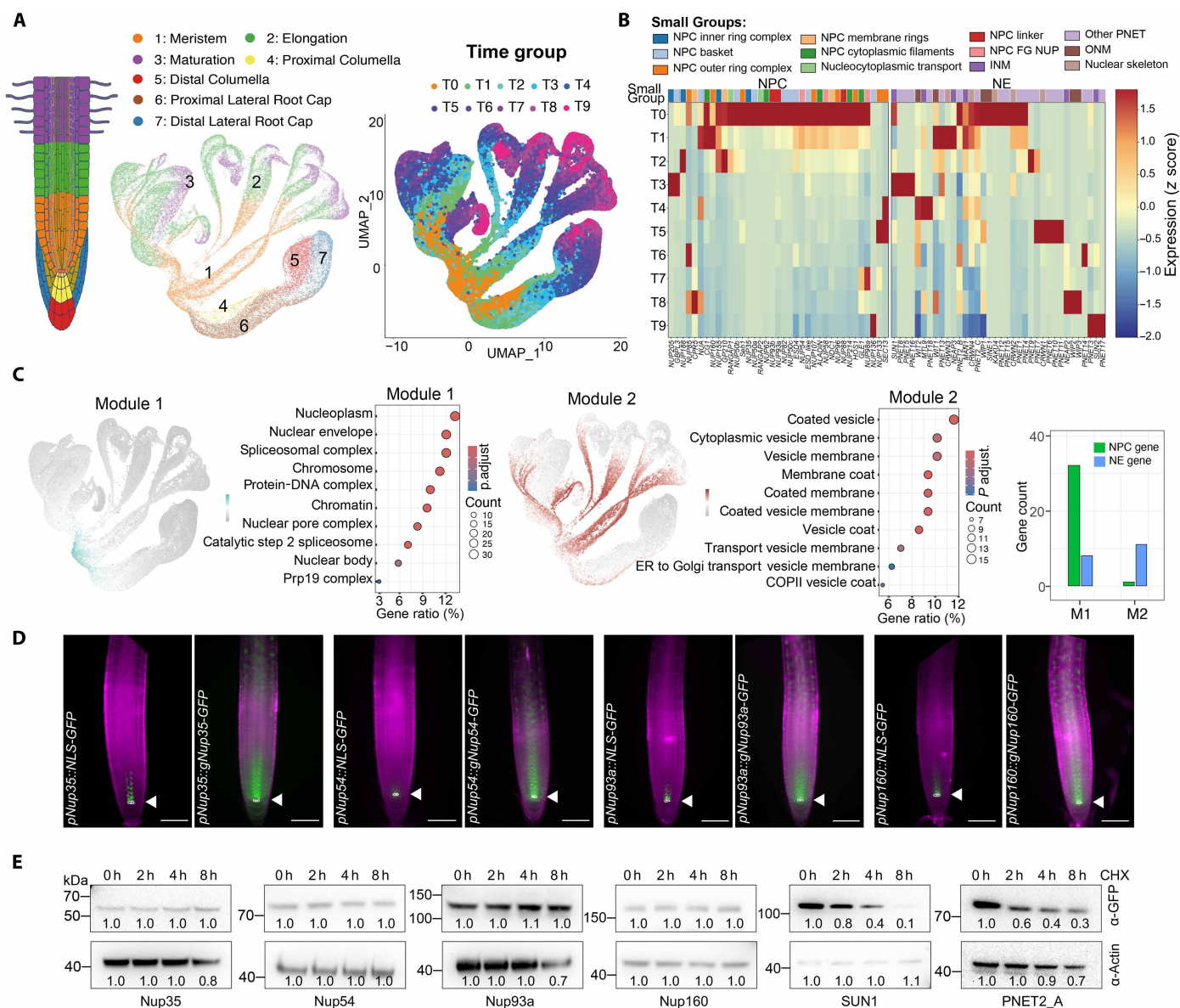


Fig. 5. Spatiotemporal analysis of NE-associated genes reveals an assembly-and-maintenance model for plant NPC. (A) *Arabidopsis* root schematic is colored according to annotated cell types derived from stage-resolved root scRNA-seq analyses (GSE152766), with the corresponding UMAP shown on the left. The UMAP shown on the right is a projection of the same dataset ordered along a pseudotime trajectory (T0, youngest/least differentiated cells; T9, most mature/differentiated cells) illustrates developmental progression from the meristematic niche to differentiated tissues. (B) z score-normalized expression heatmap of NPC and PNET genes across pseudotime. (C) Coexpression modules identified by hdWGCNA are mapped to the pseudotime-ordered UMAP project in (A). Two major modules (M1 and M2) are shown, each enriched for distinct functional categories. Gene counts for NPC and PNET genes in each module are shown on the far right. (D) Representative confocal images showing spatial distribution of *Nup* promoter activity (*pNup::NLS-GFP*) and corresponding *Nup* proteins (*pNup::gNup-GFP*) in transgenic *Arabidopsis* root. The quiescent center is indicated by an arrowhead. Scale bars, 100 μ m. (E) Cycloheximide (CHX) chase assay for NPC (Nup35, Nup54, Nup93a, and Nup160) and PNET (SUN1 and PNET2_A) proteins. Seedlings were treated with CHX for indicated time before protein was extracted and subject to immunoblot using anti-GFP and anti-actin antibodies. Protein quantification was normalized to time 0 in each sample and shown under immunoblots. Similar results were obtained three times.

restricted than its promoter activity and that NPCs produced at the earliest cell stages are stably maintained throughout subsequent developmental stages (Fig. 5D and fig. S4C), providing strong evidence for the NPC assembly-and-maintenance model in plants. We observed a similar pattern for the nucleoskeletal protein CRWN2 (fig. S4C). The *CRWN1* promoter displayed activity throughout differentiated cell layers, suggesting differential regulatory control among nucleoskeleton proteins.

We then examined whether differential protein stability could account for the persistence of NPC proteins in terminally differentiated cells, where cognate transcripts are markedly reduced or undetectable. *Arabidopsis* Nup proteins displayed unusually long protein half-lives, with negligible degradation detected after an 8-hour cycloheximide (CHX) treatment (Fig. 5E). This extreme stability and negligible turnover contrast sharply with the notably short half-lives of other NE proteins, such as SUN1 (76) and PNET2_A (Fig. 5E).

This differential stability supports the notion that core NPC components are less susceptible to rapid turnover. While our CHX-chase assays do not measure multiday protein half-lives, as prolonged CHX treatment and translation inhibition is cytotoxic, the observed stabilities are consistent with the reported longevity of NPC subunits from mammalian research and provide support for the assembly-and-maintenance model of NPC organization in plant cells.

DISCUSSION

This study presents a comprehensive multiomics analysis of the plant NE by integrating proximity-labeling proteomics with single-nucleus and single-cell transcriptomics, gene coexpression network analysis, pseudotime reconstruction, and targeted cellular and biochemical assays. Unlike previous plant NE profiling studies limited by specific baits or subdomains, the employment of CRWN proteins and broad-range PNET probes allowed us to capture the full complexity of the NE. This atlas provides direct evidence that the NE is not merely a physical barrier and transport gatekeeper but a central hub integrating functions including, but not limited to, chromatin organization, RNA processing, lipid metabolism, and regulation of protein homeostasis. In addition, we created an online resource that allows the community to interactively explore NE gene coexpression patterns across *Arabidopsis* tissues.

An intriguing insight emerging from this NE multiomic analysis is a functionally segregated, “two-speed” compositional architecture, characterized by distinct protein turnover rates. First, the stable scaffold (the slow speed): the core NPC biogenesis is strictly coupled to the proliferative state, with gene expression peaking in active dividing cell populations (e.g., root initial cells and shoot apical meristems). Despite this restricted transcriptional window, protein-level analysis reveals that Nups persist as stable complexes across differentiated tissues, having exceptionally long half-lives (likely days or even weeks). This extreme longevity and low turnover may be an adaptation that mitigates the high energetic and structural cost associated with the removal and de novo assembly of the massive NPC during interphase. Second, the dynamic interface (the fast speed): In stark contrast, the surrounding NE interface—comprising PNETs, certain nucleoskeletal elements, and associated factors—is characterized by rapid turnover (half-lives measured in hours) and high transcriptomic plasticity. These components exhibit heterogeneous temporal expression profiles that align precisely with specific developmental transitions. This dichotomy allows the plant cell to maintain a constant, essential transport capacity via the long-lived NPC while rapidly remodeling the surrounding membrane environment to precisely respond to developmental cues and environmental stresses.

Our hdWGCNA analysis revealed that the NE is not a monolithic organelle but rather a “mosaic” that adopts specialized compositions depending on tissue- and cell-type context. For example, the specific expression of *Nup205* in leaf epidermal cells, the frontline of pathogen defense, supports its previously reported role in immune regulation (69) and suggests that specific NPC components can modulate immune signaling, rather than serving solely as generic transport factors, in defined cellular contexts. The restriction of *PNET7* to the root endodermis, where it coexpresses with suberin and lipid biosynthesis genes, posits a role for the NE in coordinating the production of diffusion barriers. These findings imply that “generic” NE components are frequently co-opted into tissue-specific functional modules, a

feature that may underpin the developmental plasticity and robust environmental responsiveness required by sessile organisms.

The enrichment of IDRs within the integral NE proteome provides a molecular basis for this flexibility. IDR-containing proteins are known to drive liquid-liquid phase separation (LLPS), suggesting that PNETs may form transient, membrane-associated, phase-separated perinuclear compartments, such as nuclear lamina, that serve as important signaling hubs (35, 77–79). These dynamic condensates likely facilitate the rapid, local integration of essential functions, including chromatin tethering, lipid metabolism, immune responses, and stress signaling, with rapid protein turnover ensuring timely remodeling in response to cellular demands. For instance, recent work demonstrated that the PNET2 protein interacts with membrane-bound NAM, ATAF1/2, and CUC2 (NAC) transcription factors and sequesters them within the nuclear lamina via phase separation. Heat stress then promotes the disruption of LLPS, releasing the NACs to initiate stress responses (79). Future work using live-cell imaging and optogenetic perturbation could directly test whether other PNET proteins form functional phase separation at the NE and how these LLPS structures contribute to tissue-specific gene regulation.

Several important limitations of this study warrant discussion. First, proximity labeling inherently captures spatially enriched neighborhoods rather than definitive interactomes, with potential for false negatives (transient or low-abundance proximal proteins) and false positives (nonspecific preys). Second, some of the functional inferences rely on transcriptomic data as proxies for protein abundance and localization. While powerful for detecting spatial and temporal trends, this approach does not capture posttranslational modifications, protein stability differences, or the extended half-lives characteristic of many NE proteins, such as the Nups examined here. The coexpression modules identified here should therefore be considered hypothesis-generating frameworks requiring protein-level validation. Third, current NE proximity labeling does not incorporate spatiotemporal resolution in response to environmental stimuli, which would illuminate how NE composition dynamically remodels. Also, because reproductive initiation involves extensive transcriptome reprogramming, the NE proteome in reproductive tissues may differ substantially from that in vegetative tissues, warranting further investigation.

MATERIALS AND METHODS

Plant material and growth conditions

All *Arabidopsis thaliana* plants used in this study were of the Col-0 background. Seeds were surface-sterilized and stratified at 4°C for 3 days before being sown on half-strength Murashige and Skoog (1/2 MS) agar plates. Seven-day-old seedlings were then transferred to soil. *Arabidopsis* and *Nicotiana benthamiana* plants were grown under a 16-hour light/8-hour dark cycle at 22°C. The *crwn1*, *crwn2*, *crwn3*, and *crwn2 crwn3* mutant seeds were provided by E. Richard from the Boyce Thompson Institute. The *pCRWN1::CRWN1-M-TurboID-3HA* transgenic lines are in *crwn1* mutant background, and *pCRWN2::CRWN2-M-TurboID-3HA* and *pCRWN3::CRWN3-M-TurboID-3HA* transgenic lines are in *crwn2 crwn3* double mutant background. *35S::PNET7-BioID2* and *35S::PNET9-BioID2* transgenic lines are in wild-type background. All transgenic plants were generated by floral dip transformation using *Agrobacterium tumefaciens* GV3101 and selected with Basta herbicide.

Plasmid construction

In-fusion technologies (Vazyme, ClonExpress II One Step, catalog no. C112) were used for cloning unless specified otherwise. To generate constructs for protein localization, coexpression, complementation, and proximity-labeling assays, *mCherry*, *mEGFP*, and *TurboID-3HA* were cloned into the pEG100 vector. The genomic DNA of *CRWN1*, *CRWN2*, *CRWN3*, *PNET7*, *PNET9*, *PNET15*, *PNET16*, *PNET17*, *PNET18*, *PNET19*, *PNET20*, *SUN1*, *Nup35*, *Nup54*, *Nup93a*, and *Nup160* were cloned into pEG100 vector with tags. The native promoter of *Nup35*, *Nup54*, *Nup93a*, and *Nup160* amplified and cloned into pEG100 by In-fusion cloning.

Fluorescence imaging analysis

To image *Arabidopsis* plants, we used 7-day-old transgenic seedlings. Coexpression was performed using *Agrobacterium*-mediated transient protein expression in *N. benthamiana* as previously described (50). *Agrobacterium* strains carrying corresponding constructs were mixed and infiltrated into the leaves of 4-week-old *N. benthamiana* plants. Images were acquired from leaf epidermal cells 2 days postinfiltration using a Zeiss LSM710 confocal microscope.

Immunoblot analysis

Total protein was extracted from transgenic seedlings using the protein extraction buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 0.5% NP-40, 0.5% sodium deoxycholate, plant protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride, and 40 μ M MG132]. Protein extracts were separated by SDS–polyacrylamide gel electrophoresis, followed by immunoblot analysis using an anti-GFP antibody (1:5000 dilution Clontech, catalog no. 632381), an anti-hemagglutinin antibody (1:5000 dilution; Roche, catalog no. 11867431001), or streptavidin–horseradish peroxidase (1:10,000 dilution; Abcam, catalog no. 7403).

Proximity labeling and affinity purification

The BioID2- and TurboID-based proximity labeling and affinity purification of biotinylated proteins were adapted from a previous study (63). One-week-old transgenic seedlings—including *pCRWN1/2/3::CRWN1/2/3-M-TurboID-3HA*, *35S::PNET7-BioID2*, and *35S::PNET9-BioID2*—along with wild-type nontransformants were treated with 50 μ M biotin for 4 hours (TurboID-tagged lines) or 24 hours (BioID2-tagged lines) at room temperature. For each sample, 0.4 g of treated seedlings (three biological replicates per sample) was harvested and frozen in liquid nitrogen. The material was ground to a fine powder, and the total protein was extracted with the protein extraction buffer. The protein extraction was subjected to tandem PD-10 desalting columns (GE-Healthcare) to deplete free biotin. The flow-through was collected for affinity purification. For affinity purification, the eluted protein fraction was mixed with 50 μ l of prewashed streptavidin-coated magnetic beads (Thermo Fisher Scientific, Dynabeads MyOne Streptavidin C1, catalog no. 65002) and incubated on a rotor wheel overnight at 4°C, and the beads were separated on a magnetic rack and washed five times with the protein extraction buffer.

On-beads digestion, MS, and proteomics analysis

For on-beads tryptic digestion, the streptavidin beads were washed with PBS buffer three times and incubated with 1 mg of trypsin in 100 μ l of 50 mM triethylammonium bicarbonate buffer overnight at room temperature with gentle shaking. The resulting digests were separated from the beads on a magnetic rack and dried by Speedvac.

The peptides were dissolved with 10 μ l of 0.1% trifluoroacetic acid and desalted using 10 μ l of C18 desalting ZipTips according to the manufacturer's instructions. The purified peptides were then dried and redissolved with 20 μ l of formic acid before being subjected to liquid chromatography tandem MS (LC-MS/MS) analysis. MS/MS spectra were searched against the TAIR 11 database using Scaffold 5 and MSFragger 3.2 software with default criteria to harvest PSM and LFQ intensities, respectively. To analyze the proximity-labeling data, both PSM and LFQ data were used as input, and the DESeq2 and DEP packages in R were applied to enrich for *CRWN1*, *CRWN2*, *CRWN3*, *PNET7*, and *PNET9* proximal candidates. Volcano plots and heatmaps were generated by R.

Gene list curation and annotation

To reduce noise from highly expressed genes and ensure comprehensive coverage of plant NE proteins, we curated a refined gene set for all transcriptomic analyses. The gene list was constructed by integrating proximity-labeling proteomics results with additional NE proteins, including plant NE proteins *PNET2_A*, *MAN1*, *KAKU4*, *SUN1*, *NEAP1*, *WIP1*, *SINE1*, *WIT1*, and *GBPL3* (PXD026924, PXD015919, PXD015920, and PXD032906). Ribosomal genes were excluded to minimize confounding effects from potential contamination. Gene identifiers and annotations were standardized using a common gene name mapping file (table S2) to ensure consistency across analyses while maintaining information about functional categories (NPC and PNET genes) and subcategories (nuclear basket proteins, nuclear skeleton proteins, etc.).

snRNA-seq/scRNA-seq data acquisition, processing, and network construction

The snRNA-seq data from *A. thaliana* root, leaf, shoot, silique, and flower tissues were obtained from (67) via CNGB database (CNP0002614; <https://ftp.cngb.org/pub/CNSA/data6/CNP0002614/Other/>). scRNA-seq datasets were downloaded from the Gene Expression Omnibus *A. thaliana* root atlas [(72); GSE152766] and *Oryza sativa* root atlas [(70); GSE251706]. To assess cell type-specific transcriptional patterns, we reanalyzed previously published scRNA-seq and snRNA-seq datasets (67, 70, 72), integrating these transcriptomic profiles with our proximity labeling-based proteomic data. Downloaded datasets were loaded as Seurat objects (v5.0) with normalized expression matrices, quality control metrics, and cell-type annotations retained from the original studies using their established nomenclature. Data processing followed the standard Seurat pipeline (80) with default parameters unless specified. All objects were updated to Seurat v.5 before analysis.

Genes of interest were filtered on the basis of their presence in the expression matrix and availability in the mapping database. We implemented hdWGCNA following the standard protocol (68) (<https://smorabit.github.io/hdWGCNA/>), with the following modifications and parameter. Expression data were log-normalized using a scale factor of 10,000 and then scaled for genes of interest. The Seurat object was configured for WGCNA analysis using the SetupForWGCNA function with selected gene features. Metacells were constructed using the MetacellsByGroups function with *k*-nearest neighbors (*k* = 25) grouped by cell type and a maximum of 10 shared neighbors. All cell types were processed simultaneously, with those containing fewer than 50 cells excluded during metacell construction to reduce downstream noise. Metacell expression profiles were independently normalized and scaled using NormalizeMetacells and ScaleMetacells functions.

Network topology was assessed across soft-thresholding powers (1–30) using TestSoftPowers to identify the optimal power achieving approximate scale-free topology ($R^2 > 0.8$) for each Seurat object. All soft-threshold values are provided in table S7. Gene coexpression networks were constructed using ConstructNetwork with signed network type and Pearson correlation at the selected soft power. Following topological overlap measure (TOM) calculation and hierarchical clustering, modules were identified through dynamic tree cutting with dataset-specific minimum module sizes: 5 for snRNA-seq, 40 for *O. sativa* root scRNA-seq, and 50 for *A. thaliana* root scRNA-seq, with the larger thresholds (40, 50) applied to generate larger modules containing more genes for robust classification. Module eigengenes (MEs) were calculated using the ModuleEigengenes function. Module membership (kME) was quantified for each gene using the ModuleConnectivity function, and enrichment statistics were calculated as described in the GO enrichment section.

For visualization, UMAP embeddings from the original Seurat objects were used without additional dimensional reduction. Module activity patterns were visualized through multiple complementary approaches: MEs were projected onto UMAP coordinates using ModuleFeaturePlot, dotplots showing average module expression and percentage of expressing cells per cell type were created using DotPlot from Seurat, and hierarchical clustering dendrograms with TOM heatmaps were generated using PlotDendrogram from the hdWGCNA package.

Average expression heatmap from scRNA/snRNA-seq data

snRNA-seq data from *A. thaliana* root, shoot, leaf, and silique tissues [(67); CNP0002614] and scRNA-seq datasets from *A. thaliana* [(72); GSE152766] and *O. sativa* [(70); GSE251706] root atlases were processed to calculate average gene expression across cell types using the AverageExpression function in Seurat with normalized RNA data, followed by z -score normalization across genes to generate expression heatmaps. The percentage of cells expressing each gene per cell type was also calculated. Heatmaps were generated in Python 3.8 using seaborn, with genes first grouped by broad functional categories and then hierarchically clustered within groups using average linkage on Euclidean distances via `scipy.cluster.hierarchy`. Pseudotime ordering in the *A. thaliana* root dataset followed the original annotation from (72). For weighted correlation analysis, gene coexpression patterns across cell types and temporal progression were analyzed using a WGCNA-inspired approach. Weighted correlations were calculated as $adjacency = \text{sign}(correlation) \times |correlation|^\beta$ ($\beta = 6$) to emphasize strong connections while preserving network connectivity. Genes were ordered via hierarchical clustering using average linkage on weighted correlation-based distances ($1 - |weighted\ correlation|$), while cell types were arranged along an NE-to-NPC functional axis using normalized expression scores ($NPC\ mean - NE\ mean$), with high NPC-expressing types ($z\ score \geq 1.0$) anchored toward the NPC end ($pull\ strength = 0.75$). Heatmaps were generated in R with ComplexHeatmap (43). Visualizations included z score-scaled expression matrices, gene-gene Pearson correlation matrices (range -1 to 1), NPC/NE membership tracks with density plots computed using a sliding window of $n(genes)/50$ and associated gene annotations; implementation followed the author's online examples and reference materials.

Ortholog retrieval and presence/absence maps

Ortholog data were retrieved from the PANTHER Knowledgebase (v19.0) via its REST API (81). For each *A. thaliana* gene identifier,

we queried the ortholog/matchortho end point with `geneInputList` set to the query gene ID, `organism = 3702` (NCBI: txid3702), and `orthologType = all` to return both least-diverged (LDO) and other orthologs, thereby capturing 1:1 and non-1:1 relationships. Species-level taxonomy (domain/kingdom, phylum, class, order, family, genus, and species) was retrieved from the National Center for Biotechnology Information (NCBI) Taxonomy database (82, 83) via Entrez E-utilities (EFetch, `db = taxonomy`) using the NCBI taxonomy IDs carried in the PANTHER responses. Eight eukaryotic kingdoms represented in our dataset were retained as separate groups; non-eukaryotes were collapsed to “Bacteria” for visualization. The full ortholog edge list, species codes, taxonomy table, and species inventory are provided in table S3. After presence/absence matrices were then constructed, to visualize patterns as a heatmap, we applied k -means clustering ($k = 4-8$, $n_init = 10$, `random\_state = 42`) to the binary ortholog profiles, followed by within-cluster hierarchical ordering using Jaccard distance and average linkage. Heatmaps were rendered with `seaborn.clustermap` in Python 3.8.

Ortholog identification, mapping, identifier harmonization, and name conversion

To enable cross-species functional annotation between *A. thaliana* and *O. sativa*, we performed comprehensive ortholog mapping using three complementary databases. Given our focused gene set and the potential for information loss when restricting analyses to one-to-one orthologs, we included all ortholog types (one-to-one, one-to-many, and many-to-many relationships) to capture the full spectrum of evolutionary relationships. Ortholog data were obtained from three sources: PANTHER, Ensemblplants, and RGAP.

From the PANTHER database, rice-*Arabidopsis* ortholog pairs were extracted from the comprehensive application programming interface results, filtering specifically for relationships between these two species. Orthologous gene relationships between *A. thaliana* and *O. sativa* ssp. *japonica* were also identified using the Ensembl Plants BioMart web interface (83) (<https://plants.ensembl.org/biomart/martview/ece7f5bdfce2470ac632f6c6d5f689a5>), with ortholog tables containing all relationship types downloaded directly from the BioMart portal. In addition, ortholog group data were obtained from the Rice Genome Annotation Project (https://rice.uga.edu/annotation_pseudo_apk.shtml) (84), which identifies orthologous relationships across seven plant species. We filtered this dataset to retain only ortholog groups containing both our genes of interest and rice-*Arabidopsis* pairs. All rice gene identifiers were standardized to the RGAP locus identifier format (LOC_OsXXgXXXXX) using the RAP-MSU conversion table to ensure compatibility with the rice scRNA-seq dataset (70). For annotation purposes, rice orthologs were labeled using their corresponding *Arabidopsis* gene names with numerical suffixes (ortho_1, ortho_2, etc.) to accommodate multiple mappings. Complex ortholog groups, where rice genes map to multiple *Arabidopsis* genes, were annotated using multiple *Arabidopsis* orthologs separated by semicolons (a_ortho1; b_ortho1, etc.) to maintain clarity in downstream analyses.

GO enrichment analysis

GO enrichment used clusterProfiler v4.10.0 (85) against org.At.tair.db across biological process (BP), cellular compartment (CC), and molecular function (MF). Significance used Benjamini-Hochberg false discovery rate with adjusted P value and q value thresholds of 0.05. For each module (hdWGCNA) and for gene clusters defined

by the presence/absence heatmaps, the top 10 to 15 significant terms per ontology were visualized by dot plots (point size, gene ratio; color, adjusted *P* value). All parameters were held constant across analyses to ensure comparability. To mitigate sparse functional annotation in rice, we performed orthology-based GO enrichment by mapping rice hdWGCNA coexpression modules (root scRNA-seq) to their *Arabidopsis* orthologs using the consolidated database described above. Each mapped *Arabidopsis* gene set was analyzed with identical parameters to the primary enrichment using org.At.tair.db as the reference. Statistical thresholds and visualization parameters were held constant across analyses to ensure direct comparability, and enrichment outputs were interpreted as functional proxies for the corresponding rice modules.

Supplementary Materials

The PDF file includes:

Figs. S1 to S4
Legends for tables S1 to S8

Other Supplementary Material for this manuscript includes the following:

Tables S1 to S8

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