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Recruitment, rewiring and deep conservation in flowering plant gene regulation

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Transcription factors (TFs) are proteins that bind DNA to control where and when genes are expressed. In plants, dozens of TF families interact with distinct sets of binding sites (TFBSs) that reflect each TF's role in organismal function and species-specific adaptations. However, defining these roles and understanding broader patterns of regulatory evolution remain challenging, as predicted TFBSs may lack a clear impact on transcription, and experimentally derived TF binding maps to date are modest in scale or restricted to model organisms. Here we present a scalable TFBS assay that we leveraged to create an atlas of nearly 3,000 genome-wide binding site maps for 360 TFs in ten species spanning 150 million years of flowering plant evolution. We found that TF orthologues from distant species retain nearly identical binding preferences, while on the same timescales the gain and loss of TFBSs are widespread. Within lineages, however, conserved TFBSs are over-represented and found in regions harbouring signatures of functional regulatory elements. Moreover, genes with conserved TFBSs showed striking enrichment for cell-type-specific expression in 14 single-nucleus RNA atlases, providing a robust marker of each TF's activity and developmental role. Finally, we compare distant lineages, illustrating how ancient regulatory modules were recruited and rewired to enable adaptations underlying the evolutionary success of grasses.

Evolution has produced an extraordinary array of functional diversity in the plant kingdom, from subtle distinctions between neighbouring cells to large-scale anatomical novelty. Underlying this variation is a dynamic system of gene expression orchestrated by transcription factors (TFs), proteins that promote or repress transcription by binding DNA at specific binding sites (TFBSs) containing preferred sequence motifs. Yet despite the vast morphological and physiological breadth of flowering plants, the same TF families regulate gene expression, raising the question of whether changes in TF binding preferences (*trans* evolution) or changes in TFBSs (*cis* evolution) serve as key drivers of plant diversification. Few large-scale efforts have tackled this question directly[1], lacking the requisite TF binding maps from multiple plant lineages. In bacteria, a multiplexed DNA affinity purification (multiDAP) method[2] successfully identified both conserved and lineage-specific TFBSs, building on DNA affinity purification sequencing (DAP-seq)[3] by assaying the binding of in-vitro-expressed TFs to genomic DNA

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fragments from multiple pooled species. Here we apply the multiDAP method in ten flowering plant genomes including both close relatives and distant lineages separated by 150 million years of evolution[4], generating a broad resource of TF binding specificities and genome-wide TF binding maps. The resulting 'pancistrome' allows us to dissect the relative contributions of *trans* and *cis* regulatory divergence within and between distant plant lineages.

Our cross-species TF binding maps further offer an unprecedented framework for comparative regulatory genomics. Defining core sets of conserved target genes for each TF, we track their expression across single-nucleus transcriptomes spanning multiple tissue types and developmental stages of five plant species, unravelling the regulatory programs controlling cell-type identity across distantly related lineages. We further uncover clear patterns of TF network rewiring and recruitment to novel tissues, revealing how ancient and lineage-specific gene networks shape developmental programs and drive phenotypic adaptations.

Results

A plant pancistrome

In this study we optimized multiDAP for large eukaryotic genomes by refining experimental conditions to achieve low background and high sensitivity. We then applied it to investigate the function of 360 TFs representing 33 major TF families and 49 distinct motifs[3], mapping their binding profiles across the genomes of ten plant species simultaneously (Supplementary Table 1). This set of plants included *Arabidopsis thaliana* plus three other members of the Brassicaceae family (hereafter termed 'brassica'), *Arabidopsis lyrata*, *Capsella rubella* and *Brassica oleracea* (wild cabbage); four additional members of the eudicots, *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato), *Fragaria vesca* (wild strawberry) and *Populus trichocarpa* (black cottonwood); and two members of the monocots in the grass family Poaceae, *Oryza sativa* (rice) and *Sorghum bicolor* (sorghum) (Supplementary Table 2). To capture all TFBSs genome-wide, we used PCR-amplified genomic DNA fragments in the multiDAP assay to remove any tissue- or condition-specific DNA modifications such as DNA methylation that could impact TF binding. These comprehensive maps of TFBSs across ten plant genomes allowed us to investigate drivers of regulatory evolution, assessing the relative contributions of *trans* and *cis* divergence.

Minimal divergence of TF binding specificity

We first selected orthologous TFs from different species and examined the divergence of their preferred motifs and genome-wide binding properties. We selected 35 *A. thaliana* TFs representing 21 different families and 26 distinct motifs, and we identified the closest orthologue of each in five additional species, of which 108 performed well enough in duplicate multiDAP experiments to generate high-quality binding maps: 21 from *A. lyrata*, 22 from *C. rubella*, 21 from *B. oleracea*, 21 from *S. lycopersicum* and 23 from *O. sativa*. Across all orthologous TFs tested, we observed nearly identical motifs (Fig. 1a). The binding of *A. thaliana* TFs was highly predictive of the binding profiles of their orthologues in a given species' genome (mean Pearson $r = 0.88$; Extended Data Fig. 1a,b). This was true even between *A. thaliana* and *O. sativa* orthologues ($r = 0.85$), which on average share only 52% amino acid identity. These correlations were nearly as high as between technical replicates of the same TF ($r = 0.92$) and substantially higher than those measured between *A. thaliana* TFs within the same family ($r = 0.35$), even when only considering TFs that also have highly similar consensus motifs ($r = 0.55$; Extended Data Fig. 1c). This suggests that the divergence of binding specificities between orthologous TFs plays a limited role in plant regulatory evolution.

Cis evolution across timescales

We next assessed TFBS conservation and divergence between closely related species and across diverse flowering plant lineages. The results above show that the binding of *A. thaliana* TFs in a different plant genome can serve as a strong predictor for the binding of the corresponding native TFs. Accordingly, we generated a more comprehensive multiDAP dataset of *A. thaliana* TFs covering the full range of families and distinct motifs. To dissect *cis* evolution at shorter timescales, we focused on the four closely related brassica species, using 244 *A. thaliana* TFs that performed well in our previous DAP-seq study[3]. We verified that multiplexing four genomic DNA samples did not significantly alter the binding profiles or decrease the signal (Extended Data Fig. 2a). To explore the opposite extreme of regulatory conservation, we measured the binding of 74 representative *A. thaliana* TFs in the genomes of all ten plants. This subset was selected to include well-studied TFs that showed distinct binding profiles in *A. thaliana*, and while not all have a direct orthologue in all ten profiled species, each represents the set of native TFs with the same motif preference. Although running the multiDAP assay with ten genomes simultaneously introduced a slight decrease in signal for some TFs, the overall binding profiles remained similar ($r = 0.87$; Extended Data Fig. 2a).

We detected TFBSs throughout all genomes, including in genes, promoters and distal intergenic sites upstream and downstream of genes. While the total number of TFBSs per genome was highly variable and dependent on genome size, the density of TFBSs near gene starts was more stable (Extended Data Fig. 2b). To quantify *cis* evolution at both short and long timescales, we first developed a reliable approach to identify TFBSs shared across species. We focused on TFBSs near the start of protein-coding genes, from 2,000 bp upstream to 500 bp downstream of protein start codons. We assigned all protein-coding genes from all species to orthogroups (an assignment based on shared amino acid sequences)[5]. Then, for each *A. thaliana* TF–target gene relationship, we generated a conservation score (c score), representing the number of species in which an orthologous gene had a binding site for the same TF (Fig. 1b). Target genes scored as c1 represent genes where a binding site for a given TF could be found only in *A. thaliana*, whereas target genes scored as c4 represent genes where binding sites for a given TF were found in *A. thaliana* plus orthologous genes from three other species. Our approach allowed us to detect conservation even across highly diverged species where intergenic sequence alignments cannot reliably identify conserved TFBSs[6–8].

Using our brassica atlas, we assigned c scores to 1.1 million total TF–orthogroup interactions represented in the *A. thaliana* genome. Of these, 24% were conserved across all four brassica genomes (c4), with the next largest category at 21% that were unique to *A. thaliana* (c1) (Extended Data Fig. 2c). By comparing the number of TF target genes shared between two or more (c2+), three or more (c3+) or all four species (c4) to a background frequency calculated from the overlap between sets of randomly generated TF target orthogroups, we found that all conserved TF target gene sets were significantly larger than expected by random chance ($P < 0.01$). Furthermore, the enrichment and significance increased as the c score increased (Extended Data Fig. 2d). This indicates that while considerable *cis* evolution has occurred since these brassica diverged 21 million years ago[9], a large set of core TF target genes were retained.

The 150-million-year evolutionary distance between the monocots and eudicots[4] within our flowering plant multiDAP atlas enabled the identification of smaller ultra-conserved TF target gene sets (Supplementary Table 3). Among the *A. thaliana* target orthogroups conserved in the four tested brassica species, 7.1% were further conserved across all ten species (c10 target genes). Many are consistent with previously elucidated TF functions and capture ancient pathways shared across flowering plants. For example, MYB family TF MYB107, along with several NAC and WRKY family TFs, consistently targets a core set of genes associated with suberin production[10–12], including key enzymes at all major steps in the suberin biosynthesis pathway and the upstream phenylpropanoid and fatty acid synthesis pathways, as well as transporters involved in exporting suberin monomers across the cell membrane

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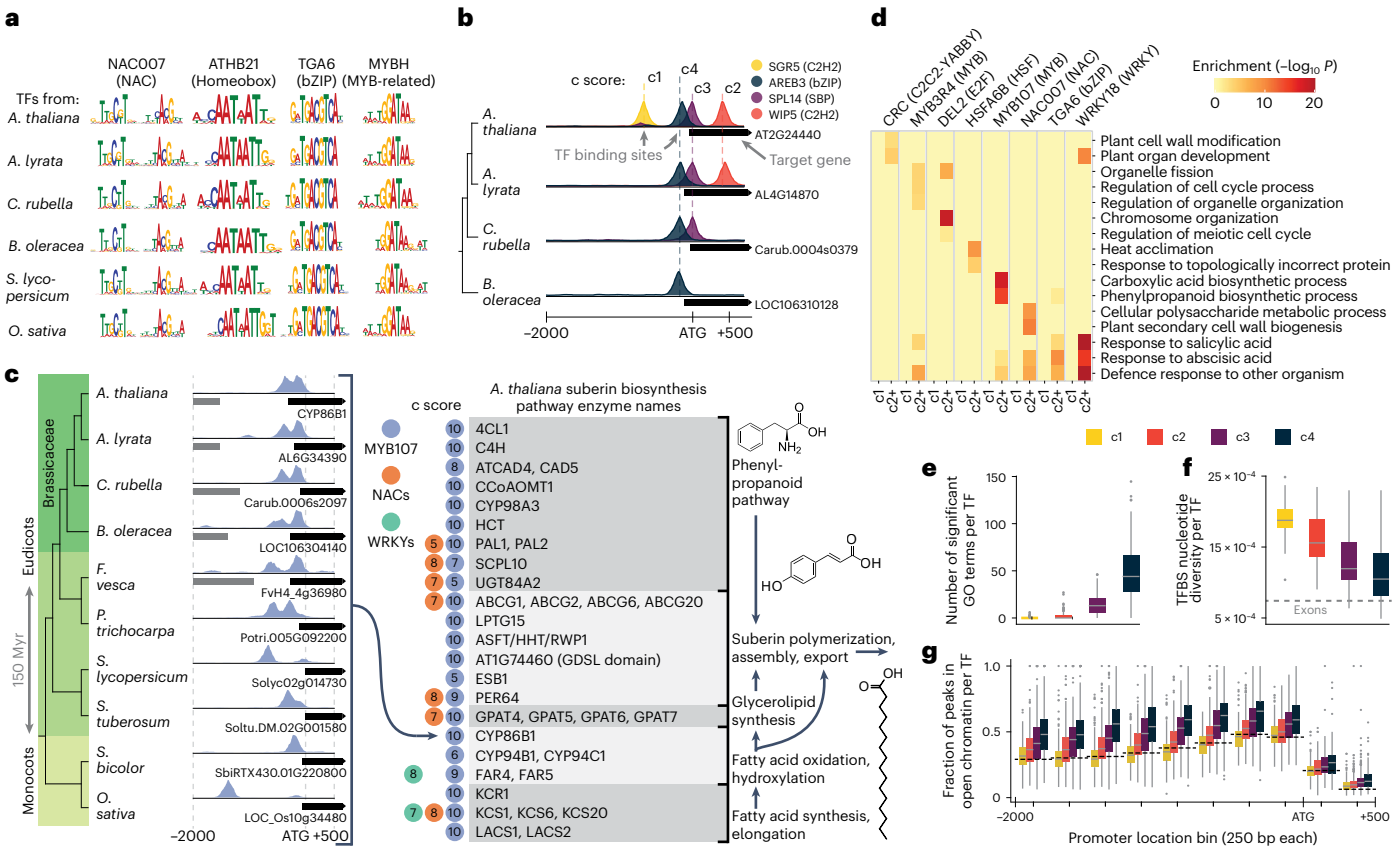

Fig. 1 | Plant multiDAP identifies motif specificities and TFBSs across 150 million years of evolution. a, High similarity of consensus motifs from four example sets of orthologous TFs from six species. **b**, Example of peak assignment to genes and c scores. **c**, Left, phylogenetic relationships between the ten species used in this study. The track plots show as an example the target gene *CYP86B1* (AT5G23190), which encodes an enzyme in the suberin pathway in *A. thaliana*, and its orthologues, all targeted by MYB107. Right, key enzymes in suberin biosynthesis, which are ultra-conserved targets of MYB107 and other TFs in the NAC and WRKY families. The colours of the circles and the inset numbers next to each enzyme name indicate TFs and c scores, respectively. **d**, GO term enrichment of non-conserved target genes (c1) versus conserved target genes (c2+) for selected example TFs. **e**, Number of GO terms significantly enriched in target gene sets. The colours for each c score shown in the legend are used throughout **e**–**g**. The box plots in **e**–**g** are drawn according to standard conventions, where the central line represents the median, the upper and lower box bounds represent the upper and lower limits of the interquartile range, and the whiskers extend to the furthest data point within 1.5× the interquartile range from the box, with $n = 244$ TFs. **f**, Average nucleotide diversity of TFBS regions in the 1001 *A. thaliana* genomes. **g**, Enrichment of open chromatin within TFBS regions, separated by c score and binned by promoter location. The dashed lines show the expected frequencies of open chromatin overlap in each bin calculated from a random background distribution. See also Extended Data Figs. 1 and 2 and Supplementary Tables 1–5.

(Fig. 1c)[10,13,14]. We further uncovered many ultra-conserved yet previously unknown MYB107 target genes with predicted suberin-related functions, which may serve as attractive targets for future characterization. Other TFs that have retained a core set of ultra-conserved target genes play key roles in coordinating development or environmental responses, such as PC-MYB1/MYB3R1 in cell division[15], GTL1 in root hair development[16], Dehydration Response Element 1A (DREB1A) in drought response[17] and CAMTA1/EICBP.B in stress hormone responses[18]. Overall, while TFBSs have diverged more rapidly than TF binding specificities, our atlas revealed substantial conservation of TF target genes in closely related species, along with a smaller set of ultra-conserved core regulons that have persisted across 150 million years of flowering plant evolution.

Conservation highlights functional TFBSs

We hypothesized that TFBSs regulating critical gene functions would be among those conserved between closely related species, reflecting the selective pressure to maintain key regulatory interactions, while TFBSs with little impact on gene expression would not be subject to the same selective pressure. To investigate this, we applied several independent approaches to test whether TFBSs conserved within the brassica dataset exhibited signatures of functional *cis*-regulatory elements (CREs). First, we found that while c1 target gene sets were rarely enriched for functional Gene Ontology (GO) terms, conserved *A. thaliana* target gene sets with c scores of c2 or greater were significantly enriched for GO terms that are consistent with known TF functions, suggesting that conservation across even two closely related species highlights functional binding sites (Fig. 1d; see also Supplementary Table 4). Among these were TFs spanning key developmental and environmental response pathways, with GO terms related to organ development for CRC[19], cell division for MYB3R-4 (ref. 20) and DEL2 (ref. 21), heat stress for HSFA6B[22], suberin synthesis for MYB107 (ref. 10), and response to pathogens and stress-related hormones for TGA6 (ref. 23), WRKY18 (ref. 24) and NAC007/VND4 (ref. 25). The number of significantly enriched GO terms increased as the conservation level increased (Fig. 1e). Some of these functional associations extend past brassica and were strongly enriched among the ultra-conserved c10 target genes in the ten-species dataset (Supplementary Table 5). Second, we found evidence of purifying selection acting to preserve the sequences immediately underlying conserved peak summits across wild *A. thaliana* populations[26]. We observed decreasing nucleotide diversity at peaks with higher c scores, with significantly lower nucleotide diversity underlying c4 TFBSs than all other TFBS sets (*t*-test, $P < 0.05$), nearly as low as that found in protein-coding exonic regions (Fig. 1f). Finally, conserved

binding sites for most TFs showed enrichment in accessible chromatin regions identified by ATAC-seq[27]. Since both TFBSs and accessible chromatin regions are known to be concentrated near transcription start sites[27], we split *A. thaliana* promoter regions into 250-bp bins and found that conserved TFBSs in all promoter bins were enriched in accessible chromatin regions, while c1 TFBSs were not (Fig. 1g). Taken together, these results demonstrate that conserved TFBSs are enriched for CREs and highlight the power of using conservation to identify functionally important TF target genes.

Charting TF activity in single nuclei

As conserved TFBSs are enriched for the hallmarks of functional CREs, TF binding at these sites is likely to impact expression of the associated target gene. We therefore explored whether quantifying the expression of each TF's conserved target genes would allow us to chart TF activity across cell and tissue types in multiple species. To do so, we generated single-nucleus RNA sequencing (snRNA-seq) atlases for seedling root and shoot, mature leaf, and flower bud tissues of all four brassica species, profiling in total 163,982 high-quality single-nucleus transcriptomes, with a median of 899 unique transcripts and 742 genes per nucleus (Supplementary Table 6). Using published reference atlases[28–30] and marker gene lists[31], we assigned all *A. thaliana* nuclei to 61 unique cell-type labels (Fig. 2a, Extended Data Fig. 3a–c and Supplementary Table 7), including a small number of seedling labels that were not present in published protoplast-based atlases but were supported by the integration of an external seedling root nucleus dataset[32]. We used SAMap[33] to lift over labels from the *A. thaliana* atlases to those of the other three species (Fig. 2b), resulting in 58 labels with at least ten nuclei from all four species (Supplementary Table 8).

We first confirmed that the expression profiles of conserved target genes generally reflected regulation by the associated TFs. In the *A. thaliana* seedling atlas (the *A. thaliana* tissue with the deepest per-cell transcript coverage), co-expression of TF–target gene pairs was significantly higher than background and strongest among the most conserved (c4) target genes, regardless of TF binding affinity (Extended Data Fig. 4a). Furthermore, in every species, c4 target gene sets showed expression patterns that were significantly more cell-type-specific than those of c1, c2 or c3 target genes (Extended Data Fig. 4b), as would be expected of genes subject to strong transcriptional control by distinct TFs.

As a measure of TF activity, we then quantified the enrichment or depletion of expression of each TF's c4 target genes in each cell type (Supplementary Table 9 and Methods). The resulting set of TF activity scores yield an expansive map of TF regulatory roles in cell growth, differentiation and function, with 241 of 244 tested TFs demonstrating significant activity in at least one cell type. While many associations reflect known regulatory biology (Fig. 2c), such as the role of WRKY TFs in stress responses and senescence[24], the G2-like TF MYR2 in seedling and leaf phloem companion cells[34], and PC-MYB1/MYB3R1 and TCX2 in cell division[35,36], numerous previously uncharacterized TF roles were identified as well. For example, mesophyll cells at all developmental stages in every tissue (cotyledon, leaf and flower) were strongly and specifically associated with both HY5 (bZIP) and TRFL1 (MYB-related). While HY5 is known to be active in mesophyll cells[37], a role for TRFL1 or any close paralogues in mesophyll cells has not been previously documented. Extending our analysis to examine ultra-conserved TF target sets suggested that certain core regulons represent ancient cell-type-specific processes; for example, c9 target genes of secondary cell wall biosynthesis regulator NAC007/VND4 (ref. 38) were more specifically expressed in mature xylem cells than TF target sets at any other conservation level (Extended Data Fig. 4c). Accordingly, a TF's conserved target genes often retained the same cell type specificity across multiple species' atlases (Fig. 2c). For example, MYB107 target genes were strong and specific markers of late-stage endodermis cells (including lignified and suberized endodermis) in the seedling atlases of all four species. As several MYB-family TFs are known to contribute to suberin deposition[11], untested family members with similar binding specificity could be responsible for activating target gene expression. However, MYB107 expression was detected specifically in endodermis cells in multiple species' seedling atlases, supporting a previously unrecognized role for this TF beyond the seed coat[10]. bZIP52 target genes were specifically expressed in elongating endodermis, cortex and atrichoblast in all four species, consistent with recent work demonstrating the role of Group I bZIP TFs in the elongation of multiple root cell types[39]. Similarly, AP2/EREBP TF RAP2.9 target gene expression marked epidermal leaf cells in all four species, reflecting a potential role in leaf cuticular wax biosynthesis[40], and the target genes of ASR3 (Trihelix) showed petal-specific expression in all four species, consistent with Trihelix regulation of petal initiation[41]. In total, we identified key regulators of cell-type-specific expression (false discovery rate (FDR), <0.01; Cohen's $d \geq 0.8$) in 57 of 61 cell types, with all but 5 showing significant activity from more than one TF family. These results underscore the extensive coverage of our TFBS atlas and open new avenues for engineering targeted cellular responses in plants.

Regulatory networks define cell types

Our map of conserved TF target gene expression enrichments suggests that each cell type has a unique fingerprint of TF activity, often involving multiple TFs acting together. We next asked whether TFs with enriched target gene expression in the same cell type operate through discrete or overlapping regulons. We visualized each cell type's regulatory network, connecting enriched TFs to c4 target genes among the cell type's top 100 marker genes. In some cell types, a single TF family appeared to be acting as a master regulator. For example, in dividing cells, 77 marker genes were targets of MYB3R5 (Extended Data Fig. 5a), a 15-fold enrichment over the expected background. Of the remaining 23 marker genes, at most 5 were targeted by any other single tested TF, suggesting that MYB3R5 and TFs with similar binding specificity are the primary drivers of gene expression in the cell division pathway. We hypothesized that the regulation of such an ancient and ubiquitous pathway would be tightly conserved beyond brassica, and indeed, close to half (48%) of the markers targeted by MYB3R5 in *A. thaliana* had orthologues targeted by MYB3R5 in all ten profiled plants. In most cell types, however, the strongest marker genes were frequently co-targeted by multiple TF families. For example, in procambium, 64 of the top 100 marker genes were targeted by one or more TFs from the BBR/BPC and C2C2-DOF TF families, and 25 of these were co-targeted by both (Extended Data Fig. 5b), significantly more than expected by chance given the number of markers targeted by each individually ($P = 6 \times 10^{-5}$). Similarly, co-targets of both NAC- and MYB-family TFs were enriched among suberized-endodermis markers ($P = 0.007$) (Extended Data Fig. 5c), and co-targets of both Homeobox and MYB-related family TFs were enriched among mature-atrichoblast markers ($P = 8 \times 10^{-7}$) (Extended Data Fig. 5d). Across the 23 seedling cell types with multiple enriched TF families, 706 (30.7%) of the top marker genes were co-targeted by TFs from more than one enriched TF family. These results suggest that cell-type-specific regulatory networks are deeply intertwined, with multiple non-redundant TFs acting both independently and in concert to regulate gene expression.

We next tested how well the complete set of all measured TF activity scores characterizes a cell type, regardless of species, by calculating correlations between pairs of TF activity score profiles from cell types in different brassica species. We found the highest correlations between matching cell types in different species (Extended Data Fig. 5e), indicating that the core regulatory architecture of developmental programs has remained largely stable across the 21 million years separating these species, with the same TF families active in the same cell types across brassica. These findings confirm that TF activity score profiles capture the salient features of cell-type-specific regulation.

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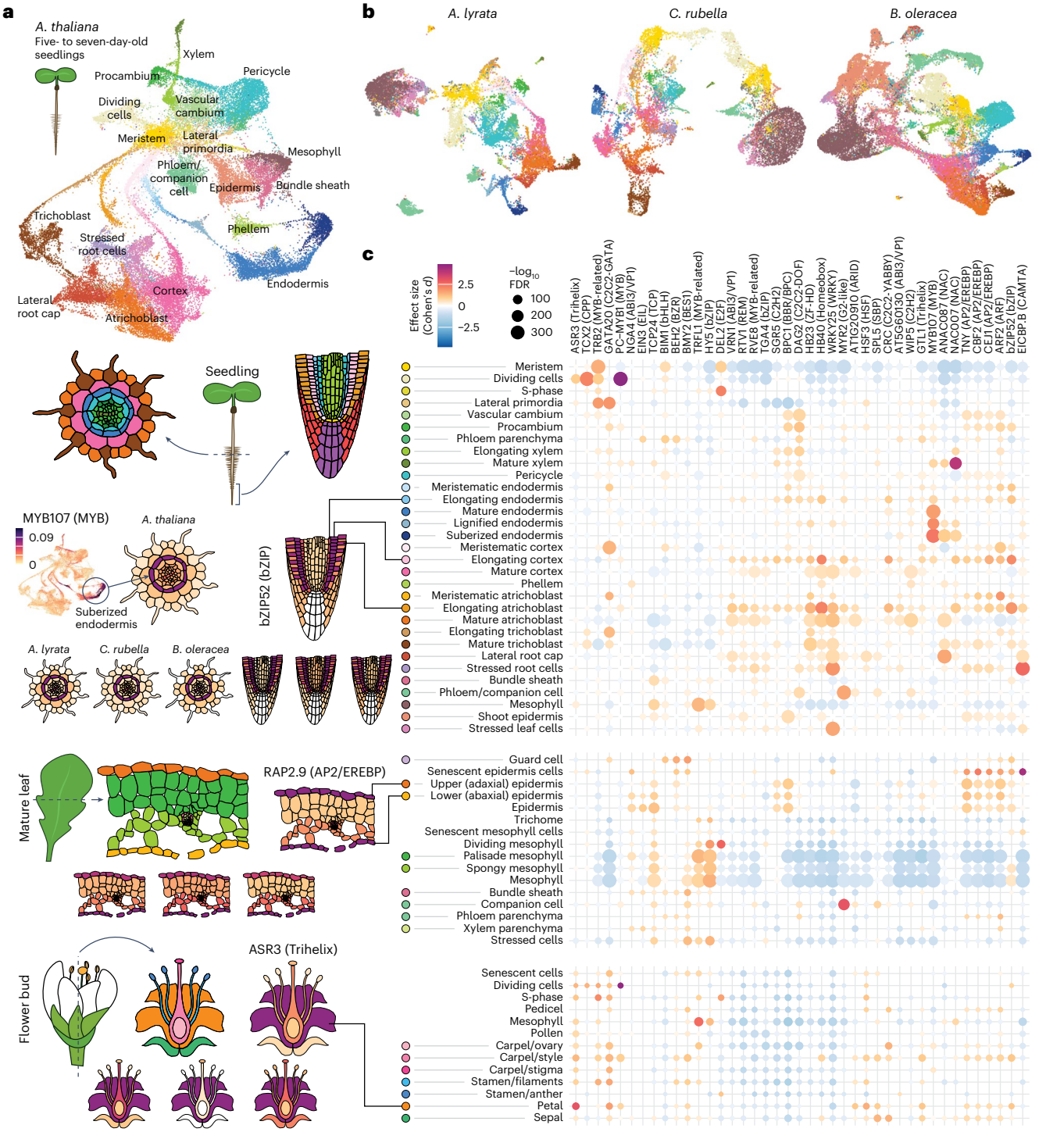

Fig. 2 | Expression of TF target genes across snRNA-seq seedling atlases enables the construction of a multi-tissue regulatory map of cell-type-specific gene expression programs. a,**b**, UMAP representation of the transcriptomes of >45,000 single *A. thaliana* seedling nuclei corresponding to 32 labelled cell types (**a**) and UMAP representations of corresponding data from three brassica relatives, with cell-type labels transferred from the *A. thaliana* atlas (**b**). The colours in **a** and **b** match the coloured circles shown beside the cell-type labels in the seedling portion of the heat map below. **c**, The heat map on the right shows enrichment or depletion of c4 target gene expression for representative TFs (horizontal axis) across labelled cell types in *A. thaliana* seedling, leaf and flower bud snRNA-seq atlases (vertical axis). Dot size represents the FDR-corrected $-\log_{10} P$ value from a Wilcoxon rank sum test of summarized target expression scores for each cell-type label versus all other cells, and dot colour represents the effect size of the expression enrichment (warm colours) or depletion (cool colours). Enrichments with FDR-corrected $P < 0.01$ are shown. The left side shows detailed plots for one example TF in each of four different anatomical regions. The spatial arrangement of cell types in a cross section of mature root is shown at the top far left, with cell types coloured according to the circles beside the cell-type labels on the vertical axis of the seedling portion of the heat map. Summarized expression of TF MYB107 c4 target genes in each cell is shown in the *A. thaliana* UMAP plot directly below, with the module score values for each cell mapped to colours as shown in the colour bar to the left of the UMAP (see Methods for details of module score calculations). Strong enrichment of target expression in suberized endodermis cells is highlighted with a labelled circle. Module scores were averaged across each cell type and then projected onto the spatial map of mature root cell types, shown directly to the right of the UMAP. Projections for the other brassica are shown directly below. The subsequent examples use the same structure but highlight bZIP52 target genes in root tips, RAP2.9 target genes in mature leaves and ASR3 target genes in flower buds. See also Extended Data Figs. 3–5 and Supplementary Tables 6–9.

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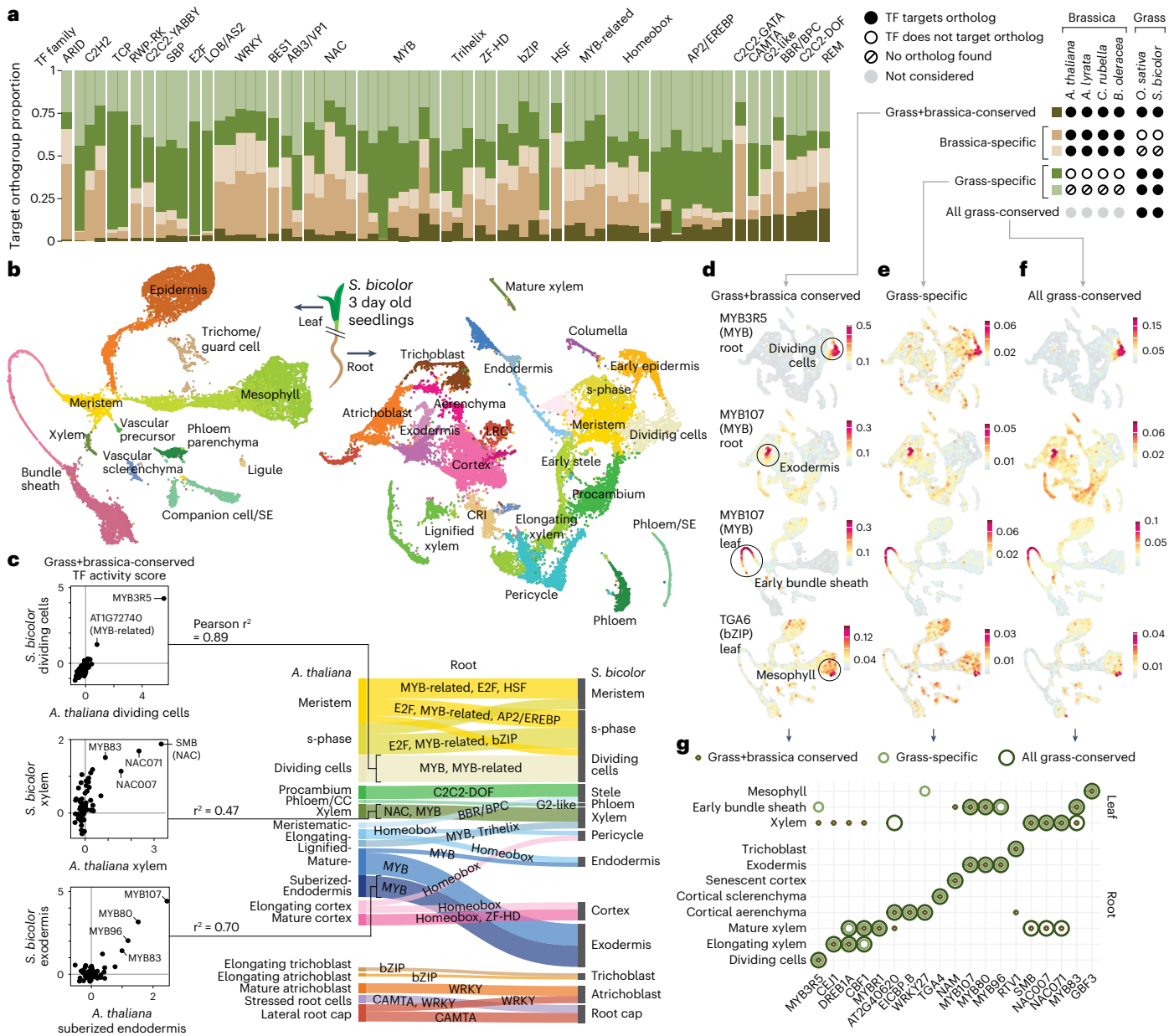

Fig. 3 | Single-nucleus transcriptomes of *S. bicolor* seedlings reveal conserved and grass-specific regulatory networks driving cell-type identity. a, Relative proportions of target orthogroups conserved within and between grass and brassica lineages, for each TF (horizontal axis). The colours correspond to the categories defined in the legend on the right: grass+brassica-conserved, TF target genes conserved in both grasses and all four brassica; brassica-specific, conserved in all four brassica but neither grass; grass-specific, conserved in both grasses but none of the four brassica; all grass-conserved, all TF target genes conserved in both grasses, regardless of brassica conservation. **b**, UMAP representation of the transcriptomes of >32,000 single sorghum nuclei corresponding to 43 labelled cell types. SE, sieve element; LRC, lateral root cap; CRI, crown root initials. **c**, Correlations of TF activity scores calculated for sorghum and *A. thaliana* cell types using target genes conserved in both grass and brassica lineages. Left, three examples shown as scatterplots, where each dot represents the activity score (measured by Cohen's *d*) of a TF in an *A. thaliana* cell type (*x* axis) versus a sorghum cell type (*y* axis). Right, diagram linking root cell types between *A. thaliana* and sorghum, where the widths of the connections correspond to the TF activity score profile correlations (Pearson r^2) between each pair. Only correlations with $r > 0.3$ are shown, after filtering to the top two correlations per sorghum cell type. **d**, Cross-lineage conserved target genes identified via multiDAP using *A. thaliana* TFs display cell-type-specific expression patterns in sorghum. The module score values for each cell were mapped to colours as shown in the colour bar to the right of each UMAP (see Methods for details of module score calculations). **e**, Expression patterns of grass-specific TF target gene sets for the same TFs shown in **d**. **f**, Expression patterns of all TF target genes conserved in both grasses for the same TFs shown in **d** and **e**. **g**, Examples where strong enrichments for all three TF target gene subsets appear in the same sorghum cell type. Only enrichments with FDR < 0.05 and Cohen's $d > 0.5$ are shown, after filtering to the top two enrichments per TF across all root and leaf cell types. See also Extended Data Figs. 6–10 and Supplementary Tables 10–12.

Recruitment and rewiring of TF networks

Having established that (1) *A. thaliana* TFs are strong predictors of orthologous TF binding in distantly related species, and (2) TFBSs conserved within the brassica family underlie regulatory networks that drive cell identity, we next leveraged the binding maps of the 74 *A. thaliana* TFs profiled in our ten-species multiDAP dataset, representing a wide breadth of TF families and binding specificities, to investigate the fate of brassica-conserved TFBSs across the 150 million

years separating brassica from monocots. We first assigned grass-c2 scores to TFBSs shared by two model monocots in the grass family, rice and sorghum, and then looked for overlap with TFBSs conserved in all four brassica species (brassica-c4 TFBSs), differentiating persistent core regulons from lineage-specific TFBSs that we ascribe to TF rewiring (Fig. 3a). Overall, we found that 9,027 (21%) brassica-c4 target orthogroups were also conserved in both grasses, constituting core regulons of target genes controlled by the same TF in all six species. An additional 50% of brassica-c4 target orthogroups were absent in both grasses; half of these (10,530) corresponded to orthogroups lacking grass genes, while most of the rest represented orthogroups with lineage-specific TFBS loss or gain. Across the TFs, we observed a wide range of cross-lineage conservation, with anywhere from 4% to 80% of brassica-c4 target orthogroups conserved in both grasses, yet the vast majority (87%) had core regulons significantly larger than expected by chance (FDR < 0.05; Extended Data Fig. 6). Overall, the widespread enrichment of cross-lineage TFBS conservation suggests that the majority of TFs control core regulons that persist across flowering plants, alongside substantial rewiring.

To explore the functional consequences of conservation and rewiring, we generated snRNA-seq datasets from leaves and roots of three-day-old sorghum seedlings, comprising a total of 13,000 and 19,000 nuclei, respectively, after stringent quality filtering, and we identified 43 cell types (Fig. 3b, Extended Data Fig. 7 and Supplementary Table 10). We first asked whether the same TFs were active in brassica and grass cell types, using core regulon target genes as markers of TF activity. Comparing activity scores across 74 TFs for each *A. thaliana* and sorghum cell type (Supplementary Table 11), we found the highest correlations between many corresponding cell types (Fig. 3c, Extended Data Fig. 8 and Supplementary Table 12). These correlations were primarily driven by small collections of TF families controlling different cell layers, including MYB, MYB-related and E2F in meristematic and immature cell types; C2C2-DOF, G2-like and NAC in vascular layers; and WRKY, bZIP and CAMTA in terminal developmental stages of the epidermis and root cap. Furthermore, many core regulons for individual TFs showed strong cell-type-specific expression in sorghum. For example, the expression of MYB3R5 target genes was enriched in sorghum dividing cells, while MYB107 target genes displayed strong expression in bundle sheath and exodermal layers, and TGA6 target genes marked a discrete set of cells in the mesophyll (Fig. 3d).

We also observed several striking examples of regulatory rewiring reflecting distinct patterns of cell-type evolution. For example, the genes coding for *MYB83* (AT3G08500) and *NAC007* (AT1G12260) TFs are strongly expressed in mature xylem cells of both species (Extended Data Fig. 9), where their target genes indicate high TF activity, consistent with the known roles of NAC and MYB families in secondary wall biosynthesis[42,43]. However, our data revealed that the dominant family shaping xylem identity is reversed in the two plant lineages (Extended Data Fig. 10). While NAC007 and related factors play the primary role in *A. thaliana* (with 27 of the top 100 xylem marker genes carrying a brassica-c4 NAC007 TFBS, compared with 4 with a MYB83 TFBS), MYB83 and related factors appear to dominate in sorghum xylem (where only 7 of the top 100 xylem markers have a grass-c2 NAC007 TFBS, yet 14 have a grass-c2 MYB83 TFBS). Moreover, we found ample evidence of direct TFBS switching within individual pairs of orthologous genes. Among the top 50 *A. thaliana* xylem markers, we identified 7 cases in which the *A. thaliana* gene is a target of NAC007 but not MYB83, while the sorghum orthologue is a target of MYB83 but not NAC007, an 81-fold enrichment compared with all *A. thaliana* genes with a sorghum orthologue. Six of these seven sorghum orthologues, including *IRX6* (SbiRTX430.02G374300), *IRX3* (SbiRTX430.02G210900), *LAC17* (SbiRTX430.03G380300) and *NAC073*, were still strong markers for xylem in sorghum. In fact, *NAC073* has two sorghum orthologues: one orthologue (SbiRTX430.09G246200) mirrors the *A. thaliana* gene with a NAC007 TFBS and no MYB83 TFBSs and is moderately enriched in xylem ($\log_2$(fold enrichment) = 1.8). The second (SbiRTX430.03G271900) shows the reverse TFBS profile (no NAC007 TFBS and a grass-specific MYB83 TFBS) and is more than twice as enriched in xylem ($\log_2$(fold enrichment) = 4.1). Importantly, these lineage-specific differences cannot be attributed to technical artefacts, as with the multiDAP approach, all species are jointly incubated with each TF and sequenced as a pool.

A second example revealed recruitment of developmental pathways that may contribute to sorghum's ability to survive in hot, dry conditions. Drought tolerance is in part enabled by an exodermal cell layer that produces suberin, a waxy substance that regulates water and nutrient transport and provides a protective barrier to roots[44]. While this layer is absent in *A. thaliana*, it instead suberizes an internal cell layer, the endodermis. Consistent with this, we observed a strong correlation of TF activities between sorghum exodermis and *A. thaliana* endodermis (Fig. 3c), primarily driven by MYB family TFs, which regulate suberin and lignin production[45]. Furthermore, we found evidence that this MYB-controlled network has been recruited to the bundle sheath of sorghum leaves (Fig. 3d), where suberin forms the gas-tight layers that support the more efficient C_4 carbon fixation pathway employed by many grasses[46]. Recruitment of the MYB107 regulon was accompanied by substantial regulatory rewiring. Thirty-one of the top 100 sorghum exodermis markers and 24 of the top 100 early bundle sheath markers were associated with MYB107 TFBSs that were detected in both grasses but none of the brassica species. In both cases these grass-specific target genes outnumbered MYB107 core regulon genes among the top cell-type markers. While the majority lacked brassica orthologues altogether, in both cell types the subset with known brassica orthologues showed clear evidence of regulatory divergence, as none of these orthologues were strongly expressed in *A. thaliana* endodermis.

In fact, we found a large number of grass-specific TFBSs for almost every TF. If these represent true TF target genes, we reasoned that their expression should generally mirror that of core regulon target genes. In addition to MYB107, several TFs including MYB3R5 and TGA6 showed very clear alignment between the expression patterns of grass-specific and core regulon target genes (Fig. 3e,f). A more comprehensive search revealed 17 additional examples of TFs where the strongest enrichments aligned in the same cell type (Fig. 3g). In some cases, these grass-specific target genes outnumbered core regulon genes by a factor of 10:1, yet strong evidence of shared transcriptional control was observed. Together, this suggests that many grass-specific TFBSs reflect functional CREs that place new target genes under the control of TFs driving core developmental programs. Importantly, these results hinge on the ability of *A. thaliana* TFs to identify grass-specific target genes that may not even exist in any brassica genome.

Collectively, these findings suggest how selective repurposing of ancient gene networks may support the emergence of new cell types and functions, which together with widespread *cis*-regulatory rewiring give rise to lineage-specific traits. In conjunction with complementary studies in systems spanning photosynthetic cell types[47] to human neurons[48], this supports an emerging model of how ancestral TFs are harnessed during the evolution of specialized phenotypes.

Discussion

Our study presents a comprehensive account of how TF regulatory networks shape cell-type identity and evolution, leveraging new binding maps for 360 TFs and 14 new single-nucleus transcriptomic atlases across multiple tissues and both major lineages of flowering plants. Together, these provide a foundational resource for unravelling regulation through the lens of comparative genomics. By integrating these atlases, we demonstrated that conserved target genes are powerful predictors of TF pathway functions and that core TF regulons persist alongside ample rewiring throughout the course of plant evolution.

Further work is required to fully understand the complexities of gene regulation, including the impact of epigenetic marks, protein domains outside the TF's DNA binding region and cofactor interactions. While we chose to focus on promoter regions near protein-coding genes, our plant multiDAP atlas includes distal binding sites, which may also contribute to gene regulation. In addition, overlapping binding specificities of multiple TFs within a family complicate the definitive attribution of regulatory outputs to specific TFs. Nevertheless, we observed many cases where conserved binding sites for specific TFs known to play roles in a developmental response program are highly predictive of relevant gene expression patterns even in divergent species. Finally, while we focused here on conserved TFBSs, both our previous bacterial multiDAP study[2] and a DAP-seq study of two maize strains[49] identified strain-specific binding sites that impact expression and phenotype. Future studies combining our atlas with genetic, epigenetic and functional resources may help identify patterns associated with novel functional binding sites. Expanding this atlas to include more species, developmental stages and environmental conditions will deepen our understanding of how regulatory network evolution generates phenotypic diversity, with broad implications for synthetic biology and agricultural biotechnology.

Methods

Plant germplasm and reference genomes

Reference genome sequences and annotations for *A. thaliana*[50], *A. lyrata*[51,52], *C. rubella*[53], *B. oleracea*[54], *F. vesca*[55], *P. trichocarpa*[56], *S. lycopersicum*[57], *S. tuberosum*[58], *S. bicolor* (these sequence data were produced by the US Department of Energy Joint Genome Institute) and *O. sativa*[59] were downloaded from Phytozome or NCBI RefSeq. Germplasm was obtained from ABRC, USDA, TGRC or in-house collections. See Supplementary Table 2 for a full list of germplasm and reference genome sources.

DAP-seq experiments

The DAP-seq and multiDAP assays were performed using the reagents as previously described[2,3,60], but with modifications to optimize sensitivity. All 96-well plate operations took place on a Hamilton Vantage liquid handler. Genomic DNA was extracted from each of the ten plant species using a CTAB protocol, after which genomic DNA fragment libraries were constructed with an average shearing size of 150 bp achieved via ultrasonic shearing on a Covaris LE220-plus with the following settings: water temperature, 5–9 °C; time, 420 seconds; peak power, 450 W; duty factor, 30%; cycles per burst, 200. Different species were labelled using unique Illumina i5 index adapters. Upstream of the DAP-seq assay, fragment libraries were amplified for ten cycles to remove any DNA modifications with the same primers as previously described[2].

The coding sequences (CDSs) of plant TFs were cloned into the pIX-HALO-PaqCI vector, a modified vector optimized for chewback cloning but resulting in identical assembled plasmid sequences as the original pIX-HALO[3,60]. The HaloTag-fused TF CDSs were amplified with the primers pIX-Halo-T7-fwd (5′-GTGAATTGTAATACGACTCACTATAGGG) and pIX-Halo-AfterPolyA-rev (5′-CAAGGGGTTATGCTAGTTATTGCTC) using KAPA HiFi HotStart ReadyMix (Roche KK2602). The resulting PCR products were cleaned up using the Mag-Bind TotalPure NGS Kit (Omega M1378-02), and correct amplicon sizes were verified on a Fragment Analyzer system (Agilent) using the HS NGS Fragment Kit (Agilent DNF-474-1000). A total of 10 µl at a minimum concentration of approximately 100 ng μl^{-1} of each PCR amplicon was used to express TF proteins in vitro using the TnT T7 Quick for PCR DNA System (Promega L5540) with an incubation at 30 °C for 2 h. Each 96-well plate of protein included four negative control wells containing a mock in vitro protein expression reaction without any DNA expression template, to be used for background signal subtraction in the downstream peak-calling step.

For each sample, 20 µl of Magne HaloTag Beads (Promega G7282) were first washed and resuspended in 50 µl of binding/wash buffer (1× phosphate buffered saline and 0.005% NP40 detergent). To bind TFs to the magnetic beads, the washed beads were combined with 100 µl of each protein expression reaction and incubated at room temperature for 1 h on a rotator. The HaloTag–TF-bound beads were gently washed in 150 µl of the same binding/wash buffer four times, using a magnetic plate adapter to pellet the beads and discard the wash buffer between each wash. Genomic DNA libraries in a volume of 20 µl 10 mM Tris-Cl (pH 8.5) were brought to a total volume of 100 µl using binding/wash buffer, combined with the beads, and incubated on a rotator at room temperature for 1 h. Input genomic DNA library amounts were titrated on the basis of genome size to target equal sequence coverage of each species: *A. thaliana*, 100 ng; *A. lyrata*, 172 ng; *B. oleracea*, 407 ng; *C. rubella*, 112 ng; *F. vesca*, 183 ng; *O. sativa*, 312 ng; *P. trichocarpa*, 327 ng; *S. lycopersicum*, 652 ng; *S. tuberosum*, 618 ng; *S. bicolor*, 565 ng. In addition, 10 µg of salmon sperm DNA (Invitrogen 15632011) was added alongside the genomic DNA libraries to reduce background signal caused by non-specific binding. After incubation, the beads were again gently washed with the same wash buffer four times, after which the wash buffer was removed and discarded. We found that the wash conditions are critical to achieving high sensitivity. At each wash step, the bead pellet should be fully resuspended via gentle pipetting of the wash buffer stream directly at the bead pellet. After the final wash, the beads were resuspended in 22 µl of low EDTA TE buffer (10 mM Tris-Cl, 0.1 mM EDTA, pH 8.5) containing i7 index primer mix at a concentration of 1 µM each[2]. This solution was heated to 95 °C for 10 min, after which 20 µl of eluate from each well was transferred to a new plate and mixed with an equal volume of KAPA HiFi HotStart ReadyMix. The plate was then placed on a thermocycler running the following program: an initial denaturation step of 95 °C for 3 min, followed by 10 cycles of 98 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s, with a final extension time of 72 °C for 1 min then a hold at 4 °C. Equal volumes of each reaction were pooled across rows of the plate and then purified via gel purification. The pools were sequenced on either an Illumina NovaSeq 6000 S4 flow cell or a NovaSeq X plus 10B or 25B flow cell, using 2×150 bp paired-end sequencing, targeting ten million reads (five million fragments) per TF for *A. thaliana*, and proportionally scaled to larger genomes as dictated by the relative genomic DNA library input mass. For the four-species brassica dataset, we identified libraries from that first round of sequencing with low sequencing yield and selectively pooled these for a second round of sequencing to increase coverage.

Single-nucleus experiments

Plant growth conditions. *A. thaliana*, *A. lyrata*, *C. rubella* and *B. oleracea* seeds were surface-sterilized before being planted vertically on agar plates containing 3 mM $Ca(NO_3)_2$, 1.5 mM $MgSO_4$, 1.25 mM $NH_4H_2PO_4$, 1 mM KCl and 1× micronutrients (Murashige and Skoog Micronutrient Salts 100×, MSP18-10LT), in 0.8% agar at pH 5.7. The seeds were sterilized by soaking in 75% ethanol for 1 min, 50% bleach for 5 min and 25% bleach with 0.2% Triton for 4 min, then rinsed with sterile water eight times. After sterilization, seeds in 2-ml Eppendorf tubes were wrapped in tin foil and put into a 4 °C fridge for cold stratification. The seeds were left in the fridge for three days for *A. thaliana* and *C. rubella* or five days for *A. lyrata*. Cold stratification was not needed for efficient germination of *B. oleracea* seeds. We staggered the planting of the four species to account for their different germination times, allowing us to harvest seedling root and shoot tissues of plants at the same five- to seven-day-old growth stage of all species on the same day. *A. lyrata* was planted on the agar plates three days before *A. thaliana*, *C. rubella* was planted one day after *A. thaliana* and *B. oleracea* seeds without cold stratification were planted two days after *A. thaliana*. The conditions of the growth chamber (Percival Scientific) were set to 16 h light at 22 °C and 140–150 µmol m^{-2} s^{-1} illumination followed by 8 h dark at 19 °C, with the light turning on at 5:00. Roots, shoots and whole seedlings were sampled between 10:00 and 12:00.

To enable sampling of mature leaves and flower buds on the same day for all four species, planting times were staggered and growth conditions were modulated to synchronize the growth of all four species. For mature leaves, we sampled the youngest fully expanded rosette leaf from plants grown in soil in a plant grow room or cold room. *B. oleracea* mature leaves were harvested from plants that were two months old grown at 13 °C under a light cycle of 12 h light/12 h dark. *A. lyrata* was three months old at harvest, grown at 4 °C with a light cycle of 12 h light/12 h dark. *A. thaliana* and *C. rubella* were grown under conditions of 16 h light at 28 °C and 8 h dark at 23 °C and harvested at two months old. Mature leaves were sampled between 10:00 and 12:00.

For floral buds, we sampled young buds on the main stem from plants grown in soil, where the petal was just emerging (~1 mm visible). All four plant species were grown at 16 h light at 22 °C and 8 h dark at 19 °C when the floral buds were collected. *A. thaliana* and *B. oleracea* flowered at about two months old without cold treatment. To induce flowering in *A. lyrata* and *C. rubella*, two-month-old plants were transferred into a 4 °C cold room for two months and then transferred back to conditions with 16 h light at 22 °C and 8 h dark at 19 °C for one month. In this way, all four plant species were synchronized to flower on the same day and sampled between 10:00 and 12:00.

S. bicolor seeds were sterilized, planted vertically on agar plates as above and placed in a growth chamber (Percival Scientific) set to 28 °C with 800 µmol m^{-2} s^{-1} illumination, 16 h light/8 h dark, with the light turning on at 8:00 daily. Samples from three-day-old seedlings were collected between 10:00 and 11:00.

Nuclei isolation. Plant tissue samples were either processed immediately after harvest or flash-frozen in liquid nitrogen and stored at −80 °C for up to one year before nuclei isolation. Nuclei isolation from tissues was performed as follows.

Buffer 1 (lysis buffer) consisted of 0.275 M sorbitol (Sigma-Aldrich S6021), 0.1% Triton X-100 (Sigma-Aldrich 93443), 0.01 M $MgCl_2$ (Ambion AM9530G), 1× protease inhibitor cocktail (Sigma-Aldrich 4693132001), 0.3 U $µl^{-1}$ RNase inhibitor (Roche 03335399001) and 1 mM DTT (Teknova D9750). Buffer 2 (wash and resuspension buffer) consisted of 1× phosphate buffer saline (without Mg and Ca), 1% BSA, 0.4 U $µl^{-1}$ RNase inhibitor and 1 mM DTT.

For isolation of nuclei from mature leaves containing high levels of chloroplasts, an extra modified wash and resuspension buffer (buffer 3) was used to eliminate chloroplasts before the final wash and resuspension step by buffer 2. It consisted of 0.275 M sorbitol, 0.2% Triton X-100, 0.2% NP40 (Thermo Fisher Scientific PI28324), 0.01 M $MgCl_2$, 1× protease inhibitor cocktail and 1 mM DTT.

All nuclei isolation steps were carried out in a cold room at 4 °C. For each sample, 20–100 mg of fresh or frozen tissue was chopped for 3 min using a razor blade on a glass plate with 100–200 µl of buffer 1, then transferred to a petri dish with 1.5 ml of cold buffer 1 and allowed to rest on ice with gentle shaking for 2 min. For multiplexed samples, tissues from different species were weighed separately before being combined and chopped together. The samples were then transferred to a 48-well filter plate (25 µm, 4 ml, Agilent, 201003-100), which was pre-wet with 1 ml of cold buffer 1 before it was placed on the receiving plate attached to a QIAvac96 (Qiagen). Pressure was maintained below 100 bar during filtration. The filtered nuclei solution was centrifuged at 500 *g* for 10 min at 4 °C to pellet the nuclei, after which the supernatant was discarded.

For samples with low or no chloroplast content, the pellet was resuspended via gentle flicking and pipetting, washed with 2 ml of cold buffer 2 and centrifuged at 500 *g* for 5 min, and the supernatant was discarded. For nuclei isolation from mature leaves with high chloroplast content, the pellet was resuspended via gentle flicking and pipetting and 10 ml of cold buffer 3 was added; the sample was then centrifuged at 500 *g* for 5 min, and the supernatant was discarded. The pellet was washed with 2 ml of cold buffer 2 and centrifuged at 500 *g* for 10 min, and the supernatant was discarded. For both protocols, pellets from the final centrifugation were resuspended in 100 µl of cold buffer 2 for snRNA-seq.

Nuclei quality and quantity were assessed via flow cytometry and microscopy before the single-nucleus partitioning and barcoding step. A BD Accuri C6 plus flow cytometer was used to analyse the quality and quantity of nuclei stained by propidium iodide (Sigma-Aldrich P4864), added to a final concentration of 50 µg ml^{-1}. The stained samples were incubated on ice in the dark for 5–10 min prior to analysis. The analysis was conducted using light-scatter and fluorescence signals produced by a 20-mW laser illumination at 488 nm. Signals corresponding to forward-angle scatter (FSC), 90° side scatter and fluorescence were collected. Fluorescence signals (pulse area measurements) were screened using the following filter configurations: (1) FL-2, with a 585/40-nm band-pass filter, and (2) FL-3, with a 670-nm long-pass filter. Threshold levels were set empirically at 80,000 for FSC-H to exclude debris commonly found in plant homogenates. Templates for uni-parametric (FL2-A and number of nuclei) and bi-parametric (FL2-A and FL3-A) frequency distributions were established. Upon identifying the region corresponding to nuclei, we collected data to a total count of 5,000–10,000 nuclei. The flow cytometer was operated at the Slow Flow Rate setting (14 µl sample per minute), with data acquisition for a single sample typically taking 3–5 min. Different plant species exhibited varying patterns of somatic endoreduplication within most of their tissues and organs. For example, in young *A. thaliana* roots and cotyledons, this variation is reflected in the form of multiple clusters in the distribution, corresponding to nuclei forming a 2C, 4C, 8C, 16C and so on, of the endoreduplicative series[61]. All the nuclei of all the endoreduplicative series were counted and divided by the actual volume analysed in the flow cytometer to obtain the concentration of the nuclei. An EVOS M5000 microscope was also used to validate nuclei quality and quantity by analysing 7 µl of diluted nuclei sample stained by 1 µl of diluted SYBR Green dye (Thermo Fisher Scientific S7585 diluted 1:10,000 in water) using a C-Chip Disposable Hemocytometer. For nuclei prepared from fresh tissue samples, most of the nuclei in each sample showed well-resolved edges without obvious evidence of blebbing. In the case of nuclei prepared from frozen samples, where cell debris around the nuclei can interfere with these microscopy observations, nuclei quality was primarily assessed by comparison to fresh nuclei's distribution and the endoreduplicative series pattern on the flow cytometer.

Single-nucleus library preparation. Single-nucleus RNA-seq libraries from *A. thaliana*, *A. lyrata*, *C. rubella* and *B. oleracea* tissues (two flower bud, two mature leaf, two seedling shoot and four to seven seedling root samples for each species, plus four whole-seedling samples for *A. thaliana* and *B. oleracea*; Supplementary Table 6) were prepared as follows. The nuclei suspension was diluted to a concentration of 700–1,000 nuclei per µl using cold buffer 2. Single nuclei were partitioned and barcoded using a Chromium system (10x Genomics) with the Chromium Next GEM Single Cell 3′ Reagent Kits v.3.1 (Dual Index). Approximately 20,000 nuclei in total were loaded for single-species samples, and up to 60,000 nuclei for multiplexed species samples. Library creation was carried out following the manufacturer's protocol. Sequencing was performed on an Illumina NovaSeq 6000 S4 flow cell or NovaSeq X plus 10B or 25B flow cell, using 2× 150 bp paired-end sequencing, targeting 200,000,000 fragments per species for RNA-seq samples.

Single-nucleus RNA-seq libraries from *S. bicolor* tissues (four *S. bicolor* root samples and three *S. bicolor* leaf samples; Supplementary Table 6) were prepared as follows. Note that some samples were multiplexed on the same lane of the cartridge with samples from several other plant species, which were resolved independently via mapping to their respective reference genomes after sequencing. For each sample, the pooled nuclei suspension was diluted to a concentration of 600–900 total nuclei per µl using cold buffer 2. Single nuclei were partitioned and barcoded on a BD Rhapsody system (BD Rhapsody HT Xpress, BD Rhapsody Scanner) using the BD Rhapsody WTA V3 kit and

cartridge, loading 50,000–150,000 total nuclei per lane. Library creation was carried out following the manufacturer's protocol. Sequencing was performed on an Illumina NovaSeq X plus 25B flow cell, using 2× 150 bp paired-end sequencing and targeting an average of at least 50,000 reads (25,000 fragments) per nucleus.

DAP-seq analysis

Primary DAP-seq data analysis pipeline. Fastq files were quality-filtered and adapters were trimmed using BBTools v.38.96 bbduk.sh[62] with the parameters k, 21; mink, 11; ktrim, r; tbo; tpe; qtrim, r; trimq, 6; maq, 10, and then aligned to the corresponding reference genome using bowtie2 v.2.4.2[63] with the parameters no-mixed and no-discordant. Bam files from negative control wells were merged using the merge command in samtools v.1.15.1[64] to generate a single background file for each 96-well plate. The callpeak command in MACS3 v.3.0.0a6[65] was used to generate narrowPeak files, using the merged background as the control, with the parameters call-summits; keep-dup, 1; and gsize with the total fasta genome size. Bigwig files were generated using deeptools v.3.5.2 bamCoverage with the parameters extendReads; binSize, 1; ignoreDuplicates; maxFragmentLength, 600; normalizeUsing RPGC; and effectiveGenomeSize with the total fasta genome assembly size of each species. Motifs were called from the top 100 strongest peaks identified in each library using the command meme in MEME suite v.5.3.0[66] with the parameters -dna; -revcomp; -mod, anr; -nmotifs, 2; -minw, 8; -maxw, 32, and with a background model (bfile) generated from the entire reference genome file with fasta-get-markov -m 0 -dna.

The resulting datasets were filtered to exclude those TFs and replicates with poor performance in the DAP-seq assay. As a measure of successful binding site enrichment, we calculated the fraction of reads in peaks (FRIP) scores and only used data from DAP-seq assays that generated FRIP scores of at least 0.05. For experiments including multiplexed species, we considered only those assays where each of the multiplexed species independently produced FRIP scores of 0.05 or greater. We found that multiplexing larger sets of species with varying genome sizes in a single multiDAP experiment sometimes resulted in large differences in read coverage between different TFs and in some cases differential enrichment of species. To mitigate the effect of low coverage resulting in false negatives in the peak-calling step (and thus lowering apparent c scores), we only used datasets that yielded at least 200,000 read pairs (400,000 reads) for each of the multiplexed species.

Assignment of DAP-seq peaks to target genes and c scores. DAP-seq peak files generated via the above analysis pipeline were further processed to identify target genes for each TF and in each species using a set of custom scripts. For each species, the corresponding gene annotation file (gff format) was filtered to retain only features corresponding to a single transcript per gene, for which the primary (or longest) transcript was selected. The script 1_assign_peaks_to_gff_features.sh (a wrapper for bedtools[67] v.2.31.0) was used to assign peaks to mRNA, exon and CDS features individually. The output was further processed using the script 2_annotate_peak_targets.py, which integrates information from the different assigned feature types to assign each peak to up to two gene targets and categorize their locations with respect to the target gene as upstream, downstream, within the 3′ or 5′ untranslated region (UTR), within the CDS or intronic. Peaks falling in intergenic regions were assigned to up to two gene targets, while peaks falling within the transcript (5′ UTR, CDS, intron or 3′ UTR) were assigned to only a single gene target.

The output of the above scripts was further processed with the script 3_filter_annotated_peaks_and_calculate_c_scores.ipynb, which was used to filter for peaks falling within the regions spanning −2,000 bp to +500 bp with respect to the target gene's start codon. Peaks were considered only if they had a strength of at least fivefold over background (as defined in the seventh signalValue column of the narrowPeak file format), and if they fell in one of the following regions: upstream, 5′ UTR, CDS or intron. Each target gene was then mapped to an orthogroup, as defined by applying OrthoFinder v.2.5.5[5] with the default parameters to the protein sequences of all species. To improve orthogroup resolution within the more closely related species, separate tables of orthogroups were generated and used to analyse the four-species datasets and the ten-species datasets. Finally, a c score was assigned to each TF–orthogroup assignment, representing a count of species in which the given TF also targets at least one gene within the same orthogroup. To exclude non-nuclear genes from the analyses, orthogroups containing one or more *A. thaliana* genes originating from the chloroplast or mitochondrial chromosomes were ignored.

For comparative analyses of the brassica and grass lineages, we used OrthoFinder with a manually curated species tree to call hierarchical orthogroups at each node in the species tree. To identify lineage-specific TF target genes within the brassica and grass families, we used the hierarchical orthogroups called at the base node of the corresponding clade.

See the code availability statement for scripts, reference genome files and sample data files.

DAP-seq signal comparisons. To assess DAP-seq signal similarity across individual DAP-seq assays, we compared bigwig files using the multiBigwigSummary bins command in deeptools[68] v.3.5.2 with the parameter binSize 50. To reduce computation time, this analysis was restricted to only the first chromosome of the *A. thaliana* genome using the flag region Chr1. The resulting output was processed to generate a correlation matrix with the deeptools command plotCorrelation and corMethod pearson.

GO term enrichment. We used clusterprofiler v.4.6.2[69] to perform GO enrichment analysis on the list of target genes for each TF and c score. We first converted the TAIR gene names into the Entrez IDs. We then calculated enrichment values for Biological Processes and filtered for enriched terms with level six to avoid broad GO terms. We then used the simplify function to combine terms with a similarity cut-off of 70%.

Nucleotide diversity in TFBSs. We used the 1001 Genomes polymorphism data[26] to calculate nucleotide diversity with vcftools v.0.1.17[70]. We only considered regions flanking peak summits (±30 bp from the peak summit location) that were located within 2,000 bp upstream of the assigned gene's CDS start. We did not include regions downstream of the start codon or regions overlapping CDSs in the 2,000 bp upstream of the assigned gene.

snRNA-seq analysis

snRNA-seq atlas construction. Raw fastq files for each library were filtered using BBTools v.38.96 bbduk.sh[62] to remove read pairs with 31-mers in read 2 matching common ribosomal 31-mers. Nuclear reference genomes for all species were downloaded from Phytozome where available and supplemented with organellar genomes from NCBI. For brassica species (profiled with 10X Chromium), rRNA-filtered reads were pre-processed with Cellranger count v.7.0.1, and the resulting bam file was run through Velocyto[71] to generate spliced and unspliced count matrices. Sorghum libraries (profiled with BD Rhapsody) were pre-processed with the BD Rhapsody CWL pipeline (v.2.2, available at bitbucket.org/CRSwDev/cwl) and in parallel pre-processed with STAR with the parameter soloFeatures Velocyto to generate spliced and unspliced count matrices. After pre-processing, quality control metrics were compiled for each sample by (1) calculating the total unique molecular identifiers (UMIs) and genes from each cell barcode (CBC), (2) calculating the proportion of UMIs from each CBC mapping to organellar genomes, (3) associating each CBC with an overall rate of unspliced versus spliced transcripts and (4) running the R package

diem[72] to estimate the rate of ambient RNA in each CBC. The CBCs were then filtered to those with at least 400 nuclear UMIs, 200 nuclear genes, 90% nuclear UMIs, 10% unspliced UMIs and debris score ≤2. The 400-UMI threshold was relaxed to 300 UMIs for flower bud samples to obtain enough cells for meaningful clustering, but all other filters remained unchanged. In some experiments, nuclei from multiple species were pooled together, as inter-species sequence divergence is sufficient to distinguish transcripts on the basis of genome alignment, and we did not observe any reads multi-mapping to annotated genes from different species. For these mixed-species samples, additional filtering was performed by defining background proportions for each species using all UMIs in the raw expression matrix and then running a chi-squared test for each CBC to determine whether the species proportions calculated from the CBC's associated UMIs were significantly different ($P < 0.01$) from background. CBCs passing this test were assumed to contain nuclei from one or more species. To determine which species were present, a per-species UMI threshold was defined as the 99th percentile of per-species UMIs across all CBCs failing the chi-squared test. Each CBC was then assigned an estimated ambient RNA proportion by comparing total UMIs from present species to total UMIs from all species, and CBCs with >50% estimated ambient RNA were further filtered from downstream analysis. As the rate of unspliced transcripts proved to be an especially informative metric[73] for sorghum libraries, these libraries were further filtered by detecting the inflection point (aka the 'knee' in a knee-cliff plot) for all CBCs on the basis of unspliced rate and keeping only barcodes with a rate exceeding this threshold.

After each sample was filtered, count matrices for all remaining CBCs with organelle genes removed were loaded into Seurat (v.5.0.0)[74], and SCTransform v2 normalization was performed, followed by an initial clustering using Seurat's RunPCA (with 5,000 variable genes and 20 PCs), FindNeighbors and FindClusters (resolution 0.8) functions. Next, marker genes were identified for each cluster using FindAllMarkers (with logfc.threshold, 0.5; min.pct, 0.4; only.pos, T) filtered to those with p_val_adj < 0.05, and clusters with zero marker genes and significantly lower UMI counts than other clusters were removed as low-quality clusters. DoubletFinder[75] was then run on the remaining CBCs, and those with doublet score >0.4 were removed. To prepare the final atlases, samples from the same species and tissue (seedling, leaf or flower bud) were merged into a single Seurat object, which was then split by sequencing batch and renormalized with SCT per batch before performing a final PCA (50 PCs) and clustering (resolution 1.0 for brassica and sorghum leaf, 2.0 for sorghum root).

For the *A. thaliana* libraries, we assigned cells to unique cell types and developmental stages via label transfer from three well-annotated large-scale atlases (a root atlas[28], a leaf atlas[29] and a six-day-old seedling atlas[30]) as well as cluster-specific enrichments of known marker genes[31]. Labels were transferred from each published reference atlas to each SCT-normalized batch of the *A. thaliana* seedling and leaf datasets with Seurat's FindTransferAnchors function using 20 PCs, and each CBC was putatively annotated with the highest scoring label. Additionally, marker genes from each cluster in each of the three *A. thaliana* tissue datasets were tested for enrichment (hypergeometric enrichment test) of a large suite of previously computed marker genes downloaded from scPlantDB[31]. Final cell-type labels were compiled from these two sources, with additional manual annotation based on literature support for data-derived markers when there were discrepancies or no significant matches.

As a means of validating clustering and cell-type labelling, we directly integrated our 35,169 *A. thaliana* seedling snRNA-seq transcriptomes with an external *A. thaliana* snRNA-seq dataset[32] derived from seven-day-old seedling roots, resulting in a combined dataset of >45,000 nuclei. To combine in-house and external samples, and samples from different tissue subtypes (root, shoot and whole seedling), we tested a number of different integration techniques (CCA, RPCA and Harmony) using Seurat's IntegrateLayers function and evaluated each as to the separation of root- and shoot-derived CBCs and mixing of internal and external root-derived CBCs. The RPCA method using a sample tree to fix the integration order proved to be the most appropriate, with root-specific cell-type labels overwhelmingly composed of nuclei derived from root and whole-seedling samples (for example, trichoblast-labelled cluster 29 $\log_2$ root:shoot ratio, 5.08; Extended Data Fig. 3a), and comprising an approximately even mix of nuclei from the internal and external root datasets (cluster 29 $\log_2$ internal:external ratio, −0.65). Conversely, clusters receiving leaf-specific labels were strongly depleted for root-derived nuclei, including all nuclei from the external root dataset (for example, mesophyll-labelled cluster 13, with a $\log_2$ root:shoot ratio of −5.24). Integration also revealed mixed internal–external clusters (including clusters 35, 17 and 19) that appeared to be associated with endodermis subtypes not typically captured by protoplast-based assays. These clusters shared the expression of top endodermis-specific genes (for example, AT3G32980/PRX32; Extended Data Fig. 3c) but had distinct expression profiles. Cells in cluster 35 were mostly labelled mature endodermis via label transfer from the protoplast-based reference root atlas (Extended Data Fig. 3a), yet unlike other endodermis clusters they expressed key genes associated with lignin biosynthesis, including CASP1 and ERK1. Cells in cluster 17 received mixed low-confidence labels from the root atlas label transfer and strongly expressed GELP96 and CYP86A1, genes with known roles in suberin biosynthesis and polymerization. The vast majority of external root nuclei in this cluster were labelled "suberized-endodermis" in the original publication. The publication noted that this cell type was detected in only single-nucleus rather than single-cell datasets, potentially due to difficulty in digesting suberized cell walls. Cells in cluster 19 received low-confidence atlas labels as well, yet they included external cells from unlabelled "cluster 14b" in the original publication. This cluster was also noted in the publication as appearing only in snRNA datasets and showed tentative evidence of a role in cortex development and root epidermal patterning via expression of SCRAMBLED/STRUBBELIG. Both internal and external cells in our cluster 19 were differentiated from other endodermis cells by strong expression of FACT, a HXXXD-type acyl-transferase, and TLL1, a triacylglycerol lipase, both involved in the synthesis of root wax components. A recent study[76] identified TLL1 as a strong marker of suberized phellem in *A. thaliana*, and thus we labelled this unique cluster 'phellem'. Clustering of internal *A. thaliana* seedling cells without integration of the external dataset yielded a cluster with very similar expression. We note that a cluster was also identified in a separate published nucleus-based dataset[77] that showed highly similar expression patterns and was described as an "endodermis cluster with epidermis precursor cells". Additionally, we found a distinct subset of cells in each of our three other brassica seedling atlases with a best match to cells in cluster 19, indicating that this cell type is not unique to *A. thaliana*.

Aside from the *A. thaliana* seedling atlas, all other atlases were constructed by merging relevant libraries, as no batch effects were apparent and integration was not necessary. Final cell-type labels from the *A. thaliana* seedling, leaf and flower atlases were transferred to the respective datasets from the other brassica species using SAMap[33], selecting the top-scoring *A. thaliana* label per cell. Cell-type labels for the sorghum atlases were assigned on the basis of the expression of key experimentally validated cell-type marker genes found in the published literature (Supplementary Table 10 and Extended Data Fig. 7), as well as enrichments for maize marker gene sets identified in previously published single-cell atlases[31]. After final cell-type labels were assigned in each atlas, the top 100 marker genes per cell type were identified by filtering the results of Seurat function FindAllMarkers (with assay, SCT; min.pct, 0.1; only.pos, T) to those with p_val_adj < 0.01 and sorting by avg_log2-fold change.

TF–target co-expression analysis. SCT-normalized count data from the *A. thaliana* seedling atlas were used to calculate Spearman

correlation coefficients between the expression profiles of each assayed TF and every other gene across all nuclei. To compute final TF–target co-expression scores, we compared the squared correlation coefficient for a true TF–target pair to the squared correlation coefficient for the TF and a matched control gene. To find an appropriate control gene, all genes in the genome were assigned to one of ten equally sized bins on the basis of their average expression across all nuclei (expression-bin), as well as a bin representing the number of species (out of four) in which an orthologue was present (ortho-bin). We then compiled a list of 500 randomly chosen expressed target genes per TF and identified a matched non-target gene for each from the same ortho-bin and expression-bin. Next, we additionally binned each TF's target genes by fold change of the strongest DAP-seq peak (five equally sized peak strength bins per TF) and c score, and we calculated the average fold change between the squared correlation coefficient of true target genes and that of matched non-target controls within each group. We repeated this procedure 100 times to obtain mean and standard error values for each group.

Cell-type specificity and TF activity score calculations. The combined expression of each TF's target genes was used as a proxy for TF activity. As a first step, TF target gene expression was quantified in each cell of each atlas, using Seurat's AddModuleScore function (with assay SCT and the default parameters). For each cell, this function compares the average expression of a specified gene set to the expression of control gene sets, matched by average atlas-wide expression[74,78]. To allow these scores to represent relative differences between cells in different tissues, atlases for all tissues of each species were first merged together. Per-cell scores were computed separately for each TF's target genes at each conservation level (that is, c1, c2, c3 and c4 for brassica). For visualization, these per-cell scores were averaged over cells from each cell type, and the R package ggPlantmap v.1.0.0[79] was used to plot the averages as a heat map overlaid onto diagrams of plant tissue physiology.

To calculate an overall measure of cell-type specificity for each TF's target genes at each conservation level, an ANOVA test was conducted (using the R function aov) measuring the extent to which the variance in per-cell target gene scores across each brassica seedling snRNA atlas was explained by cell-type label (formula: score ~ cell type). The *F* statistic from this test was used as a metric of cell-type specificity. *F* statistic distributions across all TFs were plotted as box plots for each c score and each species, and paired one-sided *t*-tests (using the R function t.test) were performed for each species comparing *F* statistics from TF target sets at different conservation levels.

Per-cell target gene scores were then converted to a per-cell type measure of TF activity. A TF's activity score in a given cell type was defined as the enrichment or depletion of per-cell conserved TF target gene scores, compared to scores for all other cells. The magnitude of the enrichment or depletion was calculated using the function cohen.d from the R package effsize v.0.8.0[80], and separate one-sided Wilcoxon rank sum tests were run to test for significant enrichment and significant depletion. The same process was used to calculate TF activity scores in each sorghum and *A. thaliana* cell type using subsets of brassica-c4 or grass-c2 target genes from the ten-species multiDAP dataset.

Activity score profile correlations. TF activity score profiles were compiled for each cell type identified in each of the snRNA atlases. Each profile consisted of an array of values, one for each TF, representing the enrichment or depletion of the TF's target genes in that cell type, measured by Cohen's *d* as described above. Pearson correlations were then calculated between pairs of profiles. For the within-brassica comparisons summarized in Extended Data Fig. 5e, activity profiles were constructed from enrichments and depletions of c4 target genes for the 244 TFs in the four-species multiDAP dataset (Supplementary Table 9). For the comparisons between *A. thaliana* cell types and sorghum cell types shown in Fig. 3c, activity profiles were constructed from target genes conserved in both grass and brassica lineages (c6 grass+brassica-conserved target genes) for each of the 74 TFs in the ten-species multiDAP experiment (the profiles for *A. thaliana* and sorghum seedling cell types are provided in Supplementary Table 11). The top two activity score profile correlations with $r > 0.3$ for each sorghum cell type were visualized as a Sankey diagram using the sankeyNetwork function from the R package networkD3 v.0.4[81]. Edges in the diagram were labelled with TF families found within the top three TFs of both cell types, to illustrate TFs potentially driving each correlation (Supplementary Table 12).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The raw DAP-seq fastq sequence data files were submitted to the National Center for Biotechnology Information under BioProject no. PRJNA1177505. The raw snRNA-seq fastq sequence data files were submitted to the National Center for Biotechnology Information under BioProject no. PRJNA1262374. The processed DAP-seq data including peak files (narrowPeak format), coverage tracks (bigwig format), c score tables for four-species and ten-species datasets (.tsv format) and a readme, as well as processed snRNA-seq data including single-cell gene expression matrices (.mtx format) for each library and compiled seurat objects for each species tissue, were submitted to the National Center for Biotechnology Information under GEO superseries accession no. GSE299028. A compiled list of *A. thaliana* cell-type marker genes used to assist with cell-type annotation was downloaded from scPlantDB (https://biobigdata.nju.edu.cn/scplantdb/marker).

Code availability

Scripts and example data files used for the DAP-seq and single-nucleus analyses are available in a Git repository at https://code.jgi.doe.gov/LBaumgart/plant-multidap-and-single-cell.

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Author contributions

L.A.B., R.C.O. and S.I.G. conceptualized the project. A.M.-C., D.J.D., L.A.B., P.W., R.C.O., S.I.G. and Y.Z. devised the methodology. A.M.-C., D.J.D., L.A.B., R.C.O., S.I.G. and Y.Z. validated the data. A.C.G., A.M.-C., L.A.B., L.Y., R.C.O. and S.I.G. carried out the formal analysis. C.C., E.S., G.H., N.G., P.W. and Y.Z. conducted the investigation. A.M.-C., L.A.B., P.W., R.C.O., S.I.G. and Y.Z. wrote the paper. A.M.-C., L.A.B., L.Y. and S.I.G. visualized the data. C.G.D., I.K.B., L.A.B., R.C.O., S.I.G. and Y.Y. supervised the project.

Competing interests

The authors declare no competing interests.

Additional information

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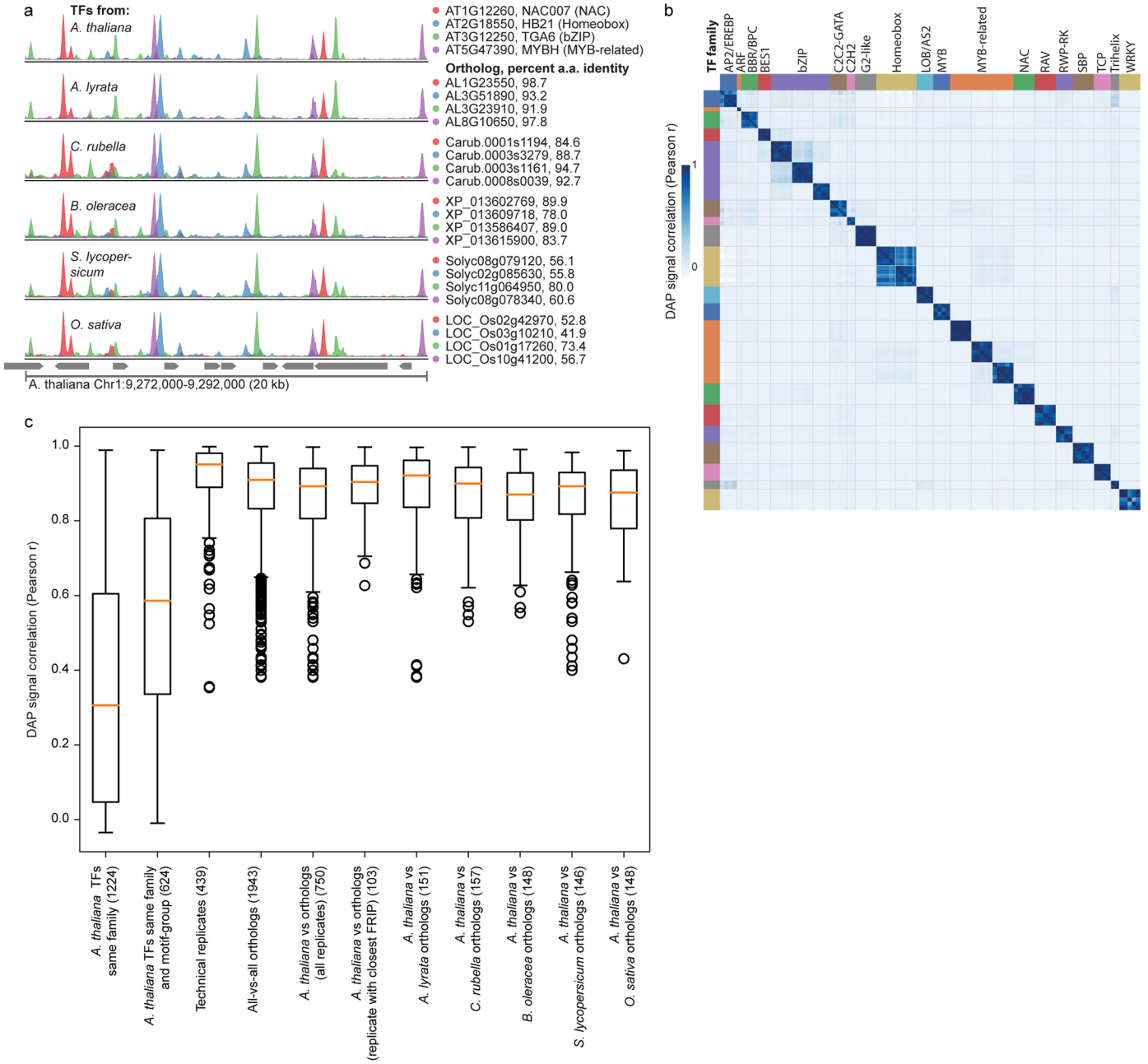

Extended Data Fig. 1 | Comparisons of genome-wide binding signal between different TFs and technical replicates. (**a**) Example 20 kilobase region of the *A. thaliana* genome shows similar TF-binding profiles of four example *A. thaliana* TFs and their corresponding orthologs from five other species. Numbers after TF IDs indicate percent amino acid identity compared to the *A. thaliana* ortholog. (**b**) All-vs-all genome-wide correlations of DAP-seq binding profiles between TFs from six species. Gray grid lines delineate individual groups of orthologous TFs and colored bars indicate TF families. (**c**) Correlations between different subsets of DAP-seq. Boxplots are drawn according to standard convention, where the box center represents the median, upper and lower box bounds represent the upper and lower limits of the interquartile range, and whiskers extend to the furthest data point within 1.5x the interquartile range from the box. Numbers in parentheses indicate the number of pairwise comparison data points shown in each boxplot. FRIP = fraction of reads in peaks, which serves as a measure of signal-to-noise in each DAP-seq library and can be used as an estimate of overall performance of the assay. Performance in the assay is variable between different TFs, and also varies slightly between technical replicates of the same TF. The category labeled 'replicate with closest FRIP' partially controls for this confounding effect on correlations: for each comparison between an *A. thaliana* TF and an orthologous TF, one technical replicate of each TF was selected such that the difference in FRIP scores between the pair being compared was minimized. In the category labeled 'same motif group', motif-groups refers to groups of *A. thaliana* TFs that fall within the same TF family and also show highly similar motifs (see Supplementary Table 1).

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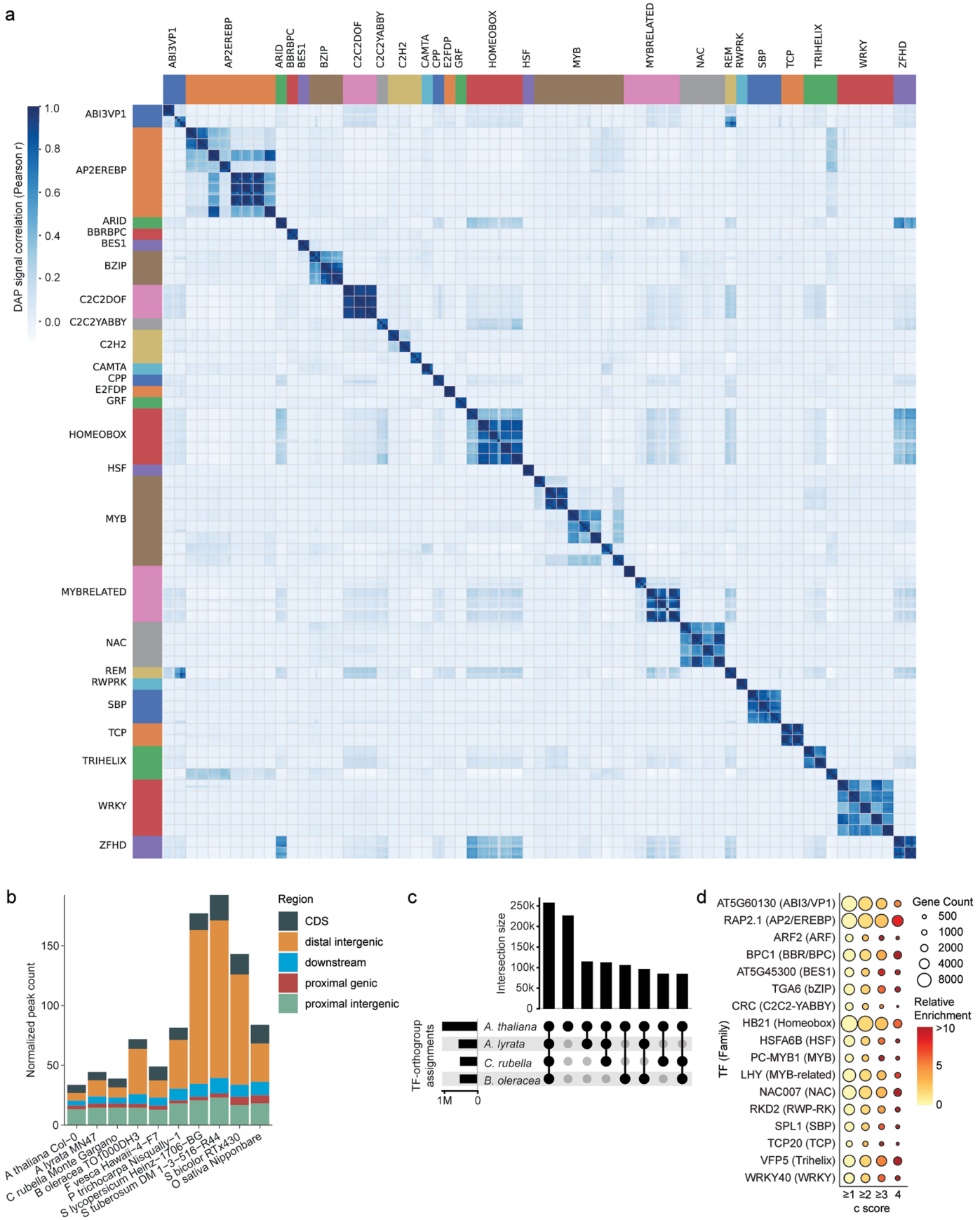

Extended Data Fig. 2 | See next page for caption.

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Extended Data Fig. 2 | Genomic TF binding maps across 10 species generated by multiDAP. (**a**) All-vs-all genome-wide correlations of DAP-seq binding profiles between four replicates for each TF using either 1, 4, or 10 multiplexed species. Gray grid lines delineate individual TFs and colored bars indicate TF families. Mean correlation between single species replicates = 0.94, standard deviation = 0.08, n = 116; mean correlation between single and four species experiments = 0.94, standard deviation = 0.06, n = 211; two-sample t-test p = 0.56. Mean correlation of 10 species vs. single species experiments = 0.87, standard deviation = 0.1, n = 163. (**b**) Count of peaks in genomic regions normalized by the number of total genes per species. CDS: coding sequence; distal intergenic: >2,000 bp upstream of any start codon; downstream: within 2,000 bp downstream of a stop codon; proximal genic: within 500 bp downstream of a start codon; proximal intergenic: within 2,000 bp upstream of a start codon (**c**) Number of TF target genes shared between *A. thaliana* and other brassica species. (**d**) Overrepresentation test for conserved sites. A subset of representative example TFs are displayed. All 244 TFs were significantly enriched ($p < 0.01$) at c scores ≥ 2.

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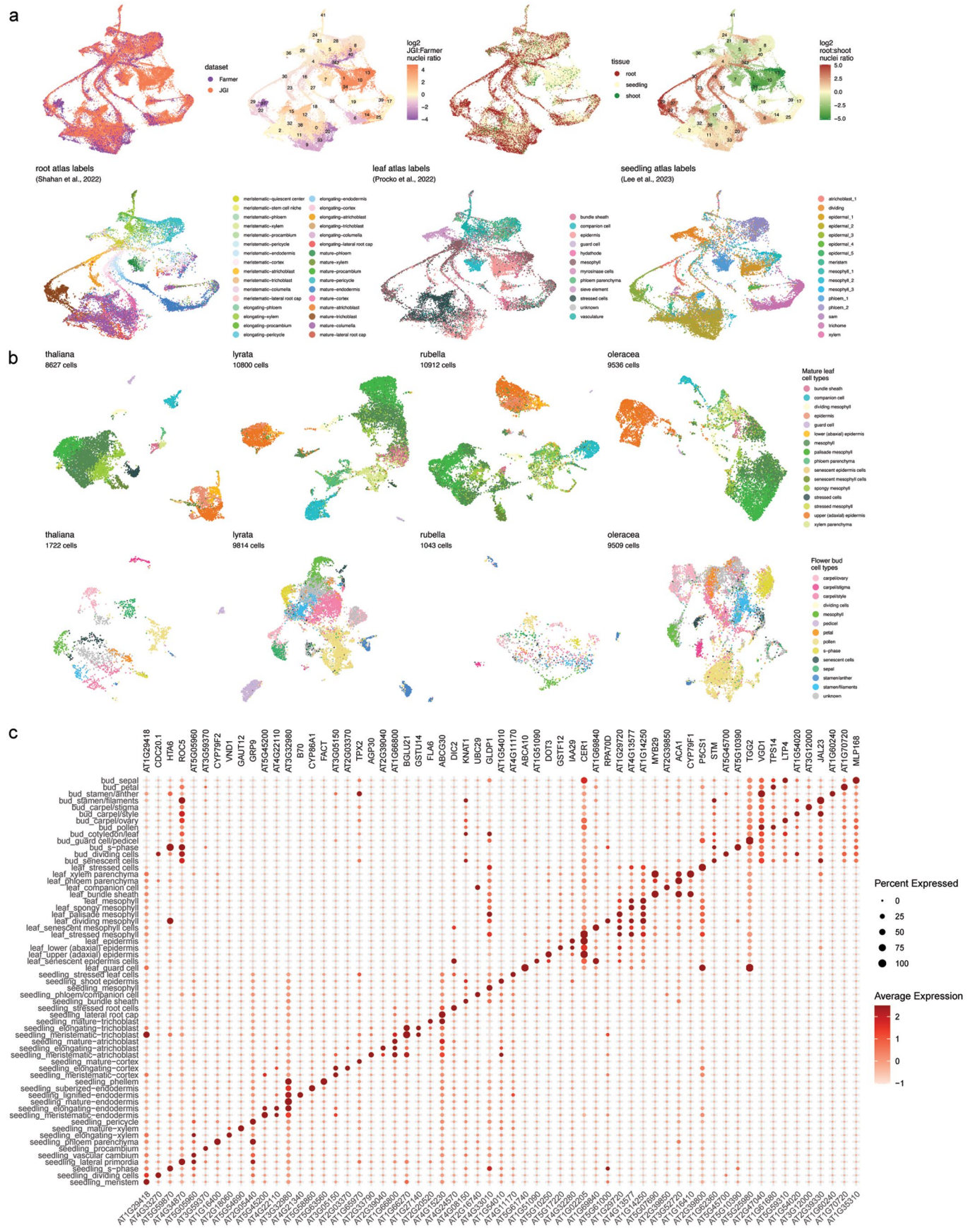

Extended Data Fig. 3 | See next page for caption.

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Extended Data Fig. 3 | Identification and labeling of cell types in snRNA-seq atlases of four Brassicaceae species. (**a**) Integration of internal and external *A. thaliana* seedling snRNA-seq datasets (first row) and results of label transfer from three reference atlases (second row). Only cells receiving a label with confidence score >0.4 are shown in these plots. (**b**) UMAP representations of mature leaf and flower bud snRNA-seq atlases from four profiled brassica species. (**c**) Dotplot of the top marker gene for each cell type, where dot size represents the percent of all nuclei with a specific cell type label showing non-zero expression of the gene, and color represents average normalized and scaled expression across these nuclei.

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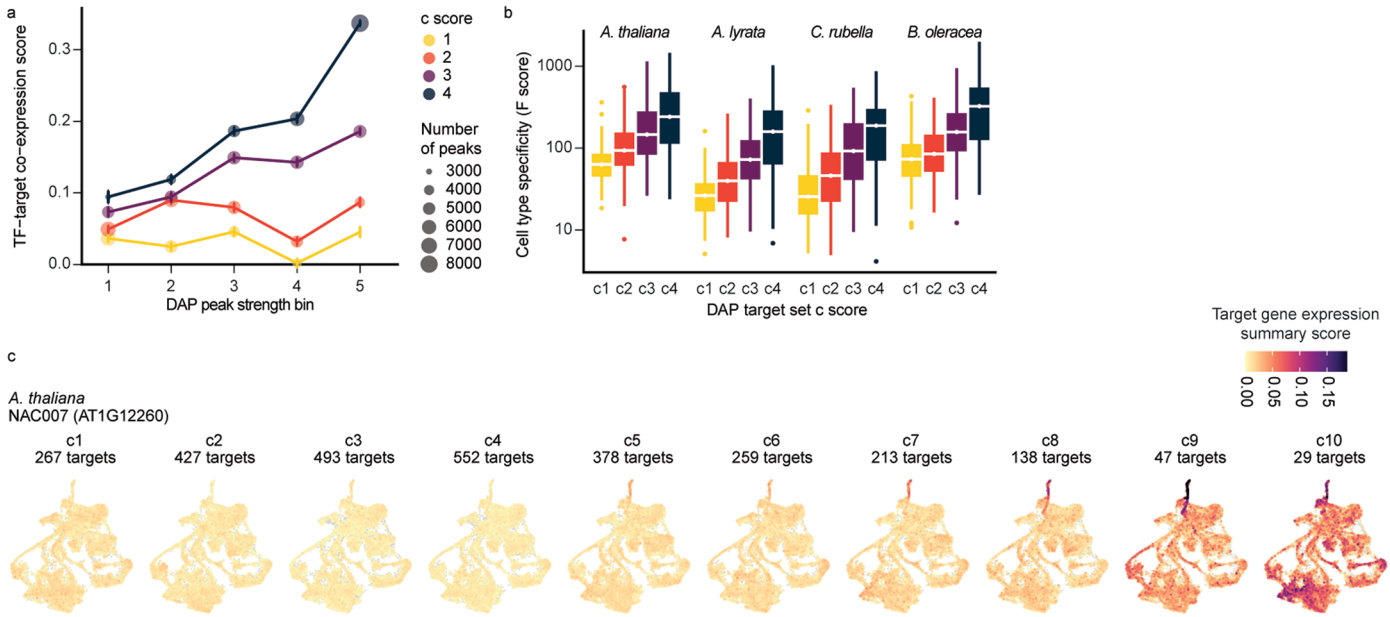

Extended Data Fig. 4 | Conserved TFBSs are predictive of target gene expression patterns. (**a**) TF-target co-expression across all nuclei in the *A. thaliana* seedling atlas as a function of multiDAP TF binding affinity and multiDAP peak conservation score. Y-axis represents the mean fold-change between true correlation of normalized TF expression with target expression vs. correlation with a matched control target gene. Point size represents the number of DAP-seq peaks considered, and error bars represent standard error of the mean. (**b**) Cell type specificity scores as a function of target conservation for 244 TFs in each of the four brassica seedling snRNA-seq atlases. Cell type specificity was defined as the F-statistic from an ANOVA test of the extent to which per-nucleus target gene expression varied systematically across labeled cell types. All distributions within a species were significantly different from each other (FDR < 0.05) using paired one-sided t-tests. Boxplots are drawn according to standard convention, where the box center represents the median, upper and lower box bounds represent the upper and lower limits of the interquartile range, and whiskers extend to the furthest data point within 1.5x the interquartile range from the box. (**c**) NAC007/VND4 target gene expression summary scores using target c scores from extended 10-species multiDAP dataset. Note that two target genes are excluded as they were not expressed in any cells in the snRNA-seq dataset.

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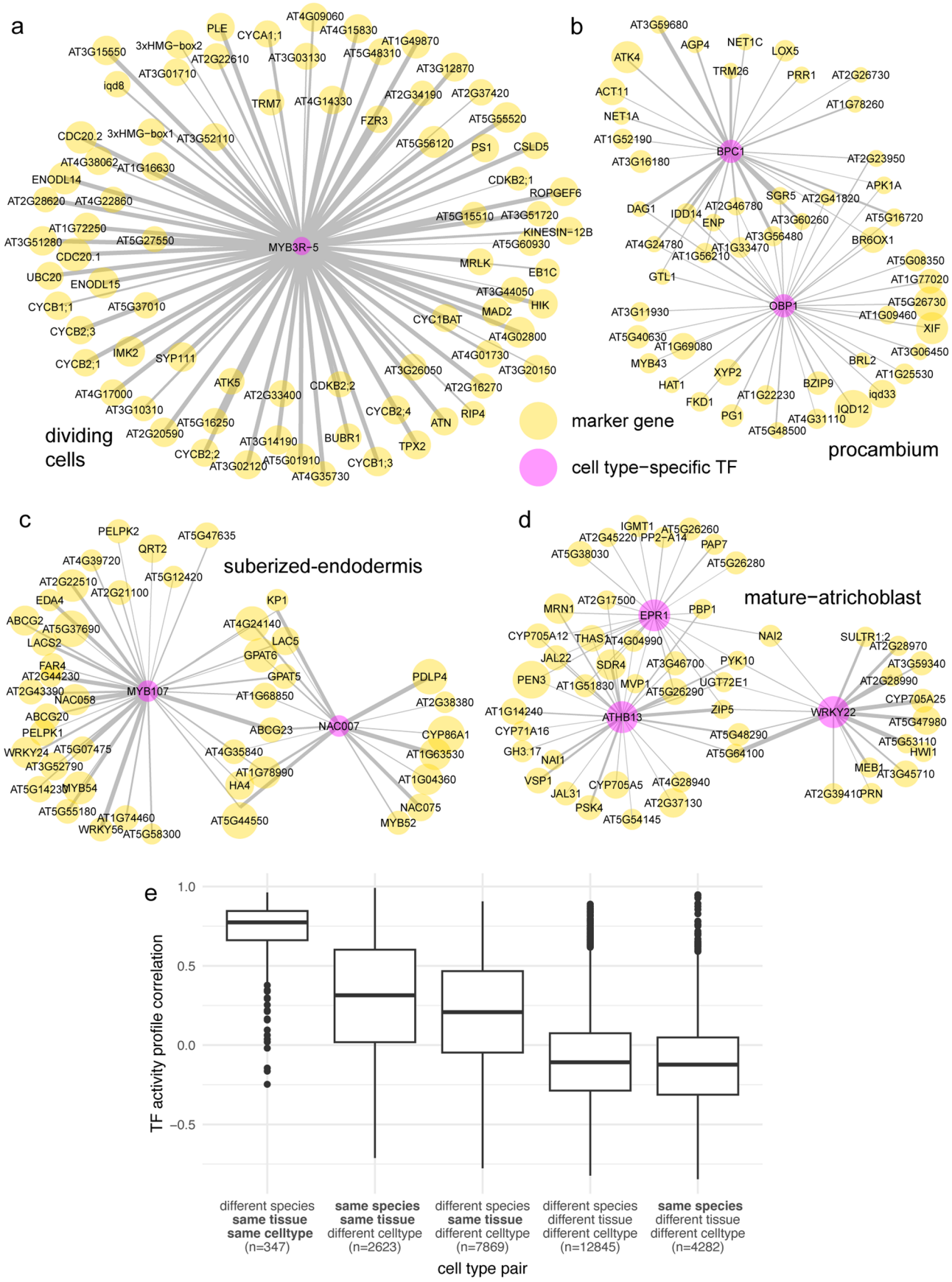

Extended Data Fig. 5 | See next page for caption.

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Extended Data Fig. 5 | Conserved TFBSs are predictive of cell type-specific gene expression. Network diagrams showing TFs with enriched activity scores (based on summarized expression of all c4 target genes) and the cell-type-specific marker genes they target for four example cell types: **a**) dividing cells, **b**) procambium, **c**) suberized-endodermis, and **d**) mature-atrichoblast. TFs are shown in pink and marker genes are yellow, with a gray line connecting them if a brassica-c4 TFBS for the TF was associated with the marker gene. Line thickness corresponds to relative binding strength (measured as DAP-seq peak height) and marker gene node size represents relative cell type specificity (measured as expression log2-fold enrichment). **e**) Distribution of Pearson correlations between pairs of TF activity score profiles, calculated for all pairs of cell types identified in the four brassica species and binned by concordance of cell type, tissue, and species. The highest correlations mark corresponding cell types from the same tissue found in different species, indicating that cell-type-specific TF activity is generally conserved across species. Number of pairs summarized by each boxplot is indicated in the x-axis labels. Boxplots are drawn according to standard convention, where the box center represents the median, upper and lower box bounds represent the upper and lower limits of the interquartile range, and whiskers extend to the furthest data point within 1.5x the interquartile range from the box.

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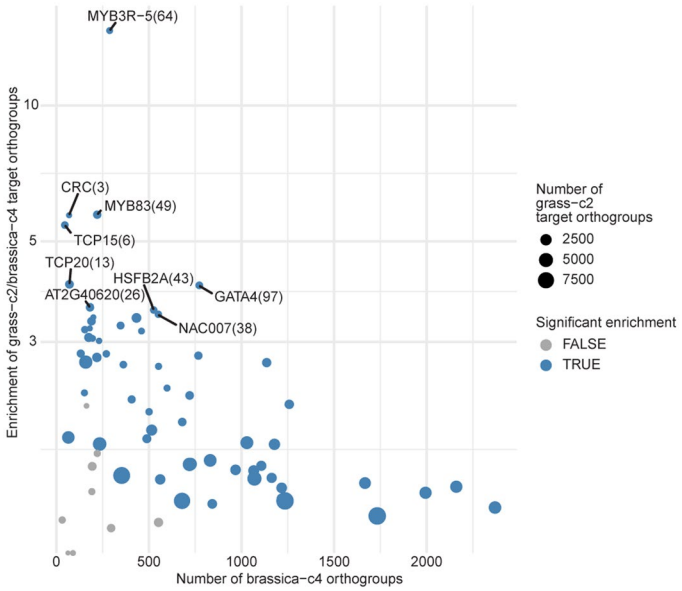

Extended Data Fig. 6 | Core sets of TFBSs are conserved across flowering plants. Scatterplot showing enrichment of each TF's core regulon (defined as target orthogroups conserved in both grasses and all four brassica), for TFs assayed in the 10 species multiDAP experiment. The enrichment scores (y-axis) indicate the extent to which the number of TF target genes shared by both grasses and brassica species was more than expected by chance, given the number of TF target genes conserved within each lineage alone. The vast majority of TFs showed significant enrichment scores (blue dots), indicating that persistent core regulons are a common feature of TF regulatory networks. To ensure meaningful statistics, only the 70 TFs targeting at least 150 total brassica-c4 or grass-c2 target orthogroups were tested. Each point represents a single TF, and x-axis shows the total number of brassica-c4 orthogroups, dot size shows the total number of grass c2-orthogroups, and the y-axis shows the enrichment of orthogroups shared between these two sets. To calculate enrichments, true counts of core regulon target orthogroups for each TF (shown in parentheses after TF name for top examples) were compared to background distributions generated by shuffling orthogroup labels for c2-grass TFBSs 1,000 times. Significance (dot color) was calculated as the number of times the shuffled core regulon count met or exceeded the actual count, followed by FDR correction. Significance was assessed at FDR < 0.05. Enrichment score (y-axis) was calculated as the fold change between the actual count and the average shuffled count.

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Extended Data Fig. 7 | Identification and labeling of cell types in snRNA-seq atlases of sorghum. Dotplots showing expression of sorghum orthologs of experimentally validated cell type marker genes compiled from published literature in each cluster of the sorghum leaf (top) and root (bottom) snRNA-seq atlas. Column labels specify the cell type(s) where gene expression was experimentally observed/validated, followed by the gene alias. LRC = lateral root cap. For gene IDs and publication references, see Supplementary Table 10.

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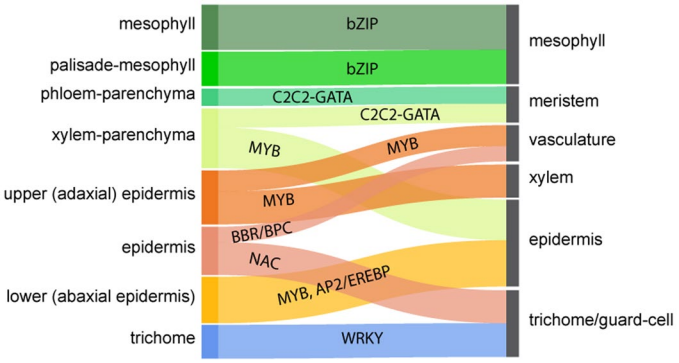

Extended Data Fig. 8 | Comparison of TF activity scores in leaf cell types of *A. thaliana* and sorghum. Sankey plot showing top activity score correlations for each sorghum leaf cell type (right) among all *A. thaliana* leaf cell types (left). A vector of TF activity scores was constructed for each sorghum and *A. thaliana* cell type, quantifying enrichment of each TF's core regulon target genes (defined as conserved in both grasses and all four brassica) using Cohen's d. Then Pearson correlations were calculated between the TF activity score vectors of all cross-species cell type pairs. Ribbons in the diagram represent the top two strongest correlations for each sorghum cell type, after filtering to correlations with $r > 0.3$. Ribbon width corresponds to Pearson r^2 value, and ribbons are labeled with TF families found in the top 5 enrichments for both the *A. thaliana* and sorghum cell type.

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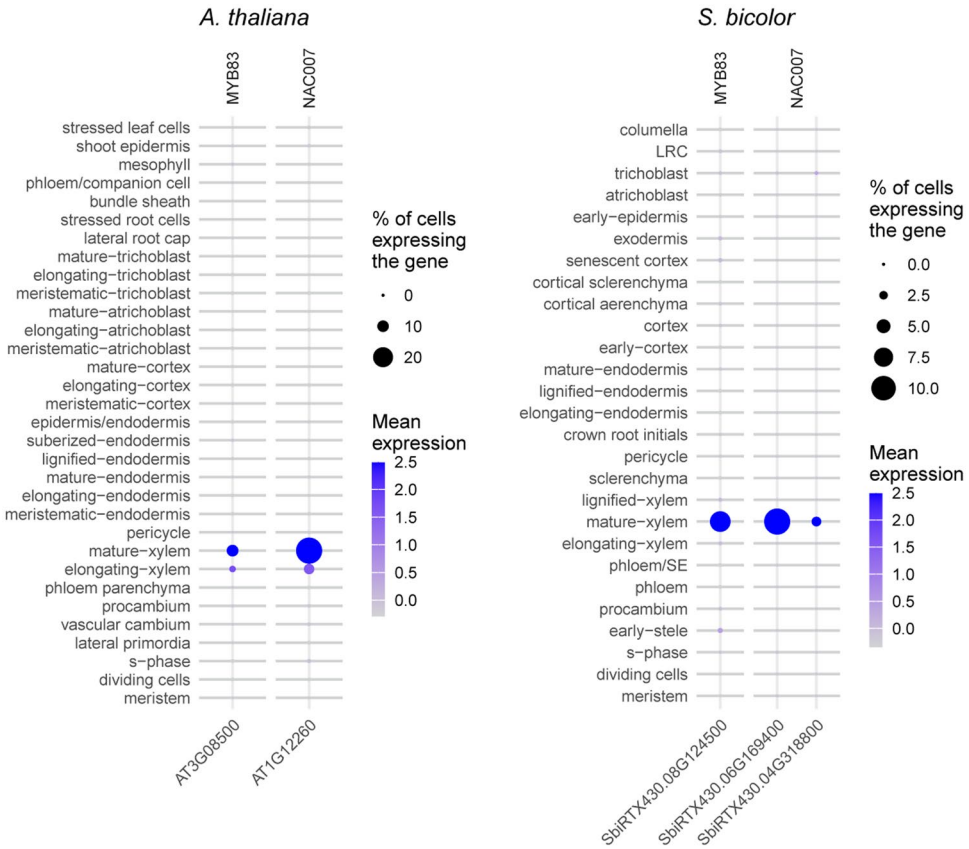

Extended Data Fig. 9 | MYB83 and NAC007 expression in roots. Dotplots showing expression of TF genes *MYB83* and *NAC007* across all *A. thaliana* seedling cell types and all sorghum root cell types.

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A. thaliana gene	alias	defline	A. thaliana xylem marker number	A. thaliana xylem log2-foldchange	S. bicolor ortholog	S. bicolor xylem log2-foldchange	MYB83 brassica cscore	NAC007 brassica cscore	MYB83 grass cscore	NAC007 grass cscore
AT2G17940	AT2G17940	WEB family protein (DUF827)	1	8.081254	NA	NA	0	0	0	0
AT5G54690	GAUT12	galacturonosyltransferase 12	2	7.839702	NA	NA	0	0	0	0
AT1G63910	AtMYB103	myb domain protein 103	3	7.582467	NA	NA	0	2	0	0
AT2G41610	AT2G41610	transmembrane protein	4	7.33454	SbiRTX430.01G456800	2.5794973	0	0	2	0
AT3G45870	UMAMIT3	nodulin MtN21 /EamA-like transporter family protein	5	7.24881	NA	NA	0	0	0	0
AT1G02335	GL22	germin-like protein subfamily 2 member 2 precursor	6	7.223052	NA	NA	0	0	0	0
AT5G60720	AT5G60720	electron transporter, putative (Protein of unknown function, DUF547)	7	7.205592	SbiRTX430.01G002800	3.7597003	0	0	1	0
AT5G37478	AT5G37478	TPX2 (targeting protein for Xklp2) protein family	8	7.165733	SbiRTX430.02G169200	2.0546143	0	0	0	0
AT5G40020	AT5G40020	Pathogenesis-related thaumatin superfamily protein	9	7.163655	SbiRTX430.01G256900	6.9794622	0	0	0	1
AT1G70500	AT1G70500	Pectin lyase-like superfamily protein	10	7.13582	SbiRTX430.04G030500	4.927484	0	0	0	0
AT5G03260	LAC11	laccase 11	11	7.121995	NA	NA	0	1	0	0
AT5G44030	CESA4	cellulose synthase A4	12	7.114725	SbiRTX430.01G231700	3.8753394	3	4	1	0
AT5G12870	MYB46	myb domain protein 46	13	7.052956	NA	NA	0	4	0	0
AT3G27200	AT3G27200	Cupredoxin superfamily protein	14	7.048906	NA	NA	0	0	0	0
AT3G51325	AT3G51325	RING/U-box superfamily protein	15	7.036542	NA	NA	0	3	0	0
AT5G51890	AT5G51890	Peroxidase superfamily protein	16	7.022842	NA	NA	0	4	0	0
AT4G35350	XCP1	xylem cysteine peptidase 1	17	7.019927	SbiRTX430.09G009200	6.310923	0	0	1	2
AT2G27740	AT2G27740	RAB6-interacting golgin (DUF662)	18	7.005147	NA	NA	0	4	0	0
AT1G43790	TED6	tracheary element differentiation-related 6	19	6.995853	NA	NA	0	0	0	0
AT1G20850	XCP2	xylem cysteine peptidase 2	20	6.988891	SbiRTX430.09G009200	6.310923	2	0	1	2
AT4G18780	IRX1	cellulose synthase family protein	21	6.965673	SbiRTX430.03G320500	3.8519224	0	4	1	0
AT2G37090	IRX9	Nucleotide-diphospho-sugar transferases superfamily protein	22	6.952028	SbiRTX430.01G430200	2.3157492	4	0	1	0
AT2G37090	IRX9	Nucleotide-diphospho-sugar transferases superfamily protein	22	6.952028	SbiRTX430.09G026700	1.8498328	4	0	1	0
AT1G13635	AT1G13635	DNA glycosylase superfamily protein	23	6.947736	SbiRTX430.07G241000	2.8414214	0	0	0	0
AT3G15050	IQD10	IQ-domain 10	24	6.941362	NA	NA	0	4	0	0
AT3G62160	AT3G62160	HXXXD-type acyl-transferase family protein	25	6.919502	SbiRTX430.03G041400	1.3082666	0	0	0	0
AT3G62160	AT3G62160	HXXXD-type acyl-transferase family protein	25	6.919502	SbiRTX430.03G047600	1.4472189	0	0	2	0
AT3G62160	AT3G62160	HXXXD-type acyl-transferase family protein	25	6.919502	SbiRTX430.09G035400	1.1362393	0	0	2	0
AT3G62160	AT3G62160	HXXXD-type acyl-transferase family protein	25	6.919502	SbiRTX430.10G191700	0.3301378	0	0	1	0
AT5G15630	IRX6	COBRA-like extracellular glycosyl-phosphatidyl inositol-anchored protein family	26	6.904182	SbiRTX430.02G374300	4.3007688	2	4	2	0
AT1G73140	TBL31	trichome birefringence-like protein (DUF828)	27	6.858102	NA	NA	4	0	0	0
AT3G24535	AT3G24535	hypothetical protein	28	6.828354	NA	NA	0	1	0	0
AT1G58370	RXF12	glycosyl hydrolase family 10 protein / carbohydrate-binding domain-containing protein	29	6.8251	SbiRTX430.02G128200	0.4698626	0	4	2	0
AT5G17420	IRX3	Cellulose synthase family protein	30	6.80161	SbiRTX430.02G210900	3.4192317	2	4	2	0
AT3G18660	PGSIP1	plant glycogenin-like starch initiation protein 1	31	6.796271	SbiRTX430.03G406200	-0.6941986	0	4	0	0
AT3G18660	PGSIP1	plant glycogenin-like starch initiation protein 1	31	6.796271	SbiRTX430.09G151400	-1.1036043	0	4	0	0
AT5G60020	LAC17	laccase 17	32	6.769086	SbiRTX430.03G380300	5.6348347	0	4	2	0
AT5G60020	LAC17	laccase 17	32	6.769086	SbiRTX430.03G380400	6.765979	0	4	1	2
AT1G29200	AT1G29200	O-fucosyltransferase family protein	33	6.724686	SbiRTX430.10G172000	1.1320747	0	4	2	0
AT5G42710	TRM30	hypothetical protein	34	6.706509	SbiRTX430.01G074100	3.0391201	0	4	2	0
AT3G62020	GLP10	germin-like protein 10	35	6.674964	NA	NA	0	4	0	0
AT1G58070	AT1G58070	WEB family protein	36	6.670475	SbiRTX430.05G071800	5.5686734	0	0	0	1
AT1G58070	AT1G58070	WEB family protein	36	6.670475	SbiRTX430.08G063100	5.4532686	0	0	0	0
AT4G27435	AT4G27435	fiber (DUF1218)	37	6.669888	SbiRTX430.02G130500	2.0108217	0	3	2	0
AT2G29130	LAC2	laccase 2	38	6.653769	NA	NA	0	0	0	0
AT2G38080	IRX12	Laccase/Diphenol oxidase family protein	39	6.635086	NA	NA	0	0	0	0
AT4G08160	AT4G08160	glycosyl hydrolase family 10 protein / carbohydrate-binding domain-containing protein	40	6.580145	NA	NA	0	4	0	0
AT1G24030	AT1G24030	Protein kinase superfamily protein	41	6.545477	SbiRTX430.02G193100	3.9772143	2	0	2	0
AT1G75090	AT1G75090	DNA glycosylase superfamily protein	42	6.510539	SbiRTX430.01G493000	7.969177	0	4	0	1
AT1G27920	MAP65-8	microtubule-associated protein 65-8	43	6.504465	SbiRTX430.07G209400	3.7789622	0	4	0	0
AT1G54790	AT1G54790	GDSL-like Lipase/Acylhydrolase superfamily protein	44	6.488345	SbiRTX430.09G137100	2.5468692	0	0	0	0
AT2G33385	arpc2b	actin-related protein C2B	45	6.431312	SbiRTX430.06G152300	4.7822646	0	2	2	1
AT2G28315	AT2G28315	Nucleotide/sugar transporter family protein	46	6.399684	SbiRTX430.01G224400	1.0572724	0	3	1	0
AT2G28315	AT2G28315	Nucleotide/sugar transporter family protein	46	6.399684	SbiRTX430.04G230900	3.1013815	0	3	0	0
AT2G28315	AT2G28315	Nucleotide/sugar transporter family protein	46	6.399684	SbiRTX430.06G157800	3.6409796	0	3	2	0
AT4G28500	NAC073	NAC domain containing protein 73	47	6.36881	SbiRTX430.03G271900	4.0598931	0	4	2	0
AT4G28500	NAC073	NAC domain containing protein 73	47	6.36881	SbiRTX430.09G246200	1.8146401	0	4	0	2
AT5G45970	RAC2	RAC-like 2	48	6.301691	NA	NA	0	3	0	0
AT1G68200	AT1G68200	Zinc finger C-x8-C-x5-C-x3-H type family protein	49	6.299533	NA	NA	0	3	0	0
AT1G66810	AT1G66810	Zinc finger C-x8-C-x5-C-x3-H type family protein	50	6.285237	SbiRTX430.03G255000	4.6781246	3	0	1	0
AT1G66810	AT1G66810	Zinc finger C-x8-C-x5-C-x3-H type family protein	50	6.285237	SbiRTX430.09G256700	5.2936119	3	0	0	0

Extended Data Fig. 10 | Differential involvement of MYB and NAC in driving cell type-specific gene expression in xylem. Table showing NAC007 and MYB83 TFBSs associated with the top 50 marker genes for the mature-xylem cluster in the *A. thaliana* seedling atlas and their sorghum orthologs. Specificity of expression of each ortholog in the mature xylem cluster of the sorghum root atlas is also shown in the column after the sorghum ortholog ID. Green shading highlights brassica-c4 TFBSs and yellow highlights grass-c2 TFBSs. Outlined boxes highlight *A. thaliana* genes with a brassica-specific NAC007 TFBS and no MYB83 TFBS whose sorghum orthologs have the reverse: a grass-specific MYB83 TFBS but no NAC007 TFBS.

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Software and code

Policy information about availability of computer code

Data collection	All DNA sequence data files were produced using manufacturer's standard software integrated with Illumina Novaseq 6000 and Novaseq X plus sequencers.
Data analysis	BBTools v38.96 (bbduk.sh) for quality filtering and adapter trimming; Bowtie2 v2.4.2 for aligning reads to reference genomes; Samtools v1.15.1 for merging BAM files; MACS3 v3.0.0a6 for peak calling; MEME suite v5.3.0 for motif analysis; v2.5.5 for orthogroup assignment; deeptools v3.5.2 for comparing DAP seq bigwig files and signal visualization; clusterProfiler v4.6.2 for gene ontology enrichment analysis; vcftools v0.1.17 for calculating nucleotide diversity; BEDTools v2.31.0 to assign peaks to genomic features; Cellranger count v7.0.1 for pre-processing 10X Chromium libraries; velocyto v0.17.17 for generating spliced and unspliced count matrices from 10X data; BD Rhapsody CWL pipeline v2.2 for pre-processing BD Rhapsody libraries; STAR v2.7.10b for generating spliced and unspliced count matrices from BD Rhapsody data; Seurat v5.0.0 for clustering, normalization, and marker analysis of snRNA-seq data; DoubletFinder v2.0.4 for detecting and removing doublets in the snRNA-seq datasets; SAMap v1.0.15 for transferring cell type labels between species; ggPlantmap v1.0.0 for visualizing heatmaps projected onto plant diagrams; cohen.d from the R package effsize v0.8.0 for calculating cell type enrichments; sankeyNetwork function from R package networkD380 v0.4 for creating Sankey diagrams. All custom analysis code with example input and output files is available in a public git repository at https://code.jgi.doe.gov/LBaumgart/plant-multidap-and-single-cell.

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

Raw DAP-seq fastq sequence data files were submitted to the National Center for Biotechnology Information under BioProject no. PRJNA1177505.

Raw snRNA-seq fastq sequence data files were submitted to the National Center for Biotechnology Information under BioProject no. PRJNA1262374.

Processed DAP-seq data including: peak files (narrowPeak format), coverage tracks (bigwig format), c score tables for 4-species and 10-species datasets (tsv format) and a readme, as well as processed snRNA-seq data including: single-cell gene expression matrices (.mtx format) for each library, and compiled seurat objects for each species tissue were submitted to the National Center for Biotechnology Information under GEO superseries accession GSE299028.

A compiled list of A. thaliana cell type marker genes used to assist with cell type annotation was downloaded from scPlantDB (available at https://biobigdata.nju.edu.cn/scplantdb/marker).

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	Not applicable
Reporting on race, ethnicity, or other socially relevant groupings	Not applicable
Population characteristics	Not applicable
Recruitment	Not applicable
Ethics oversight	Not applicable

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	No predetermined sample size calculations were performed. Ten plant species were selected to cover multiple representative clades of flowering plants based on availability of sequenced genomes and germplasm resources. 360 TFs were selected to cover multiple representatives of all major plant TF families and which had performed well in previous DAP-seq studies. snRNA samples were chosen to allocate at least two biological replicates of each selected tissue in each species of interest. DAP-seq: 2890 total DAP-seq/multiDAP libraries passing quality filters resulting from a total of 796 DAP-seq reactions, including 180 negative control libraries resulting from 48 negative control reactions where a mock in vitro protein expression was used in place of TF. Of those reactions including a real TF, 166 were run with a single genome (84 unique TFs); 204 with two species (104 unique TFs); 244 with four species (244 unique TFs); 134 with 10 species (134 unique TFs). snRNA-seq: Single nuclei RNA-seq libraries were prepared from A. thaliana, A. lyrata, C. rubella, and B. oleracea tissues including two flower bud, two mature leaf, two seedling shoot, and 4-7 seedling root samples for each species, plus four whole seedling samples for A. thaliana and B. oleracea. Four S. bicolor root samples and three S. bicolor leaf samples were used. Total dataset included 196,196 single nuclei: 36,689 from Arabidopsis lyrata; 55,743 from Brassica oleracea; 26,032 from Capsella rubella; 45,518 from Arabidopsis thaliana; 32,214 from Sorghum bicolor.
Data exclusions	As a measure of successful binding site enrichment, we calculated the fraction of reads in peaks (FRIP) scores and only used data from DAP-seq assays that generated FRIP scores of at least 0.05. For experiments including multiplexed species, we only considered those assays where

	all of the multiplexed species independently produced FRIP scores of 0.05 or greater. We found that multiplexing larger sets of species with varying genome sizes in a single multiDAP experiment sometimes resulted in large differences in read coverage between different TFs and in some cases differential enrichment of species. To mitigate the effect of low coverage resulting in false negatives in the peak-calling step (and thus lowering apparent c scores), we only used datasets that yielded at least 200,000 read pairs (400,000 reads) for each of the multiplexed species.
Replication	Not all attempts at generating snRNA datasets were successful for various technical reasons. In these cases, we repeated experiments so that we obtained at least two successful datasets from biological replicates of each tissue type and species of interest. DAP-seq is an in vitro assay and as such biological replication does not apply. Instead, technical replication rate was assessed using a subset of the TFs used throughout the study. 83 TFs were selected and tested in duplicate, of which 82 yielded successful results passing quality filters (99% successful replication rate). The mean genome-wide binding signal correlation between these successfully replicated datasets was 0.94.
Randomization	randomization was not applicable as there were no treatment groups included in this study
Blinding	blinding was not applicable as there were no treatment groups included in this study

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	Arabidopsis lyrata, MN47, Germplasm source: ABRC, Germplasm collection ID: CS22696; Arabidopsis thaliana, Col-0, Germplasm source: ABRC, Germplasm collection ID: CS6673; Brassica oleracea, TO1000DH3, Germplasm source: ABRC, Germplasm collection ID: CS29002; Capsella rubella, Monte_Gargano, Germplasm source: ABRC, Germplasm collection ID: CS22697; Fragaria vesca, Hawaii-4-F7, Germplasm source: USDA, Germplasm collection ID: PI 664444; Oryza sativa, Nipponbare, Germplasm source: USDA, via Sundar Lab UC Davis, Germplasm collection ID: GSOR 312005; Populus trichocarpa, Nisqually-1, Germplasm source: JGI inhouse clone; Solanum lycopersicum, Heinz-1706-BG, Germplasm source: TGRC, Germplasm collection ID: LA4345; Solanum tuberosum, DM1-3-516-R44, Germplasm source: USDA, Germplasm collection ID: Chromosome doubling of a monoploid (1n = 1x = 12) derived by anther culture of a heterozygous diploid (2n = 2x = 24) S. tuberosum group Phureja clone (PI 225669); Sorghum bicolor, RTx430, Germplasm source: USDA, Germplasm collection ID: PI 655996.
Novel plant genotypes	Not applicable
Authentication	Not applicable