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Peer Review File

An environmentally regulated dichotomy in cell lineages of the root

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
Summary:
Cell identity has been shown to be a major determinant of transcriptional responses to environmental stimuli, however, most studies to date have investigated this process in populations of cells. In this manuscript, Olivia et al. utilize high resolution single cell transcriptomics and whole-mount in situ hybridization chain reaction (HCR) to present a well-resolved atlas of transcriptional states present in cells of the Arabidopsis root. The improved map allows them to discover that on top of the cell-identity-dominant transcriptional signatures that distinguish cells, within each cell lineage two divergent transcriptional states exist, which are associated with genes implicated in responses to various abiotic stresses as well as response to the hormone Abscisic acid (ABA). The authors annotate cells following this alternate developmental trajectory as in an environmentally responsive state (ERS) and show that the proportion of ERS+ cells changes depending on nutrient availability. This alternate developmental trajectory is marked by the expression of some genes that are specific to particular cell types, however, there are also gene sets that mark this alternate transcriptional state across multiple cell types. ERS+ associated genes tend to function in environmentally responsive pathways such as ABA and circadian rhythms.

The manuscript in several ways is a technical tour de force and presents novel use of the HCR method to validate in situ expression patterns of genes. This allows the authors to improve cell type annotations, though the details of how this improvement is achieved is not well described. The most important advance is the discovery of the ERS cell state, which appears to create diversity in the transcriptional state of cells in a particular lineage. If well validated, this discovery would open up many new avenues of research. Are ERS+ cells poised for rapid response to changes in environment? Do they reflect “noise” in the signaling systems that become amplified or reduced upon shifts in the environment? Do plants adapted to stressful environments have different proportions of ERS+ and ERS- cells?

The primary issue is that the significance of ERS cell state is not well supported in the current manuscript. The evidence that such cells exist is demonstrated by scRNAseq, but only briefly surveyed by HCR. The authors also identify several transcription factors and signaling pathways that are expressed in ERS+ cells, but the authors do not test the function of such regulators in the emergence or functions of ERS+ cells. The manuscript would be enhanced by adding clarification to the methods used to build the developmental trajectory (such as the calculation of stemness scores for different cell types), quantification of images where a particular expression pattern is observed, and some experiments testing the relevance of the developmental trajectory that involves the ERS+ cellular state.

Major comments:
1. Abstract line 6-9: The authors oversimplify the current view of root cell identities by stating that the field considers it to be ontogenetically driven “with no impact from the environmental conditions.” It may be useful to consider Dinneny et al., 2008, Science where the authors show that a large portion of the transcriptional state of specific cell types is environment dependent with a smaller core transcriptome that is maintained under different environments. The authors should also explicitly state what they define as “cell identity”. The totality of genes expressed in a particular cell type under one specific environmental condition may be an overly constrictive definition.
2. Greater clarity on why previous studies had not identified the ERS lineages is needed. How did the HCR results inform

this discovery? What bioinformatic steps are different? If the authors process their data in the same way as in other comparable studies, is the ERS state no longer revealed?
3. Roots growing on the surface of agar media experience two different microenvironments (contact with the agar surface and water film, and contact with air). Could these local environmental cues serve as the basis for the ERS+/- states?
4. Abstract line 5: “The formation of one of the trajectories is driven by a strong and specific activation of genes involved in responses to environmental stimuli” To claim this, it would be necessary to test mutants of genes involved in the formation of this environmentally responsive trajectory and show that these mutants lack two developmental trajectories for different cell types.
5. In Extended Data Fig.2b, the gene (AT3G25980) used as a marker for Cluster 28 is expressed in dividing cells of all cell types. And the cluster 16 marker (AT3G23830) is also expressed in the meristematic zone of all cell types and not just in the epidermis. It is unclear why the clusters are not named according to the expression pattern observed by whole mount in situ HCR but are annotated as specific to the epidermis/lateral root cap. If these clusters were used to calculate the stemness score for the epidermis-root cap lineage, how was the stemness score calculated for the other lineages? How were the early markers for other lineages identified for the pseudotime analysis? Why were clusters 9 and 12 included for the identification of “early markers”? There is no whole mount in situ HCR data showing expression patterns of genes in these clusters.
6. Are the genes with expression patterns presented in cross sections for cortex and endodermis in Fig. 2c specific to the differentiation zone of the root? Or are these genes present in the meristem as well? It would be important to include the longitudinal images as well to see how the genes are expressed along the different developmental zones of the root. This would also help to confirm results of the pseudotime analysis.
7. The non-homogenous expression of AT3G47510 in images represented in Extended data 4c is not obvious. From the images it appears that while XTH21 is an early XPP marker, AT3G47510 is expressed later in the XPP cells. Quantification of the number of roots in which this non-homogeneous pattern was observed and/or quantification of signal intensity variation across different pericycle cells would be important here to observe these differences. The authors should also show expression of XTH21 at the developmental region at which AT3G47510 expression is observed.
8. The presentation of the confocal data is obscured by the way the micrographs are processed. Making the cell boundaries channel opaque prevents the signal from the inner tissues from being apparent, except in cross section. Perhaps the authors can make the cell boundary channel transparent or show a single longitudinal cross section to reveal expression along this axis more clearly. In general, the cross sections are far too small to evaluate the tissue or cell type specificity, especially for those HCR data showing expression in stele cells. Perhaps a magnified view of the stele can be shown instead of the whole root when this tissue is the primary focus.
9. Extended data 4d and e. CASP1 has been shown to be involved in deposition of Casparian Strip in all endodermal cells including the (future) passage cells. The passage cells only lack suberin lamellae. It is possible that the passage cells deposit Casparian strip at a later developmental stage compared to other endodermal cells. Are there any published data that would suggest these differences in Casparian Strip deposition between passage and non-passage endodermal cells? How does the PHO1;H3 expression differ along the root? Are key endodermal developmental regulators such as SCR present in both cell fate trajectory branches?
10. The authors identify genes that mark the ERS+ state in several cell lineages and use HCR to analyze the expression pattern of one marker for this state, VDOF1. However, the data are not convincing here. In Figure 2b, it would be important to show how the root is oriented. I believe that the cross sections represented at the top are closer to the root tip and the cross sections below are shoot ward? Is the expression of VDOF1 observed in the root apical meristem as is suggested by the scRNAseq data? To validate the ERS+ state, showing dual labeling of ERS+ and ERS- markers would be important to confirm that they are mutually exclusive states, as the data suggests. Again, the resolution of the images needs to allow for the discrimination of individual cells with and without expression of the marker gene.
11. It may be that there are multiple pathways that create heterogeneity in the transcriptional states of cell lineages including actual differences in cell identity (passage cell vs non-passage cells in the endodermis), or differences in signaling state due to heterogeneous microenvironments (see comment #3), or differences that arise from biological noise. The authors should more clearly define the potential sources of such variation and establish which variable patterns are the consequence of which phenomenon.
12. Extended data Fig 6a. It is unclear how the different cell types were assigned in this plot. What is defined as “stele” in this plot? How is this different from xylem and phloem?
13. Page 10, Line 14: The branch 1 cells are classified as ERS+ based on the presence of genes related to ABA signaling pathway. It has been shown that ABA signaling is important for endodermal and xylem differentiation even under unstressed conditions. Perhaps, the role of basal levels of ABA in the development of these tissues is revealed by the branch 1 cellular state? What proportion of cells of a particular cell type are ERS+ vs ERS-?
14. The data provided in Supplementary Figure 6b is not very convincing. The enrichment of the expression programs for the ERS+ pathway seems limited. What is the statistical basis for determining whether a gene is biased towards one lineage or another? HCR would be useful to validate genes showing marginal enrichment like ABF1 and PYL8.
15. Does Ws-2 have a higher or lower tolerance to heat stress compared to the other accessions? What is the consequence of the DEGs in the ERS+ cells?
16. The authors observe a depletion of ERS+ cells in half MS compared to full MS. If half MS represents the nutrient limited conditions, wouldn’t one expect the cellular states that express ABA signaling components to increase? For cell types in which there is no change in proportion of ERS- cells but ERS+ cells are depleted (metaxylem, phloem pole pericycle, procambium), do the authors observe fewer cells in the half MS grown plants compared to full MS? An alternate hypothesis is that in half MS condition, the cells progress through differentiation faster (more/longer root hairs) and hence the ERS+ cell states are not resolved as in the full MS.
17. Have the authors compared whole mount in situ HCR images of genes in ERS+ cell state in plants grown under full MS vs half MS?
18. The TF-binding analysis presented is correlative and should be followed up with a mutant analysis to test whether they show more or less ERS+/- cells or show differences in transcriptional state of the ERS cells.

19. The hypothesis that columella and pericycle cells have altered cell type identity in scr-3 should be validated using reporter genes, HCR or physiological assays.
20. The authors state that 20% of DEGs responsive to MS concentration are in ERS+ cells. Is this greater than expected by chance?

Minor comments:
21. Results line 9: “Protoplasts were isolated from root tips of two different Arabidopsis ecotypes, mixed at the same concentration” Can authors explain why there is a large variation in the number of cells from the different ecotypes (Col: 31611 cells and Ler: 6356 cells) and how this could influence the comparison of differentially expressed genes between accessions?
22. Page 10 Line 21-22: The statement that the ERS signature is “independent from the developmental lineages” is not quite accurate since it seems to occur predominantly in the endodermis and stele.
23. Fig. 5b, Number of root hairs plotted instead of root hair density. The authors should also provide the root length data for the two conditions tested.
24. Extended Data Fig. 10b. Do the authors observe a higher number of columella cells in half MS grown plants compared to full MS or does the change in relative proportion reflect a difference in protoplasting efficiency for this cell type?
25. Page 18 Line 20, No data has been presented that involve changes to osmotic pressure. Are the authors referring to the difference in the concentration of salts from the MS media? What is the difference is osmotic pressure between ½ and full strength MS?
26. The authors should state whether the cells for scRNAseq were collected at the same time of day.
27. Line 24, page 19: What is meant by “gatekeeper cells”. This is suggestive of a regulatory role for ERS+ cells that has not been demonstrated.

Referee #2

(Remarks to the Author)
The single cell Arabidopsis root atlas reported in the manuscript is an informative reference data set, because it also includes information from Arabidopsis ecotypes. The observation of two developmental trajectories within the root lineages (branch I and II), caused in response to environmental stimuli such as nutrient availability, is indeed novel and promises an improvement in the quality and diversity of single cell datasets. The data and methodology are described in detail and presented clearly.

However, a major concern of the results reported in this manuscript is the lack of in vivo validation and therefore the lack of evidence for the conclusions. Without additional in vivo validation, the novel aspect between environmental responses and cell identity is not efficiently proven, reducing the originality and significance of the findings reported in this manuscript.
One major concern with the observation of the two-branch-theory reported in this manuscript is that the cell content varies very dramatically between all cells in branch I compared to branch II (in all lineages where the effect was observed). This suggests that the branches are caused by cell quality, rather than biological significance. Although this problem has been mentioned and partly addressed by the authors through a sub-clustering approach according to the median UMI/cell (Extended Data Fig7); indicating that the overall cell cluster distribution is robust and that gene expression between branch I and II is not influenced by protoplast generation; this approach did not include a separate trajectory analysis which would be necessary to observe, if branch I and branch II are still detectable even in the remaining cells.

Other points that should be addressed to provide solid evidence for the robustness of the two developmental branches and the stated conclusions:
1) Validation of branch I and branch II-specific genes were provided by VDOF1 and AT3G47510 (branch I) and CASP1 (branch II). While the provided HCR pictures indeed indicate a spatial expression pattern along a developmental trajectory, it only proofs that their expression is more enriched in mature cells. There is no direct evidence provided that would connect an environmental response (observed for branch I cells) influences the pattern. Because the novelty of this manuscript lays in the report of an environmental influence on cell developmental stages, it is crucial to provide in vivo validation.
The authors further characterize the branches into environmentally responsive state ERS+ cells (=branch I) and ERS- cells (=branch II), indicating an ERS+-specific enrichment of ABA-responsive genes and genes related to a function in circadian rhythm. Again, no in vivo validation is provided that would support the observed transcription patters. Further, it would also be informative, if the spatial expression pattern of VDOF1 (or others) is indeed associated with ABA or circadian rhythm.

2) Figure 2b: There is no quantification of signal intensity given from the HCR pictures. A quantification of gene expression is important, because the reported expression pattern of e.g. VDOF1 (similar to XTH21 and other genes shown in Extended Fig.4) follows a sensitive gradient and is not completely absent vs. strongly induced.

3) Comparing the cell content between cells of both branches in previously published datasets (Ext. Fig. 5) suggests less difference of median UMI content between branch I and II in some references (pointed out in Ext. Fig 5; e.g. Wendrich et al.; Shulse et al.; Denyer et al.;) compared to this study. A statistical analysis would help in determining the significance of the difference in UMI content. Why is there no difference in cell content observed between both branches in some datasets, even though the sequencing depth or overall UMI count in all those studies are like the here reported dataset? How many cells were identified in each branch? Are the here observed transcriptional responses (e.g. enrichment of ABA-responsive genes) also observed in branch I cells found in the other references?

4) What would be the biological relevance of having different transcriptome content in cells of the same cell identity? The

authors have not provided any evidence that would answer why cells that respond to an environmental stimulus would show lower transcript abundance?

5) Cell filters: the material and methods sections did not suggest that the enrichment of mitochondrial or chloroplast gene expression was used as filter for low quality/dying cells. This is a standard filter, generally accepted in animal and plant scRNA-Seq reports, to improve data quality and removal of stressed cells.

Have chloroplast genes (which are in generally strongly suppressed in roots) been included in the cluster analysis and developmental trajectories? Were there any other criteria (in addition to the mentioned criterial on page 29 line 7-8: “Cells with <200 expressed genes were removed, and genes expressed in <3 cells were removed”) set that could help in the identification of low-quality cells caused by stress and/or cell death?

6) No in vivo validation is provided for the observation of the ERS+-specific ABA signaling or circadian clock-related gene expression on the developmental trajectories (Fig. 2); branch I/ERS+-enriched DEGs in Arabidopsis accessions (Fig. 4) or the nutrient availability single cell dataset (Fig. 5). The authors only provide in silico analyses that provide candidates involved in the potential molecular mechanism (Extended Data Figure 8). As stated already in point 1), in vivo validation is required to support the findings and their originality.

Additional comments/suggestions for improvement:

- Some references (e.g. 13) are not up to date regarding their publication status.

- Only two biological replicates are provided for the nutrient scRNA-Seq. A third replicate could provide more robustness to the observations.
Also for this dataset a validation of the observations would be required, e.g. including trichoblast markers or identified DEGs enriched in ERS+ cells. Without in vivo validation, e.g. including cell counting of ERS cells etc., there is no evidence given for the statement made on page 18 line 6-7: “This suggest that the proportion of cells affected by this superimposed state could be modulated under different nutrient availability concentrations.”

- Page 2; Line 5-6: What evidence is available that shows that cell fate is influence by environment? There are indeed multiple reports on cell number/function/chemical or physical properties that are influenced by environmental stimuli, but it would be good to add references that show changes of cell fate.

- Page 3; Line 16-18: This statement is somehow contradictory to the description the authors provide in the material and method section Page 36; line 3-6. While the authors claim that other studies “[…] overlooked potential layers of gene regulation that do not depend on the developmental origin of the cell”, the material section specifies that subsets (8) were generated based on developmental lineages (epidermis-root cap; cortex; endodermis; PPP; XPP; procambium, phloem, xylem) and that each lineage was processed independently further. In my understanding, the approach used by the authors follows the same principle as used in previous publications (e.g. Denyer et al.; Wendrich et al.; Shahan et al.). Could you please clarify?

- Page 3; Line 19-21: “[…] providing the most precise root cell atlas to date, […]” This statement is somehow confusing, given that there are already published scRNA-Seq data from Arabidopsis root with much more cells (Shahan et al., 2022) and that also all annotated cell clusters have been identified and confirmed in previous reports.

- Page 19 line 22-24: Which cells exactly should function as “gatekeeper cells”? This is a very strong statement that has too many layers that were not addressed in this manuscript. For example: What would this function include? Can you pinpoint to the exact cells that you identified in vivo with such function?

Referee #3

(Remarks to the Author)
Summary

Oliva et al. present a comprehensive single-cell transcriptome atlas, together with spatial mapping, to deeply investigate the developmental lineages of Arabidopsis roots. Impressively, the authors identified two separated trajectories (termed “branch 1” & “branch 2”) for most of the lineages, which potentially correspond to the environmental responsive state (ERS). So far as we know, this is a novel finding. However, given that this is a quite large claim, further evidence and validations are necessary to make the findings more convincing.

Major comments

1. The authors claim that 1) different developmental branches exist and 2) these branches correspond to +/- ERS. Neither of the two claims are fully convincing: the former depends on single-cell clustering and embedding on a single dataset without validations on any independent datasets and the latter highly depends on identification of differentially expressed genes followed by GO analysis. I think more direct evidence is necessary, ideally orthogonal to the way in which these patterns were discovered, given that these are major claims – some suggestions include:

-It would be better to provide some epigenetic-level evidence, for example, chromatin accessibility or methylation difference between ERS +/- states. I think the epigenetics regulations would be more sensitive to environmental responses.
-Could the two branches be identified for the other four ecotypes by repeating the same analysis on them? Also, the current findings are way unexpected, it would be better to repeat the same analysis on an independent dataset, either published or newly generated.
-Experimental validation would be very helpful, for example, knocking down a key gene, blocking the corresponding pathway, or adjusting the environmental conditions, which will potentially make the additional branch disappear. Otherwise, the current differentially expressed genes & GO analysis is not sufficient. The nutrient depletion experiment shown in the last section (Fig.5) is quite interesting, but I think such analysis based on the cell composition changes might need more replicates. HCR validation that fewer ESR+ cells exist in nutrient depleted conditions would also be nice.

2. More evidence is necessary to eliminate the possibility that these results are due to artifacts. Some suggestions:
- Coloring the UMAP in Fig.1b by 1) UMI count, 2) gene count, 3) ecotype identity, 4) sequencing or experimental batches (if available);
- Coloring the UMAP in Fig.1d by the same features above plus cell cycle phases.
- Instead of presenting the cell label transferring result, could the authors provide the integration results (i.e. UMAP with co-embedding) of the current study with the published datasets? Can the published datasets be completely analyzed separately with the author's analysis pipeline to show that the two branches can be identified in published datasets?

3. Similarly, it seems like the paper primarily focuses on "branch 1" (the ERS+ branch). Since "branch 2" (ERS- branch) seems like it could potentially be artifactual, the paper would be more convincing if branch 2 was explored in more depth throughout the paper.
- For example, when validating the branches in XPP cells using whole-mount HCR (Extended Data Figure 4C), the authors look at two marker genes, an earlier XPP marker gene that should be expressed in all XPP cells, and a marker gene that should be specific to branch 1 at a later time point. As expected the earlier time point gene is expressed in all XPP cells while the branch 1 later time point gene is not expressed in all cells, which the authors conclude "(...) confirms the branching nature of these trajectories over development, which reveals two previously unidentified in vivo alternate cellular states". While this seems to confirm an earlier and a later cell state (which I believe was previously known), it doesn't seem to compelling prove there are two branches (ERS+ and ERS-). Showing (via HCR if possible) that a subset of cells express a branch 2 marker gene that is not expressed in cells that express a branch 1 marker gene would be much more convincing.
- Are there ERS- marker genes, and if so, what are the GO-terms associated with those?
- The authors show that there are some DEGs between the two nutrient conditions tested (figure 5), and that 20% of those DEGs are enriched in branch 1 (ERS+) cells – are any DEGs specific to branch 2 and not branch 1 (hard to tell by just looking at extended data figure 10C)? More broadly put, apart from changes in the ratio of ERS+ to ERS- cells, are ERS- cells truly unresponsive to changes in the environment?
- I could be misunderstanding (or naive on my part), but it seems perhaps slightly counterintuitive that the cells that are capable of responding to the environment (ERS+ cells) would be depleted in an environmental stress condition?

4. In the published datasets, leveraging the cell-type identities transferring strategy, the authors could identify the similar two branches. I am wondering if they have performed the different expressed gene tests and GO analysis, and could the similar gene modules corresponding to environmental responses be identified as well? Also, if the two branches also existed in the previous datasets, what is the possible reason that they have not been identified?

5. Could the authors repeat the trajectory/pseudotime analysis (Fig. 1f) using some additional methods? For example, Monocle or RNA velocity based methods? Showing that these two branches can be seen when the data is analyzed with a different method (e.g. Monocle), and showing that the two branches can be found in the published datasets when those data are analyzed independently (i.e. not using cell label transferring alone) would show that these findings/branches are robust.

6. The starting point assigning strategy (Fig.1e & f) seems purely based on the computational strategies, is it possible to provide some experimental validations? For example, in the trajectory of XPP, the starting point could also possibly be the bottom rather than the middle. Again, the HCR results shown in Ext Fig. 4c only suggests the middle region is less differentiated relative to the top – it's necessary to provide more evidence about the bottom region.

7. As the remarkable difference of UMI counts between two branches (Ext Fig.5b & c), I am wondering how the authors convince the differential expressed genes between them are real rather than due to some background differences? Similarly, the high/low UMIs (Ext Fig.5b & c) between two branches might affect the strategy of finding the starting point. All the starting points seem to exist at the boundary between cells with different UMI counts.

The downsampling analysis alone doesn't seem convincing enough because genes that are never detected in branch 2 because it has too few UMIs might still be detected and called as "differentially expressed" in the branch 1 even after it has been downsampled?

8. Could the authors provide more replicates for scr-3 ecotypes? Otherwise, the cell composition analysis results are not fully convincing (Fig.3b).

9. Clusters 16 & 28 are not well annotated, which raises the possibility that they are not really distinct cell types, but rather under specific cell states or even low quality cells, for example, as shown in Ext Fig.3a, the cluster 28 is more likely driven by different cell-cycle phases.

Specific comments

- In Fig.1b, there is no further discussion on the difference between clusters within each cell type (for example, both cluster 8 and 12 are non-hair cells), why not just merge those clusters and then label them by cell type names?

- In Fig.1b, a small cell population in green color (I guess the cells belong to cluster 14) is connected to cluster 13 on the left side, any comments on it?

- In Fig.1c, some of the plots seem represented in Ext Fig.1c. In addition, 15 genes are shown in this panel, rather than 19 cell-cluster-specific marker genes talked about in the paper, which is confusing.

- In Fig.1d & f, cells are colored by sub-clusters, however, some of those colors are not easy to distinguish, especially some of them are reused in Fig.1b.

- In Ext Fig.3b-c, I tend to see how the UMAP with cells colored by cell cycle phase (like the panel a) looks like after cell-cycle regression, rather than labeled by cluster id.

- I don't fully understand what the authors wanted to indicate by Ext Fig.5a. Again, instead of showing the cell type transferring result, it would be better to directly show the co-embedding of UMAP between the current study with the published datasets.

- In Fig.3b, could the authors provide a box plot or bar plot instead, which I think would be more straightforward to see the cell composition changes?

- There appears to be a random letter "c" in the middle of the plot in extended data figure 10C

- Relatively minor, but personally didn't find the schematic in figure 5 to be super necessary in order to understand the experiment.

- Might be nice to include extended data figure 10a with Fig.5d only maybe split the two different nutrient conditions into two UMAPs so the reader can easily/qualitatively see how the proportion of cell types and the proportion of ERS+ to ERS- cells changes between the two conditions/UMAPs? There is a stark change in the number of hair cells that can be seen in the images in 5a – would be cool (though maybe not critical to the main findings of the paper) to recapitulate that with UMAP. Or, could have two UMAPs for the two conditions and highlight cells by the expression of a branch 1 gene that has a strong change in expression in response to the nutrient condition?

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have strengthened the manuscript by performing orthogonal analyses to support the presence of two transcriptional branches, incorporating additional biological replicates, and testing the role of ABA in regulating the proportion of ERS^+ cells. However, several aspects of the data interpretation and presentation would benefit from further clarification and additional controls to ensure that the conclusions are fully supported. In particular, clearer definitions of the ERS^+/ERS^- states, validation of HCR-based transcript detection, and improved visualization of comparative datasets would enhance the overall robustness and clarity of the study.

Major Comments:

1. The authors claim in the abstract that "The discovery of this alternate fate trajectory, induced by a highly active and environmentally responsive cell state, reveals an additional layer of root cell identity that may underpin the adaptability of roots to challenging environments." However, there are several aspects of this claim that were not sufficiently made. First, while cells in an ERS+ and ERS- state were identified for specific tissue types, the authors have not shown that these cells are especially responsive to the environment, only that the proportion of cells in this state increases with stress. It is not clear how cells transition into and out of the ERS+/- states and whether cells with an ERS+ state are any more responsive to stress than ERS- cells. One possible approach would be to use ABA FRET sensors (e.g., ABACUS or ABAleon) to visualize basal ABA levels in individual root hair cells, then apply an abiotic stress treatment to determine whether cells with higher basal ABA concentrations indeed exhibit faster or stronger ABA responses.
2. HCR-based transcript detection is a critical component of validating the manuscript's conclusions regarding the ERS^+ cell state. It is therefore essential to demonstrate that the observed differences in signal intensity truly reflect biological variation rather than experimental artifacts. I recommend that, in addition to detecting ERS^+ state markers, the authors include simultaneous detection of ubiquitously expressed genes that are expected to be present in both branches of the cell type as internal controls for probe performance and hybridization efficiency. It would also strengthen the analysis to present images across multiple Z-planes, confirming that the apparent absence of signal in certain cells is not due to differences in the chosen focal plane.

3. The identification of two trajectories with both PAGA and Monocle is encouraging. To strengthen the claim of a true bifurcation, I recommend quantifying agreement between methods (e.g., overlap of branch assignments and correlation of local pseudotime). And since both PAGA and Monocle are graph-based analyses, it will be better to use a graph independent approach, like RNA velocity models, to confirm the trajectories identified in PAGA and Monocle. Additionally, please include pathway enrichment for the Monocle trajectories. Do similar GO terms appear in the Monocle-based analysis as in the PAGA-inferred branches?
4. Extended Data Fig.2. It is unclear what is validated in this figure. If published reporter lines support the reported expression patterns, the corresponding references should be included. Note also that AT1G11190 is BFN1, not BNF1.
5. In Extended Data Fig.3, the images shown for genes AT2G36100, AT3G47510 can be made clear by showing optical cross sections at different distances from the root tip. Currently, it is difficult to determine, for example, whether AT2G36100 is expressed in the endodermis.
6. In the author response, the statement "ERS$^-$ does not mean that these cells are unresponsive to the environment; rather, it means they have not acquired the specific transcriptional state that we define as 'environmentally responsive' (ERS$^+$)" remains conceptually ambiguous. In the hard-agar drought stress experiment, the increase in ERS$^+$ cells is accompanied by a decrease in ERS$^-$ cells, suggesting that both states are, in fact, responsive to environmental cues. To avoid confusion, I recommend refraining from using the term "environmentally responsive" to describe the ERS$^+$ state, as it implies that ERS$^-$ cells are environmentally inert.
7. In the drought stress and full MS/half MS experiments, could the authors clarify whether seeds were germinated directly on the respective media or if seedlings were transferred to these conditions after initial germination? The method of establishing the treatment is important, as transfer-related stress or developmental stage differences could influence the proportion of ERS$^+$ cells and the interpretation of the results.
8. In Figure 4d, it would be helpful to project the datasets from the different drought stress conditions onto a single UMAP embedding. Presenting the data in a shared UMAP space would allow for a more direct comparison of the cellular distributions and ERS$^+$ cell frequencies across conditions.
9. In Extended Data Fig. 15b–d, do the authors observe that branch 1–specific genes across the different ecotypes are enriched for ABA- or circadian-related pathways? Clarifying whether this enrichment is consistent among ecotypes would help determine if the observed transcriptional divergence reflects conserved ABA/circadian regulation or ecotype-specific differences in environmental responsiveness.
10. Data on root hair number are not presented in the manuscript. The corresponding section in the Methods should therefore be removed unless these results are included.
11. Since the edge_threshold parameter in the PAGA analysis was "adapted to each lineage," it would be important to specify the exact thresholds used or provide a clear rationale for how these values were determined, as this choice can strongly influence the inferred trajectory topology.
12. The cross-dataset validation should come with supporting statistics. The authors used extensive projection-based comparisons to determine if ERS+ and ERS- populations were also present in previously published scRNA-seq/snRNA-seq datasets. However, these projections were known to vary dramatically when using different parameters. The authors need to show corresponding statistics for the stability of these projections.

(Remarks on code availability)

Referee #2

(Remarks to the Author)
I appreciate the authors' careful and thoughtful revisions in response to my initial review. The manuscript has improved substantially in providing evidence for the validation of the ERS:
- Validation of cell type and ERS+ marker genes by HCR
- Provide additional analysis that demonstrate that ERS+ cells are not analytical artifacts
- Characterization of the ERS in previous single cell/nuclei data sets
- The establishment of an ERS is modular and adaptable under varying nutrient (full MS or ½ MS) or stress (e.g. drought) conditions

My previous concerns and suggestions have been adequately addressed. The authors have provided new data and clear responses to all points raised in my initial review, incorporating the necessary revisions throughout the manuscript. The identification, characterization, and validation of the ERS as an additional cellular characteristic—beyond cell type lineage and developmental state—remain highly novel. The finding that drought stress can modulate the establishment of this state across multiple cell types, and that ABA biosynthesis and receptor mutants exhibit reduced numbers of ERS$^+$ cells, represents a particularly interesting and significant contribution.
The source genomic data are complete, and the methods are described in adequate detail to ensure transparency and reproducibility. The provided code further enhances reproducibility. The statistical analyses are appropriate, and there are no apparent data that would require additional statistical testing. The reporting of error bars and probability values is clear and consistently presented in figure legends and relevant text sections.

The following point is an optional suggestion that could further refine a point of discussion, though it does not affect the overall novelty or conclusions of the study:
-Page 16 Line 5-8: I agree with the authors` conclusion that the underlying regulatory networks of the ERS could vary between cell types, which causes the described degree of acquisition of the ERS-state between cell types. However, as the ERS is regulated by ABA, differences in ABA sensitivity or signaling among cells within and between cell types could also

offer a plausible explanation. I understand that testing this hypothesis is beyond the scope of the present manuscript, but this argument could be added to the current statement to provide a more comprehensive view of the possible regulatory mechanisms influencing ERS establishment.

Minor comments:
- Page 15 Line 9: “sRNA-seq” should be scRNA-seq
- Page 32 Line 16: “Putative TF targets genes” should be “Putative TF target genes”
- Figure 3 panel D: the y-axis should be “Cell state proportion”

(Remarks on code availability)
The authors provide detailed descriptions of the analytical code within the Methods section and make all supporting code available through an accessible GitHub repository. The repository includes a comprehensive README file with clear installation and usage instructions. In addition, the Seurat objects and FASTQ files corresponding to the data generated and analyzed in this manuscript are provided, ensuring transparency and facilitating reproducibility.

Referee #3

(Remarks to the Author)
The authors have done extensive and commendable additional work in this revision, including generating new datasets, performing new experiments, and developing new analyses under specific conditions. These efforts substantially strengthen the study. However, our main concern remains whether branch 2 represents a true biological state or a technical artifact. There are some additional analyses suggested below that could be helpful and are not “major lifts”. Depending on the authors’ take on our concerns, we could imagine these being addressed through minor revisions & textual changes that reflect the concerns or remaining uncertainty.

1. Low UMI / High Mitochondrial %

Branch 2 cells have low UMI counts and high mitochondrial percentages, a common issue in scRNA-seq where low-quality cells form spurious clusters. In Extended Fig. 9, Branch 2 appears to show higher mitochondrial % and chloroplastic % than Branch 1, despite the text stating they are comparable. The overplotted jitter makes it difficult to see the distribution clearly; a statistical comparison (and perhaps clearer violin or density plots) would help.

2. Lack of distinct gene expression features
Most of the analyses (e.g., scRNA-seq across ecotypes, snATAC-seq) primarily strengthen evidence for Branch 1. Since the central question is whether Branch 2 is real, a more direct approach would be to identify specific genes that are up- or downregulated in Branch 2 with biological interpretability. To our reading, there are still no clear gene expression features defining Branch 2. In analogous datasets, distinct clusters are characterized by specific genes that can usually be either connected to the literature or some function in a clear, believable way (e.g. known/clear markers are best, but GO modules, though not our favorite, and the like often suffice). This facilitates gaining confidence (for authors, reviewers and readers) that a given cluster is real rather than an artifact. The authors provide some explanations in their response why distinct features were not found. We found these points to be reasonable but not entirely convincing. We recognize that this concern is not really addressable if the given reasons are valid, but it is another reason for caveating Branch 2.
3. Extended Figures 9 & 10 Interpretation

We do not see how Extended Fig. 10 supports the authors’ statement, which is very confusing. From Extended Figs. 9 and 10, it seems the authors have not fully ruled out the possibility that the second branch represents damaged cells with lower UMI capture (characterized by high mito% and low UMI ***which is a a common issue*** in scRNA-seq analysis. On a related point, Extended Fig. 10 is intended to show that genes enriched in Branch 1 span a similar expression range to those expressed in both branches, suggesting that Branch 1-specific genes aren’t simply missed in low-UMI cells. Conceptually this makes sense, but the data are overplotted, and many Branch 1-specific genes appear lowly expressed. Two violin plots (one for “branch 1-specific” and one for “branch 1 + Branch 2-specific” genes) showing their average expression in Branch 1 might make this comparison clearer. Currently, this figure feels more qualitative than quantitative.

4. Integration Analysis

The evidence from the integration analysis seems somewhat arbitrary. First, the first dataset (Extended Fig. 13) did not detect Branch 2. Second, the second dataset (Extended Fig. 14) was not analyzed with the same integration approach as the first. Third, it is unclear what cell filtering criteria were applied to each dataset; applying different UMI or mito% cutoffs could probably determine whether Branch 2 appears or disappears.

5. UMI Downsampling

To test whether branch separation is driven by UMI differences, the authors downsampled cells with >1,000 UMIs to 1,000 while leaving lower-UMI cells unchanged. It’s not clear why 1,000 was chosen (perhaps it corresponds to the average of Branch 2?) but ideally, downsampling would match the full UMI distribution of Branch 2 rather than applying a fixed cutoff. Even after downsampling, Branch 2 remains slightly lower and retains a pronounced low-UMI tail not seen in Branch 1.

6. Cell Cycle Effects (Extended Fig. 6b)

The text states that branch separation is not explained by cell cycle stage, which may be true, but there appears to be a consistent difference in the proportion of G1 cells between branches. Although the two branches remain after regressing out cell cycle effects, regression may not fully remove all related variation?

7. HCR Validation (Fig. 2e)

The HCR experiments validating Branch 1–specific genes are among the most promising evidence. We suggest adding quantitative analyses and results, rather than only highlighting selected examples through visualization, as this would provide stronger support.

Also, because HCR efficiency can vary, some apparent heterogeneity could stem from technical variability rather than biological absence of expression. A useful control would be to visualize an example gene expressed in both branches (from Extended Fig. 10) at similar expression levels to the HCR targets; if uniformly detected, it would strengthen the biological interpretation. If not, it might suggest technical effects. Including quantitative data (e.g. fraction of positive cells per marker) would further strengthen the conclusion. That being said, we recognize the extent of important new experimental work that the authors have already done in the course of the revisions (so this is more of an idea/optional suggestion than a strict request for a new experiment)

(Remarks on code availability)

Version 3:

Reviewer comments:

Referee #1

(Remarks to the Author)
This round of review responds to the authors' rebuttal and the new data provided; several points are well addressed, but my principal concern — the naming of the state (point 8) — remains unresolved, and the rebuttal in some respects reinforces it. My remaining concerns are largely interpretive: the wording in several places asserts function, causation, and responsiveness that the data, which are transcriptional-state correlations, do not yet establish, together with one methodological point about the HCR validation that bears on a central claim.

Major points
1. The recurring inference of function and responsiveness from a transcriptional state need to be toned down (Abstract, p.2; Results, p.3). Several framing statements claim more than the data show.
– Abstract: "…genes involved in responses to environmental stimuli, including those activating the abscisic acid (ABA) pathway." It is unclear whether these genes are activating the ABA pathway or responding to it; the distinction matters because it is the difference between a causal and a correlative claim, and the present data support the latter.
– Abstract: "…a highly active and environmentally responsive cell state." Is this accurate? Activity (UMI/gene number) is measured; responsiveness of individual cells to environmental signals is not directly demonstrated.
– p.3: "…cell types in the root are in fact composed of subsets of cells whose function is modulated in response to environmental information." What is demonstrated is a difference in gene expression state, not a difference in function. I would recommend the authors substitute "gene expression state" for "function" here and edit comparable statements throughout.

2. The transcriptional asymmetry between the branches, and what defines ERS^- (p.8) needs revision. The statement that "branch 1 is substantially more transcriptionally active than branch 2" is not accurate as supported. The identification of branch-1-specific genes alone does not indicate that branch 2 cells are less transcriptionally active. The lack of a defined branch-2 expression program does support the asymmetry, as does the UMI/gene-count comparison the authors cite — but the sentence as written rests the claim on the module/gene identification, which is not strong support; the wording should be tightened to reflect what actually establishes the difference. This connects to a broader question about ERS^-: ERS^+ is well defined by co-expression modules and ABA/environmental-response signatures, but ERS^- remains largely defined by absence of the ERS program. Is this interpretation correct, or are there programs enriched in ERS^- cells? The possibility that ERS^- represents a lower-transcriptional or dormant state (see point 6) should be addressed directly rather than left implicit.

3. The position of the bifurcation differs between datasets (cross-dataset validation). The authors validated the presence of two branches using the Shahan et al. dataset and demonstrated the specific expression of Modules 20 and 21 in that dataset. However, the branching/bifurcation appears to be very different between the two datasets: in the authors' atlas the ERS^+/ERS^- bifurcation is found almost immediately following their defined developmental origin, whereas in the Shahan et al. dataset it appears to occur much later (likely the differentiating cells) along the developmental axis. It would be worthwhile to perform a cross-mapping between the two datasets and examine what could be the cause of this discrepancy.

4. The in vivo HCR demonstration of cell-to-cell heterogeneity is not yet convincing (pp.10–11).
– The claim that canonical cell-type markers "remained detectable in negative cells, ruling out differences in HCR efficiency"

(Ext. Data Fig. 10c) requires quantitation. Those canonical markers should show less heterogeneity between cells than the ERS markers do; otherwise the observed variability is not specifically attributable to the ERS. This can be addressed with the existing imaging data by quantifying the cell-to-cell variance of the canonical markers against that of the ERS markers; if such a comparison is not feasible, the claim should be tempered accordingly.
– The complementary-expression result in Fig. 3e–h is mainly showing hair vs. non-hair differences in expression. As presented it is not a convincing demonstration of the branch 1 / branch 2 dichotomy independent of the known hair/non-hair distinction, and the text should either show the effect within a single epidermal class or soften the claim.

5. The scRNA-seq and HCR proportions are not the same quantity, so the claimed correspondence is not established (p.16). The statement that "the positive and negative populations detected by HCR correspond to the ERS^+ and ERS^- branches… as their relative proportions vary similarly across both independent experimental approaches" is not accurate as an inference. The scRNA-seq data are characterized by the proportion of cells with expression, whereas the HCR data are shown as the proportion of cell area with positive signal. Similar trends in two different metrics do not demonstrate correspondence of the underlying populations. Expression needs to be quantified on a per-cell basis, and the distribution of per-cell expression examined across these conditions, before this correspondence can be claimed.

6. The drought-induced increase in ERS^+ may reflect global transcriptional activation rather than specific induction (p.18). Because ERS^+ cells have higher UMI/gene counts and water deficit increases overall transcript abundance, the increase in ERS^+ proportion under drought could partly reflect global transcriptional activation rather than specific induction of the ERS^+ program. This also raises the possibility that ERS^- cells represent a lower-transcriptional or dormant state (see point 2). The authors should comment on whether this is a valid alternative hypothesis and, if so, include mention of it in the manuscript. Relatedly, the framing of the result ("the extensive reassignment of cells into the ERS under drought… can be strongly modulated by environmental inputs") does not distinguish a difference in the strength of the ERS signature (and therefore the threshold for calling a cell ERS^+) from a difference in the number of cells in versus out of the state (a more binary switch). If the existing data can distinguish these, the result should say which is occurring; if not, the framing should be qualified. The authors already raise the closely related question in the Discussion — whether the ERS^+/ERS^- proportions reflect probabilities of switching between states — and I would ask that the same caveat be carried back into the Results so the drought interpretation is not stated more strongly there than in the Discussion.

7. "Functionally regulated rather than stochastic" — the basis is unclear (p.17). The authors claim that the consistency of the two populations and their modules "strongly supports the hypothesis that the ERS reflects a functionally regulated transcriptional program rather than stochastic variability"? Reproducibility across datasets does not by itself exclude a stochastic but consistently-proportioned process (this bears directly on the naming question, point 8). The logic connecting the evidence to this conclusion should be made explicit, or the conclusion softened, especially as it relates to function.

8. The name "Environmentally Responsive State" remains unresolved.
I appreciate the authors' detailed response and the new data provided. However, the core concern remains unresolved: the name "Environmentally Responsive State" still implies per-cell sensing and response. The authors' rebuttal helpfully clarifies what the data do and do not show, but in doing so it further undermines the name. First, the authors state: "ERS+ and ERS– cells are not defined as cells that are intrinsically more or less capable of responding to environmental stimuli." If the cells are not defined by per-cell responsiveness, they should not be named for it. Second, Response Figure 1 shows ERS+ cells exhibit lower transcriptional responses to water deficit than ERS– cells (UMI fold-change: 1.32 vs. 1.50), making it harder still to justify calling the state "environmentally responsive."

The authors have already made the revision in one place: the Discussion now reads "This additional transcriptional state, whose prevalence is modulated by environmental conditions." This phrasing is more accurate, and the name should reflect this description. The unknown per-cell determinant of this state is equally consistent with stochastic gene expression tuning the probability of entering the state. Therefore, ERS may represent a stochastically-accessed transcriptional state whose threshold or probability is environmentally modulated, consistent with a bet-hedging strategy. In addition, reproducibility across datasets excludes technical noise but does not rule out biologically stochastic switching, which is itself a regulated and consistent process. The revised Discussion's own definition of the ERS, "an additional layer of identity regulated by ABA," is a cell-intrinsic explanation that would be better reflected in the name than what "environmentally responsive" implies. Suggested alternative: "Environment-modulated state" or "environment-regulated state," the latter preserving the ERS acronym. This requires no new experiments.

Minor
9. "cells acquire the ERS" should be adjusted to use more neutral phrasing. Phrases like "cells acquire the ERS" imply an active switch that has not been directly observed. "Cells in the ERS" or "ERS-enriched cells" are more neutral.
10. "upwards" should be replaced with "shootward" (p.3). The field-standard directional term for displacement away from the tip is "shootward" (vs. "rootward"); "upwards" is orientation-dependent.
11. "border cells" should be replaced with "border-like cells" (p.5). Arabidopsis produces border-like cells (BLCs), which remain attached in sheets, rather than the individually detaching border cells of other species. The term should be corrected.
12. "drought" should be replaced with "water deficit" in most cases. The system is a hard-agar / reduced-water plate model; "drought" overstates it. I would refer to it as water deficit or a model of water-limitation stress and reserve "drought" for the qualified term the authors use elsewhere.

(Remarks on code availability)
NA

Referee #3

(Remarks to the Author)
The authors have addressed our key concerns and we are supportive of the publication of this manuscript.

(Remarks on code availability)

Version 4:

Reviewer comments:

Referee #1

(Remarks to the Author)
I thank the authors for the extensive consideration of my comments and for actively addressing these concerns through substantial revisions. The manuscript is now much clearer in its description of this newly discovered cell state and I look forward to further investigations on the subject.

(Remarks on code availability)

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Point by point response to comments on Nature manuscript 2022-06-09447

We extend our sincere thanks for the constructive feedback and time and effort in considering our manuscript. We believe we have addressed all comments and requested changes, which has helped us improve our manuscript considerably. Below we provide a point-by-point response to all comments and suggestions.

Referee #1

Summary:

Cell identity has been shown to be a major determinant of transcriptional responses to environmental stimuli, however, most studies to date have investigated this process in populations of cells. In this manuscript, Olivia et al. utilize high resolution single cell transcriptomics and whole-mount in situ hybridization chain reaction (HCR) to present a well-resolved atlas of transcriptional states present in cells of the Arabidopsis root. The improved map allows them to discover that on top of the cell-identity-dominant transcriptional signatures that distinguish cells, within each cell lineage two divergent transcriptional states exist, which are associated with genes implicated in responses to various abiotic stresses as well as response to the hormone Abscisic acid (ABA). The authors annotate cells following this alternate developmental trajectory as in an environmentally responsive state (ERS) and show that the proportion of ERS+ cells changes depending on nutrient availability. This alternate developmental trajectory is marked by the expression of some genes that are specific to particular cell types, however, there are also gene sets that mark this alternate transcriptional state across multiple cell types. ERS+ associated genes tend to function in environmentally responsive pathways such as ABA and circadian rhythms.

The manuscript in several ways is a technical tour de force and presents novel use of the HCR method to validate in situ expression patterns of genes. This allows the authors to improve cell type annotations, though the details of how this improvement is achieved is not well described. The most important advance is the discovery of the ERS cell state, which appears to create diversity in the transcriptional state of cells in a particular lineage. If well validated, this discovery would open up many new avenues of research. Are ERS+ cells poised for rapid response to changes in environment? Do they reflect “noise” in the signaling systems that become amplified or reduced upon shifts in the environment? Do plants adapted to stressful environments have different proportions of ERS+ and ERS- cells?

The primary issue is that the significance of ERS cell state is not well supported in the current manuscript. The evidence that such cells exist is demonstrated by scRNAseq, but only briefly surveyed by HCR. The authors also identify several transcription factors and signaling pathways that are expressed in ERS+ cells, but the authors do not test the function of such regulators in the emergence or functions of ERS+ cells. The manuscript would be enhanced by adding clarification to the methods used to build the developmental trajectory (such as the calculation of stemness scores for different cell types), quantification of images where a particular expression pattern is observed, and some experiments testing the relevance of the developmental trajectory that involves the ERS+ cellular state.

Response: We thank the referee for the constructive and thoughtful feedback, and for the encouraging comments about the potential of the discovery of the ERS cell state. In the revised manuscript we have now addressed all of these issues, as described in detail below in response to each comment.
Briefly, as suggested, we have strengthened the support for this finding through extensive independent validation, by expanding the number of ERS-specific genes tested with whole-mount *in situ* hybridization chain reaction (HCR) transcript detection, and by ruling out several plausible technical and analytical artefacts. Furthermore, we have added new quantitative analyses of experimental imaging data, and additional details and clarification of the methods used to build the developmental trajectories. Importantly, we have also conducted new experiments to functionally test regulators of the ERS+ cells, analysing both ABA-deficient and ABA-insensitive mutants, which confirmed that ABA signalling contributes to the establishment of the ERS. Finally, we now demonstrate the relevance of the ERS+ developmental trajectory, showing that this state is environmentally responsive, with the proportion of ERS+ cells changing in response to a model of drought stress, through both scRNA-seq and independent validation by HCR with quantitative image analysis of transcript abundance changes. Collectively, these additions demonstrate the robustness of this cellular state, uncover its regulators, and highlight its functional relevance under environmental stress.

Major comments:

Referee 1 - point 1: Abstract line 6-9: The authors oversimplify the current view of root cell identities by stating that the field considers it to be ontogenetically driven "with no impact from the environmental conditions." It may be useful to consider Dinneny et al., 2008, Science where the authors show that a large portion of the transcriptional state of specific cell types is environment dependent with a smaller core transcriptome that is maintained under different environments. The authors should also explicitly state what they define as "cell identity". The totality of genes expressed in a particular cell type under one specific environmental condition may be an overly constrictive definition.

Response: Thank you for pointing this out. We have modified the abstract to be more precise and cautious with our statements. The beginning of the abstract now reads as follows:

"Plant development is extremely flexible compared to animals, with growth and architecture being continuously shaped by environmental cues. Roots, for instance, adapt their development to the substrate they are anchored to, water and nutrient availability, and the local biome. This plasticity suggests that, at the cellular level, environmental inputs have a substantial impact on cell function and activity. The root is currently viewed as a collection of distinct cell types, organized in stereotypical layers, each arising from stem cells along a linear differentiation trajectory, much like animal lineages. Cells within each layer are often regarded as mostly homogeneous, with a cell type-specific transcriptional signature that gradually shifts as differentiation progresses."

Referee 1 - point 2: Greater clarity on why previous studies had not identified the ERS lineages is needed. How did the HCR results inform this discovery? What bioinformatic steps are different? If the authors process their data in the same way as in other comparable studies, is the ERS state no longer revealed?

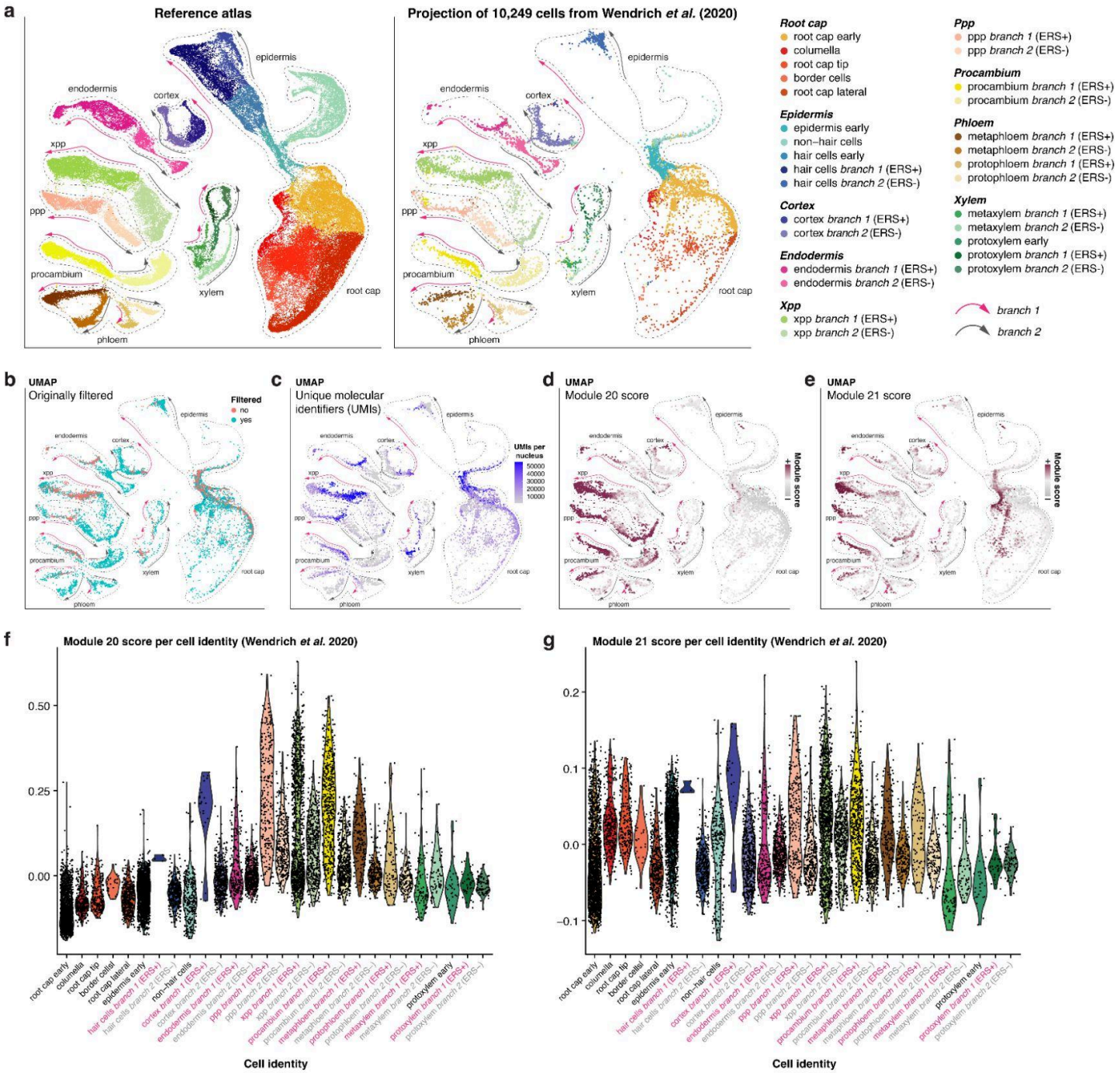
Response: We have added further details comparing our findings to previously published datasets in **Response Figures 1-3 / Extended Data Figures 13,12,14**, focusing on three representative studies, each of which either had specific limitations that likely prevented the identification of the ERS state, or observed the lineage branching but did not focus on it:

- In Wendrich *et al.*[1], the authors applied a hard UMI threshold to distinguish cells from damaged cells or background, discarding all data below this cutoff. While this was a common practice in early single-cell RNA-seq studies, it removed a substantial proportion of ERS− cells, making it difficult to detect the presence of two populations. When we re-processed the Wendrich *et al*. dataset using our pipeline, we recovered many more cells and were able to identify both ERS− and ERS+ cells across lineages, and the expected UMI and gene module enrichments in ERS+ cells (**Resp. Fig. 1 / Ext. Data Fig. 13**). Although our detailed comparison focused on this study, other datasets also applied strict filtering criteria that may have similarly led to the loss of biologically relevant information. Importantly, the bioinformatic approach we have developed more carefully discriminates real cells from damaged cells or background (**Resp. Fig. 17 / Ext. Data Fig. 1**), and we use this to demonstrate the presence of both ERS+ and ERS− cells in our datasets and others'. Crucially, we also rigorously independently validate the existence of the ERS states through *in situ* HCR imaging (see response to Referee 1 - point 10**, Resp. Fig. 10 / Fig. 2e** and **Resp. Fig. 13 / Ext. Data Fig. 18**).
- In Farmer *et al.*[2], the authors focused on characterising the different cell type populations and correlating them with chromatin accessibility. While they identified some subclusters, notably in the endodermis, they did not conduct a comprehensive pseudotime analysis across all lineages, which likely contributed to the branching events remaining uncharacterised However, our new analyses of their snRNA-seq data in the revised manuscript clearly identifies both ERS+ and ERS− states, and the ERS-specific gene modules (**Resp. Fig. 2 / Ext. Data Fig. 12**).
- In Shahan *et al.*[3], which currently represents the largest Arabidopsis root single-cell atlas and includes data from Denyer *et al.*[4] and Ryu *et al.*[5], the branching of lineages is clearly visible on the UMAP and is briefly mentioned in the main text and a supplementary figure, but not deeply explored. Nonetheless, this demonstrates that independent scRNA-seq analysis workflows can also discern the lineage branching. Our new analyses of the Shahan *et al.* scRNA-seq data in the revised manuscript also clearly shows the branching trajectories of the ERS+ and ERS− states, and the expected UMI and gene module enrichments in ERS+ cells (**Resp. Fig. 3 / Ext. Data Fig. 14**).

Ultimately, the identification of the ERS state was not informed by HCR. Rather, the HCR experiments were used to independently experimentally validate the *in vivo* expression of genes associated with this state in a subset of cells of multiple cell types, as we observe in the scRNA-seq data. The discovery itself was enabled by several key aspects of our analytical approach: (1) a more careful strategy for filtering cells, which avoided

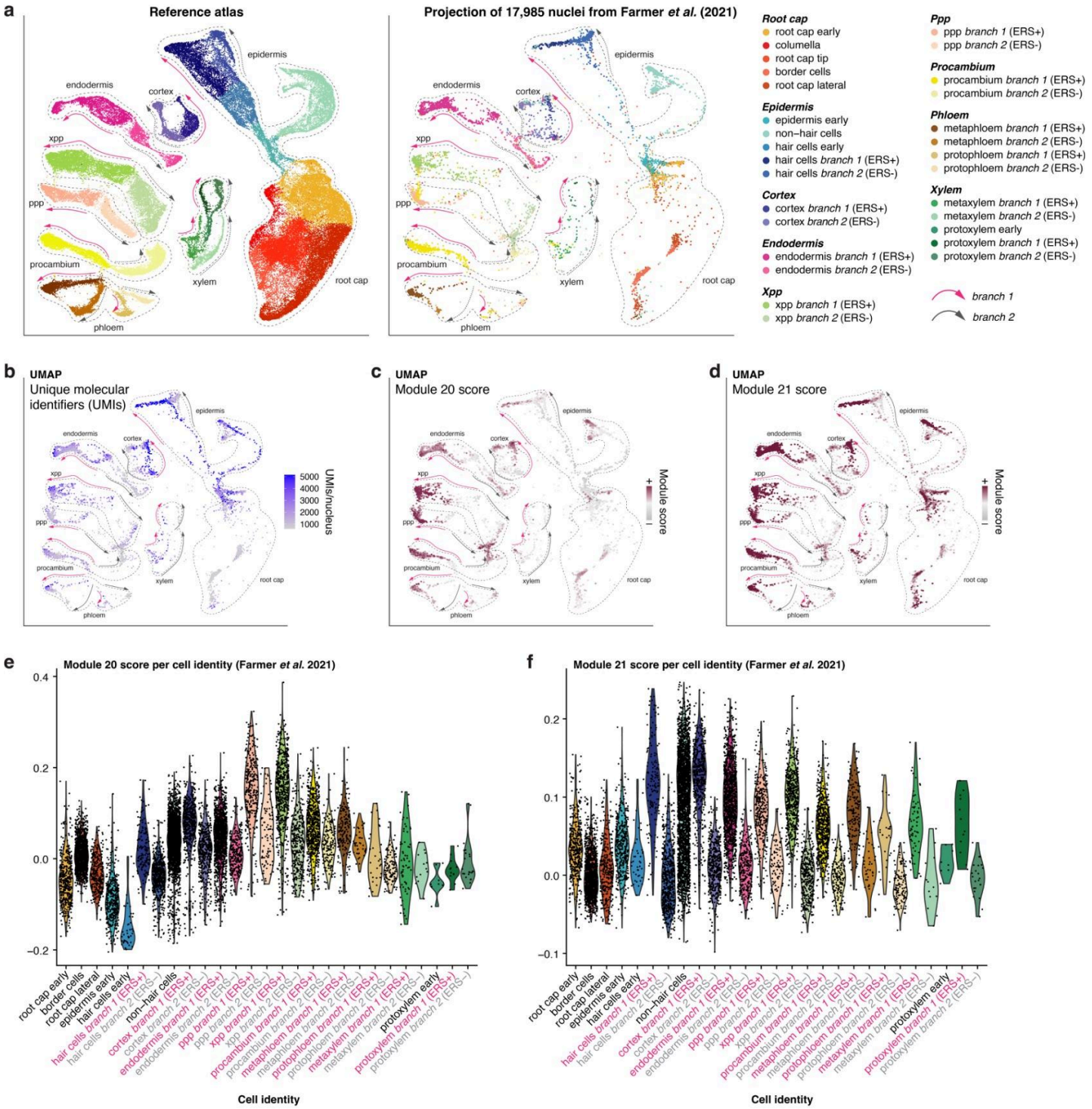
excluding potentially informative populations; (2) the use of Partition-based Graph Abstraction (PAGA) trajectory analysis, which is particularly well suited for robustly capturing branching events; and (3) the identification of gene co-expression modules across the entire dataset. This third step, in particular, allowed us to detect a shared transcriptional state across branches in multiple lineages, rather than focusing solely on differential expression along pseudotime within each lineage, as in previous studies.

Importantly, even though these two populations were not detected or deeply characterised in the original studies, we demonstrate that, upon re-analysis, both ERS+ and ERS− populations can be identified in each dataset, that they differ in transcriptional activity as indicated by UMI content, and that ERS+ cells consistently express the same co-expression modules described in this study.



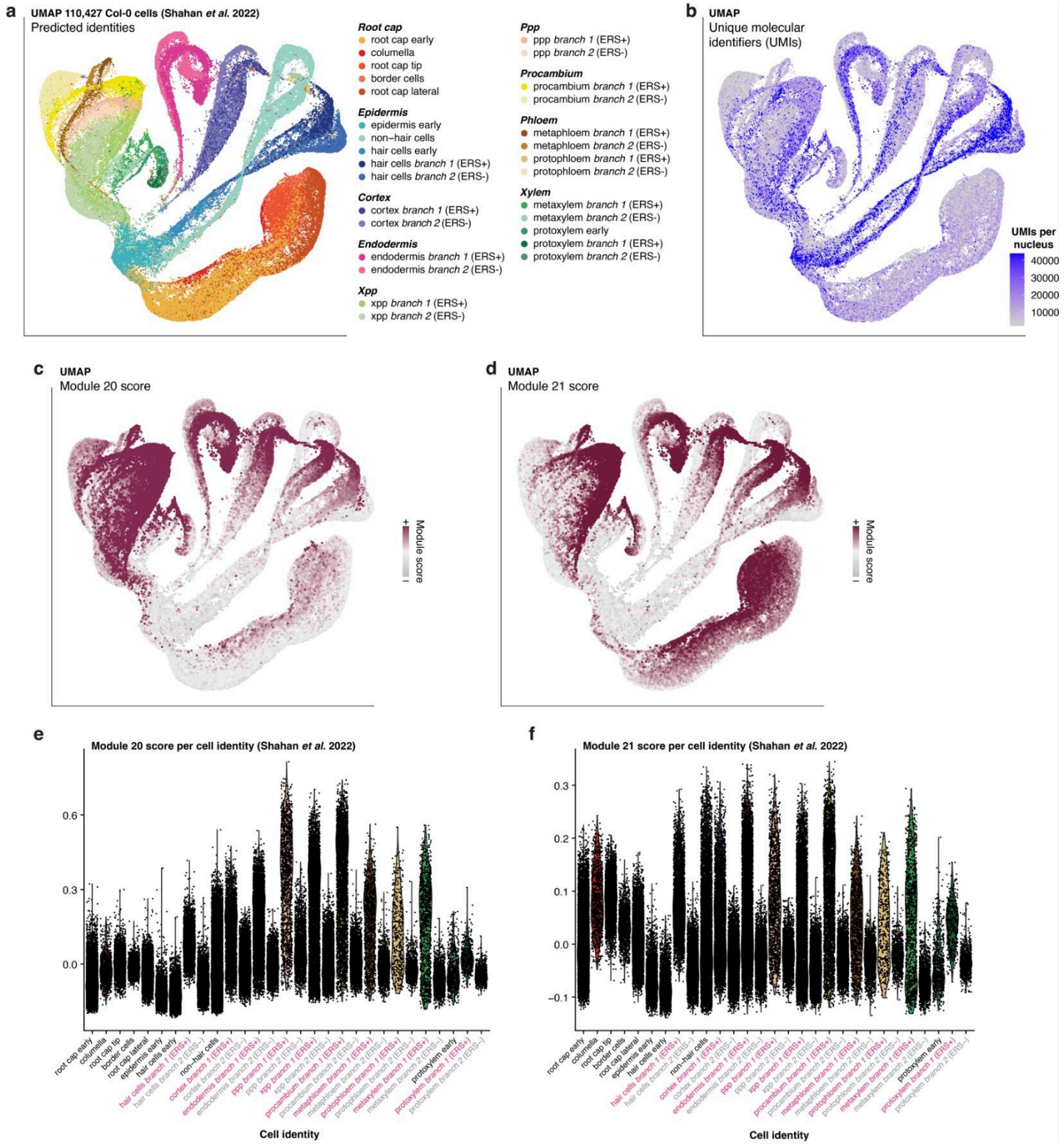
Response Figure 1 / Extended Data Figure 13: Branches and their associated ERS-specific genes are detected in the scRNA-seq dataset from Wendrich *et al.*

a, UMAPs showing our reference atlas and the projection of cells from Wendrich *et al.* into the atlas UMAP embedding, with cells coloured by their assigned identity based on our atlas. **b,** UMAP showing whether cells were originally discarded in their study based on UMI content. **c,** UMAP showing the distribution of UMI content per cell. d–g, UMAPs (d, e) and violin plots (f, g) showing the distribution of expression scores for our 'branch 1'-specific modules 20 and 21 in their scRNA-seq data.



Resp. Fig. 2 / Ext. Data Fig. 12: Branches and their associated ERS-specific genes are detected in the snRNA-seq dataset from Farmer *et al.*

a, UMAPs showing our reference atlas and the projection of nuclei from Farmer *et al.* into the atlas UMAP embedding, with cells coloured by their assigned identity based on our atlas. **b,** UMAP showing the distribution of UMI content per nucleus in Farmer *et al*. **c–f,** UMAPs (c, d) and violin plots (e, f) showing the distribution of expression scores for our 'branch 1'-specific modules 20 and 21 in the snRNA-seq data.



Resp. Fig. 3 / Ext. Data Fig. 14: Branches and their associated ERS-specific genes are detected in the scRNA-seq dataset from Shahan *et al.*

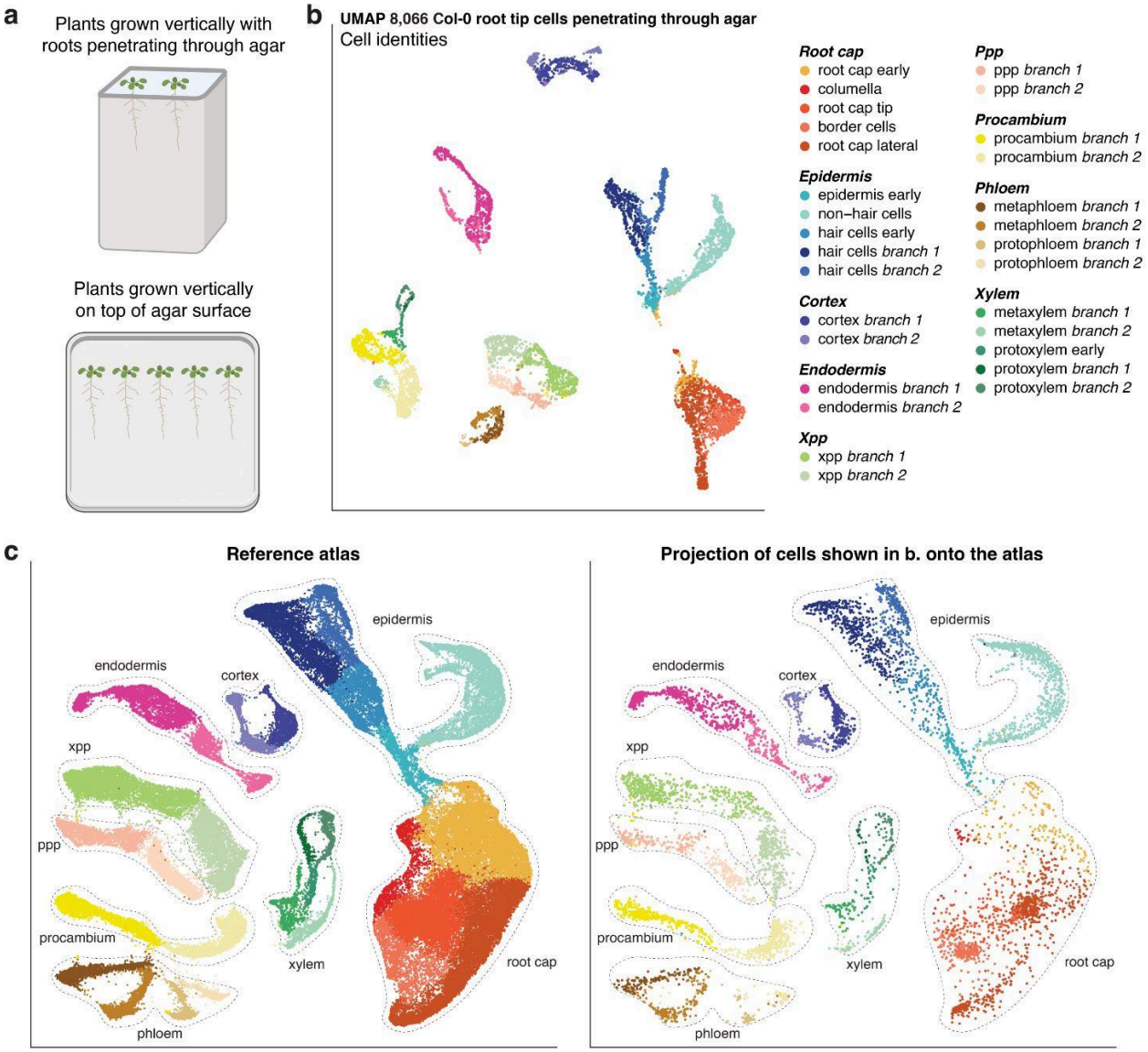
a, UMAPs showing the reference atlas embedding from Shahan *et al.*, with cells coloured by their assigned identity based on our atlas. **b,** UMAP showing the distribution of UMI content per cell in their atlas. **c–f,** UMAPs (c,d) and violin plots (e,f) showing the distribution of expression scores for our 'branch 1'-specific modules 20 and 21 in their scRNA-seq data.

Referee 1 - point 3: Roots growing on the surface of agar media experience two different microenvironments (contact with the agar surface and water film, and contact with air). Could these local environmental cues serve as the basis for the ERS+/- states?

Response: We thank the referee for this thoughtful suggestion. It is indeed plausible that the two distinct microenvironments encountered by surface-growing roots - contact with the agar and water film versus contact with air - could influence transcriptional states and underlie the ERS+/− distinction.

To test this possibility, we sowed seeds on top of a vertical agar block embedded within a plate, rather than on the agar surface. This setup allowed the roots to penetrate and grow downward through the media, rather than across its surface. We then collected and processed the same section of the root tips at the same developmental stage as all other experiments and using the same scRNA-seq library preparation protocol and analysis workflow as we did for the cell atlas.

As shown in **Resp. Fig. 4 / Ext. Data Fig. 8**, we were still able to clearly detect both ERS+ and ERS− branches in all lineages, similar to our original experiments. This demonstrates that differential exposure to air versus agar is not driving the observed dichotomy in lineage trajectories.



Resp. Fig. 4 / Ext. Data Fig. 8: Branches in the trajectories are not due to the two microenvironments experienced by roots growing at the agar surface

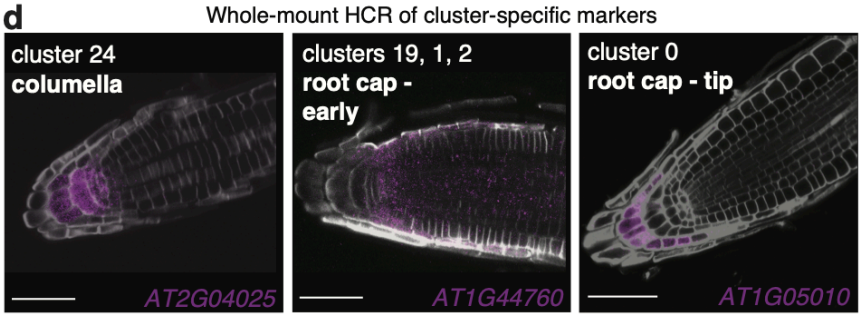
a, Diagram showing the two experimental setups: roots grown penetrating vertically downwards through the agar (top, tested here) versus roots grown along the medium surface (bottom, standard condition). **b,** UMAP plot showing the cell identity assignments for roots grown penetrating the agar, using our reference atlas. Note that the branches are detected in this atlas as well. **c,** Projection of cells from roots penetrating the agar onto the reference atlas UMAP embedding, showing that cells are distributed across all branches, similar to the reference atlas.

Referee 1 - point 4: Abstract line 5: “The formation of one of the trajectories is driven by a strong and specific activation of genes involved in responses to environmental stimuli” To claim this, it would be necessary to test mutants of genes involved in the formation of this environmentally responsive trajectory and show that these mutants lack two developmental trajectories for different cell types.

Response: We have rephrased the sentence in the abstract to better reflect our intended meaning. The revised sentence now reads: *“One of these paths is characterized by a strong and specific activation of genes*

involved in responses to environmental stimuli, including those activating the abscisic acid (ABA) pathway.". What we meant to convey is that both branches share the expression of some cell type- and pseudotime-specific genes, but one of the branches (ERS+) is uniquely characterised by the strong and specific activation of ERS-associated genes. This distinct transcriptional signature differentiates the two branches and likely explains their separation in trajectory space.

Referee 1 - point 5: In Extended Data Fig.2b, the gene (AT3G25980) used as a marker for Cluster 28 is expressed in dividing cells of all cell types. And the cluster 16 marker (AT3G23830) is also expressed in the meristematic zone of all cell types and not just in the epidermis. It is unclear why the clusters are not named according to the expression pattern observed by whole mount in situ HCR but are annotated as specific to the epidermis/lateral root cap. If these clusters were used to calculate the stemness score for the epidermis-root cap lineage, how was the stemness score calculated for the other lineages? How were the early markers for other lineages identified for the pseudotime analysis? Why were clusters 9 and 12 included for the identification of "early markers"? There is no whole mount in situ HCR data showing expression patterns of genes in these clusters.

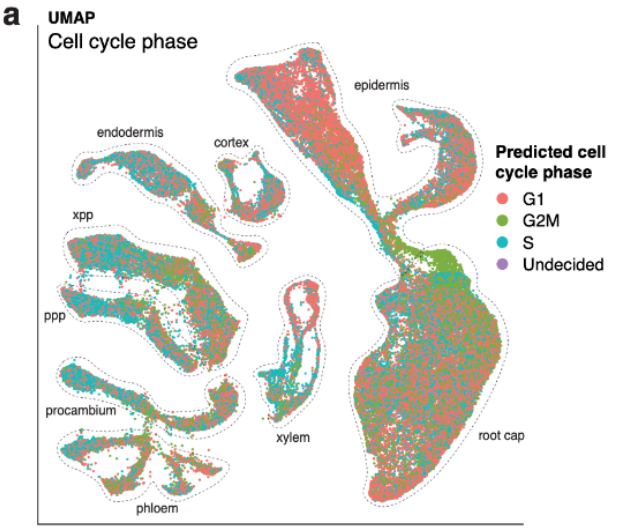


Resp. Fig. 5 / Fig. 1d *(part)*: HCR of cluster-specific markers.

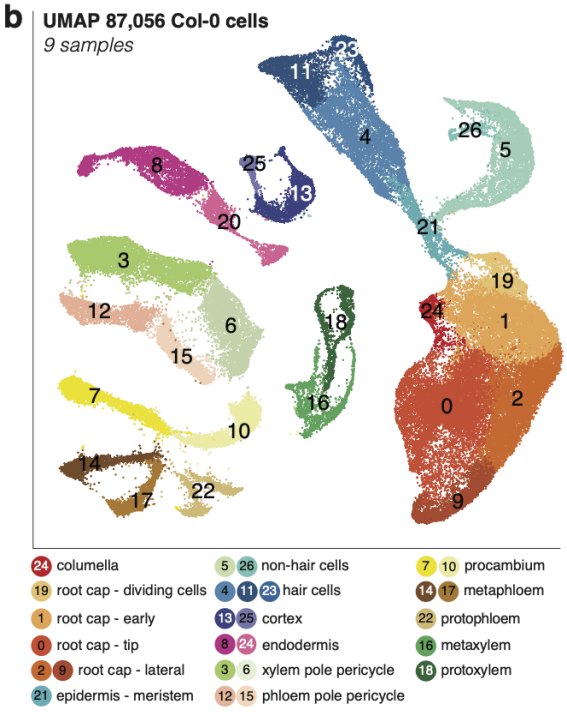
Response: Thank you for pointing this out. In the revised manuscript and updated dataset we have revised some of the cluster naming to more clearly name clusters according to the observed HCR expression patterns, as suggested. For improved clarity, we no longer use these two specific genes (*AT3G25980*, *AT3G23830*) that were originally presented in the HCR-based cluster identification, as we agree that they did not optimally represent the associated clusters, as detailed below. *AT3G25980* is indeed expressed in mitotic cells across multiple cell types, rather than being specific to the root cap, as confirmed by HCR. 'Cluster 28' presented in the original manuscript consisted almost entirely of cells in the mitotic phase and was detected specifically in the early stages of root cap development. It also expressed several root cap-specific genes. This is why we originally named this population *"root cap - dividing cells"*. In the updated manuscript, we have now included this cluster of cells (Cluster 19) in the broader *"root cap - early"* cluster group, which is supported by HCR detection of the marker gene *AT1G44760* (**Resp. Fig. 5 / Fig. 1d**). The corresponding enrichment of mitotic phase cells in the *"root cap early"* group is evident in the cell cycle analysis presented in the revised manuscript **Resp. Fig. 6 / Ext. Data Fig. 6a**).

As pointed out, *AT3G23830* is expressed in meristematic cells in all lineages in the HCR experiments. However, in our scRNA-seq dataset, only the epidermal lineage contained a cluster that expresses *AT3G23830*, which is why we initially annotated this cluster as *"epidermis-meristem"*. In the revised manuscript, we now explain the identification of this cluster based on well-established features of epidermal development, specifically, that the epidermal and root cap lineages originate from the same stem cell, and that epidermal cells early on split into two distinct populations. As observed in the scRNA-seq data, this group of cells (cluster 21, *"epidermis - meristem"*, **Resp. Fig. 7 / Fig. 1b**) bridges the epidermis and the root cap, appearing before the divergence of hair and non-hair cell fates, and therefore likely represents an early stage of epidermal development close to the meristem.

This topology - root cap-connecting cells transitioning into epidermal subtypes - is unique to the epidermal lineage in our dataset. In other lineages, we do not



Resp. Fig. 6 / Ext. Data Fig. 6a: Cell cycle phase prediction. a, UMAP plot showing the predicted cell cycle stage of cells in the reference atlas. Note a group of mitotic cells at the early stages of root cap development.

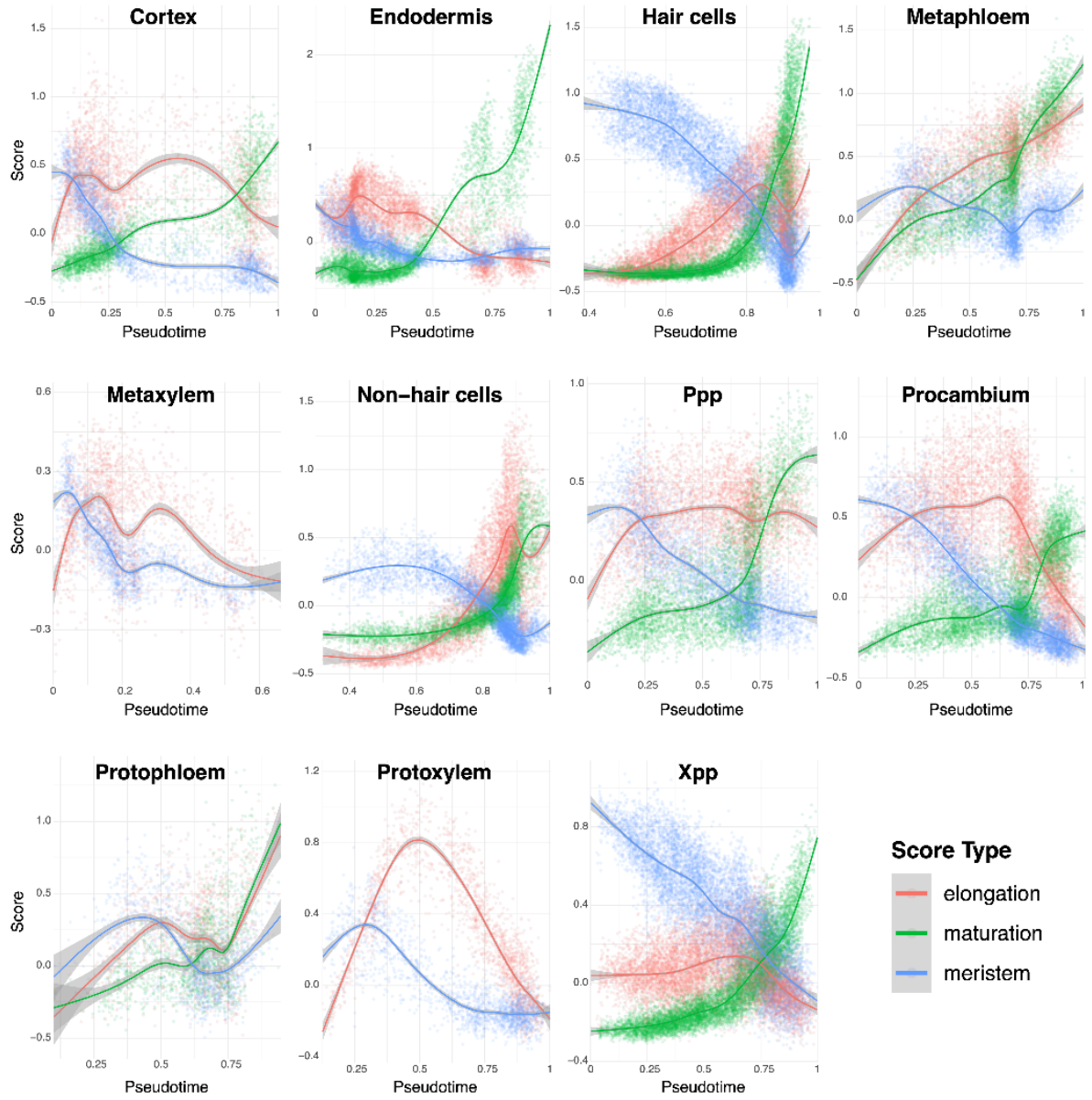


Resp. Fig. 7 / Fig. 1b: UMAP of reference atlas. Cluster 21 = *"epidermis - meristem"*.

observe a distinct group of cells that connects to the root cap or meristematic regions, which prevents us from identifying the origin of those lineages based on data topology. This could be due to technical limitations, such as the epidermis being more easily released than inner tissue layers during protoplasting, making it better sampled at these early stages. As a result, the epidermis is the only lineage for which we can confidently identify an early population based on its position in trajectory space and its connection to root cap cells.

Therefore, to assign an "early" score to cells in other lineages, which used the following strategies. We first selected genes that were specifically enriched in this *"epidermis - meristem"* cluster compared to more mature hair and non-hair cells. We then identified the cells in other lineages that co-express this gene set at the highest levels, and considered these to represent the earliest developmental stages (t = 0) for each lineage. To validate our approach, we used the top 50 genes defined as specific to the meristem, elongation, or maturation zones in each lineage by Shahan *et al.*[3] to calculate cell type-specific "meristem", "elongation", and "maturation" scores. We then assessed how these scores progressed along our pseudotime ordering (**Resp. Fig. 8 / Ext. Data Fig. 4**). We found that cells closer to t=0 had higher meristem scores, followed by a peak in elongation scores, and finally an increase in maturation scores at the end of the trajectory. This confirms that our pseudotime analysis recapitulates the developmental progression described by Shahan *et al.*, supporting the robustness of our method.

Crucially, we have now also validated our trajectories using HCR in three lineages - epidermis, endodermis, and xylem pole pericycle - by showing that genes detected closer to t = 0 in the scRNA-seq data are expressed nearer to the root tip, while genes specific to later stages are detected further away from the meristem (see response to **Referee 1 - point 6 below**, and **Ext. Data Fig. 3**).

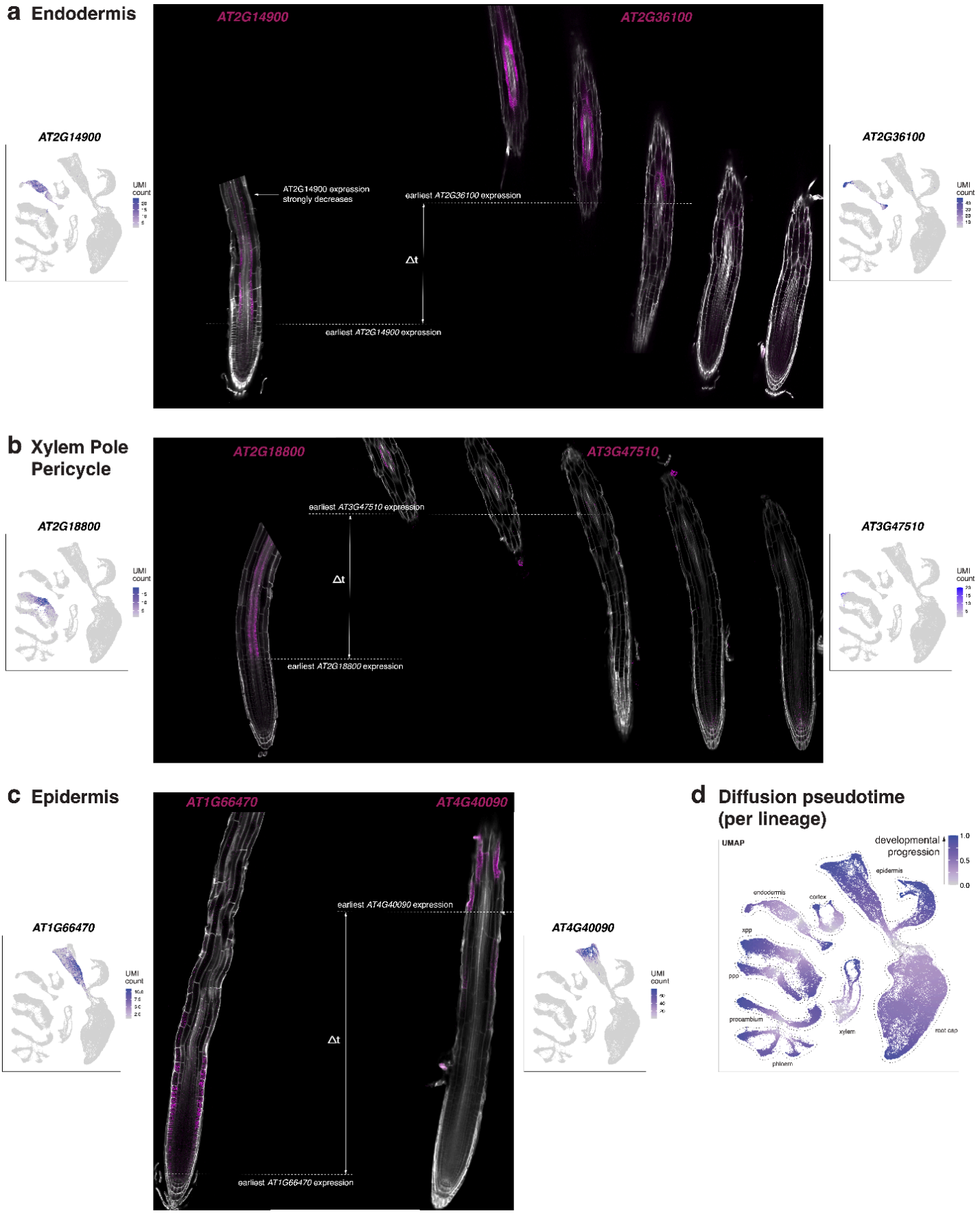


Resp. Fig. 8 / Ext. Data Fig. 4: Validation of trajectories using cell type- and developmental stage-specific markers from Shahan et al.
Scatter plots and regression lines showing the distribution of expression scores for the top 50 genes specific to the meristematic, elongation, and maturation zones defined by Shahan *et al*., plotted as a function of our pseudotime assignment for each cell type. Successive peaks of meristematic, elongation, and maturation scores are observed along the pseudotime axis, validating our trajectory analyses.

The reason we originally included clusters 9 and 12 (which correspond to early stages of root hair and non–root hair development) in the calculation of the stemless score is that, as explained above, other lineages do not physically connect to the meristem cluster on the UMAP. This suggests we may not have captured the very earliest developmental stages for those cell types. As such, their t = 0 cells may more closely resemble cells located just above the meristem cluster - namely, clusters 9 and 12 - making it reasonable to include them when defining the gene signature of cells assigned as t = 0 in different lineages. However, in the revised manuscript, we used only the “epidermis-meristem” cluster to identify genes for the stemless score and found very similar results. Overall, through our independent experimental methods and analyses, and comparison to independent studies, we are highly confident that the early developmental stages in our scRNA-seq lineages have been accurately assigned.

Referee 1 - point 6: Are the genes with expression patterns presented in cross sections for cortex and endodermis in Fig. 2c specific to the differentiation zone of the root? Or are these genes present in the meristem as well? It would be important to include the longitudinal images as well to see how the genes are expressed along the different developmental zones of the root. This would also help to confirm results of the pseudotime analysis.

Response: Thank you for the suggestion. In Fig. 1 of the original manuscript, we used HCR markers to identify cell lineages, which are most easily distinguished in cross sections (except for the root cap). As suggested, in the revised manuscript we have now used a subset of these markers to validate the pseudotime trajectories, and included longitudinal images in **Ext. Data Fig. 3a-c / Resp. Fig. 9a-c** (below) to show their expression along developmental zones.

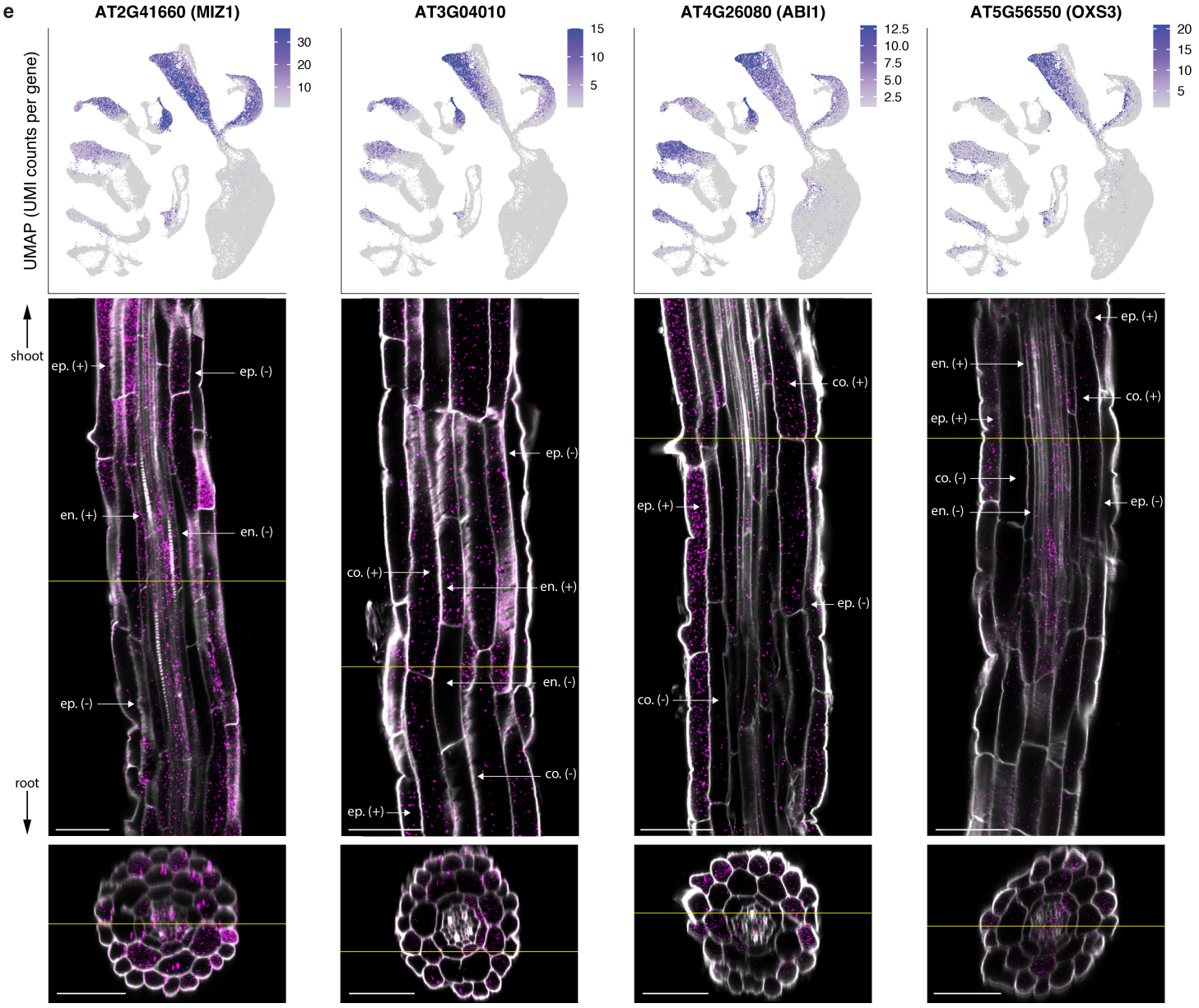


Resp. Fig. 9 / Ext. Data Fig. 3: Validation of pseudotime assignments
a, UMAP plots and longitudinal optical sections of whole-mount HCR showing the expression of two endodermal markers, *AT2G14900* and *AT2G36100*, *in silico* and *in vivo*. *AT2G14900*, which is predicted to be expressed early according to the trajectory analysis (see pseudotime assignment in d), is detected closer to the meristem, whereas *AT2G36100*, a later marker, is detected further away, validating the pseudotime assignment. Multiple optical sections are shown when a complete longitudinal section of the root could not be obtained at once due to minor root curvature during mounting. Dotted lines indicate the earliest developmental stage at which expression of each marker was detected. **b,** Similarly, in the xylem pole pericycle, *AT2G18800* (early marker) is detected nearer to the meristem and *AT3G47510* (late marker) is detected further away, consistent with the trajectory analysis. **c,** The trajectories for epidermal hair cells are also validated: *AT1G66470* (early marker) and *AT4G40090* (late marker) show concordant expression patterns *in silico* and *in vivo*. **d,** UMAP plot summarizing the pseudotime assignment per lineage in our reference atlas.

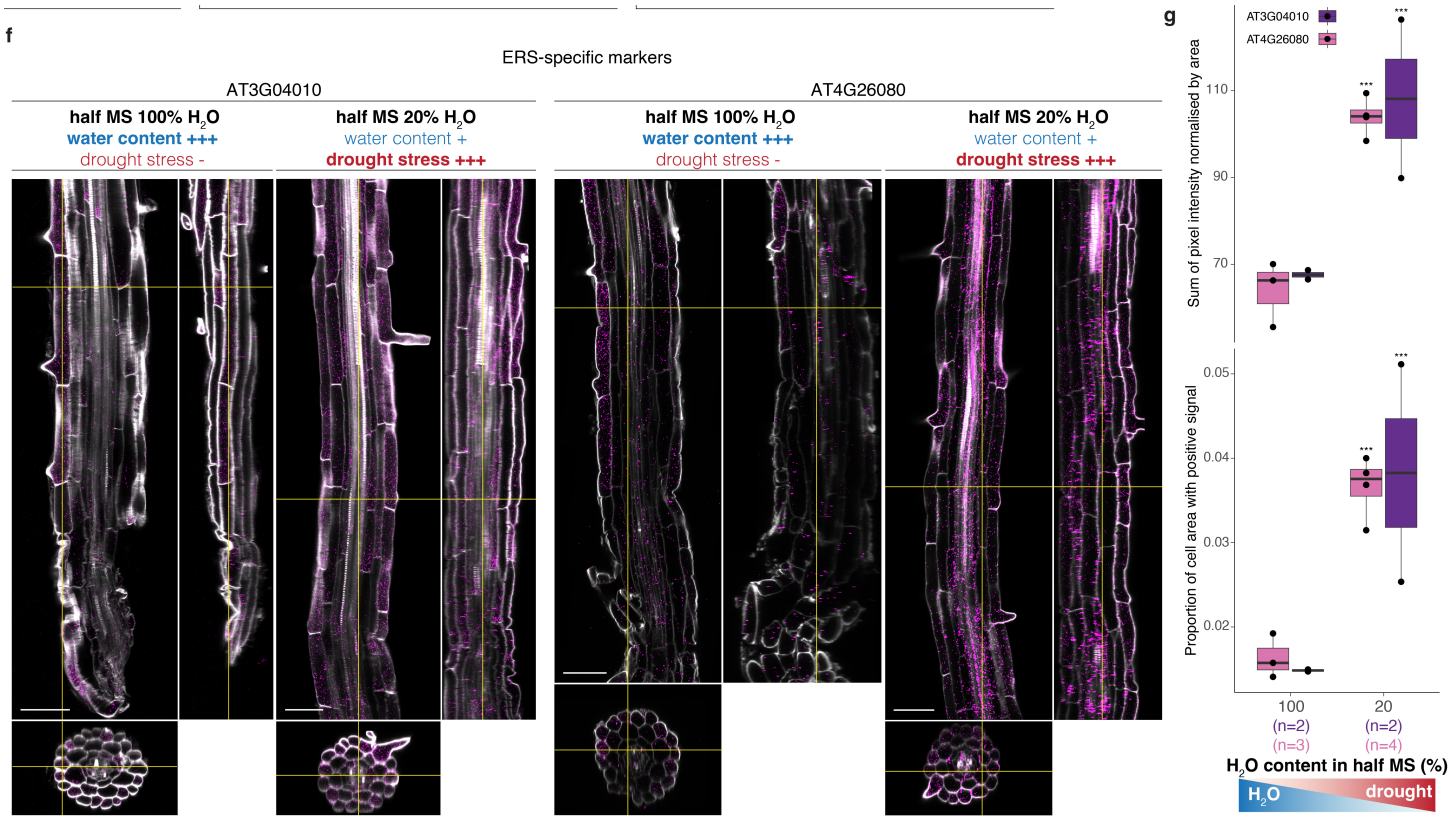
Referee 1 - point 7: The non-homogenous expression of AT3G47510 in images represented in Extended data 4c is not obvious. From the images it appears that while XTH21 is an early XPP marker, AT3G47510 is expressed later in the XPP cells. Quantification of the number of roots in which this non-homogeneous pattern was observed and/or quantification of signal intensity variation across different pericycle cells would be important here to observe these differences. The authors should also show expression of XTH21 at the developmental region at which AT3G47510 expression is observed.
Response: We observed this non-homogenous pattern in every root that was imaged. Accurate quantification of fluorescence signal per cell is challenging, as it requires reliable cell segmentation, which is particularly difficult to achieve in the inner and narrow cell layers like the pericycle. Furthermore, this would also require reliably distinguishing xylem pole from phloem pole pericycle cells, which is again challenging. To circumvent this difficulty, we have decided to use *AT3G47510* only for pseudotime validation, and to more clearly and comprehensively demonstrate and confirm the presence of the two branches, we selected a new set of four ERS-specific genes expressed in the outer layers of the root, where it is easier to distinguish between positive and negative cells. All four genes are enriched in only a subset of cells, strongly supporting the existence of two distinct populations (**Resp. Fig. 10 / Fig. 2e**).

In the revised manuscript, we have kept *XTH21* (*AT2G18800*) and *AT3G47510* as effective independent markers for pseudotime progression (i.e., “early” vs. “late”), as shown in **Resp. Fig. 9b / Ext. Data Fig. 3b**, together with additional validation markers for the epidermis and endodermis. Furthermore, to more clearly show the expression pattern along the developmental axis, we have chosen to present longitudinal sections rather than cross sections for these images.

On the broader point of quantifying expression patterns in images, we agree that this is important and have now included quantifications of signal changes for ERS-specific genes, showing a correlation with the increased proportion of ERS+ cells in response to environmental stress (see further details in response to Referee 2 – point 4**, and Resp. Fig. 11f,g / Fig. 4f,g**).



Resp. Fig. 10 / Fig. 2e: HCR validation of expression of multiple ERS-specific genes only in a subset of cells, of multiple cell types. UMAP plots and optical sections showing transcript detection of four 'branch 1' markers, *AT2G41660* (*MIZ1*), *AT3G04010*, *AT4G26080* (*ABI1*), and *AT5G56550* (*OXS3*), both *in silico* from scRNA-seq data and *in vivo* by whole-mount *in situ* Hybridization Chain Reaction (HCR). Yellow lines indicate the relative positions of longitudinal and transverse cross sections. Example cells that are positive (+) or negative (–) for each transcript in the epidermis (ep.), cortex (co.), or endodermis (en.) are indicated with arrows. Also see **Ext. Data. Fig. 17** that shows the position of these longitudinal images in the context of the root tip. Scale bar = 50µm.

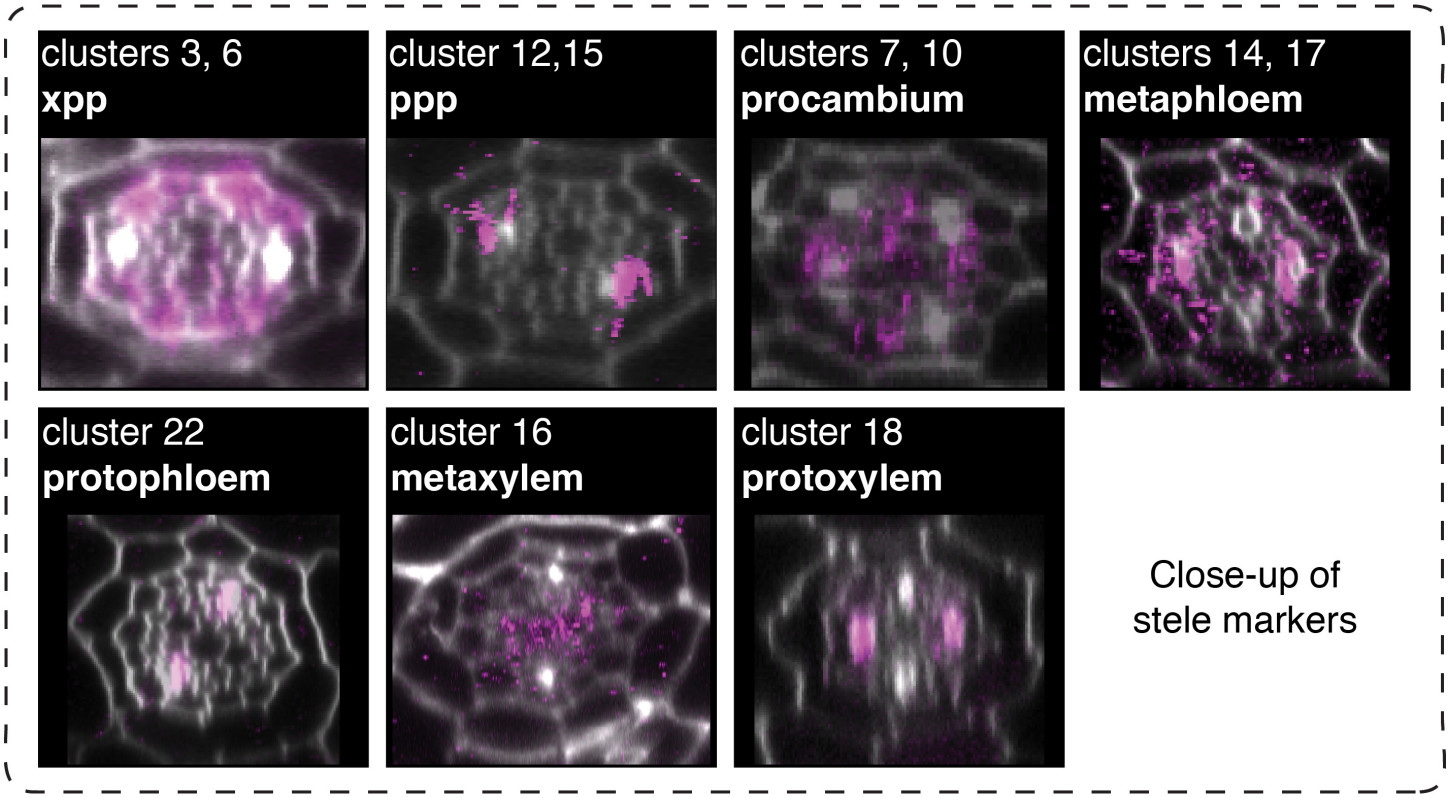


Resp. Fig. 11 / Fig. 4 *(part)*: HCR validation of the increased proportion of ERS+ cells in response to drought stress. f, Orthogonal confocal views of whole mount HCR detection of two 'branch 1'-specific markers described in Fig. 2e, *AT3G04010* and *AT4G26080* (*ABI1*), in roots grown in half MS (control, 100% H_2O) or under the harshest drought condition (half MS, 20% H_2O). Yellow lines indicate the relative positions of the three optical sections. Scale bar = 50µm. **g,** Box plots showing the increase in total signal intensity or in the area of positive signal (both normalised by total cytoplasmic area, see Methods) in the HCR detection in roots of the ERS-specific markers *AT3G04010* and *AT4G26080* (*ABI1*) under drought conditions (half MS, 20% H_2O) compared to control (half MS, 100% H_2O) (Wilcoxon rank-sum test, *** indicates a p-value < 0.001).

Referee 1 - point 8: The presentation of the confocal data is obscured by the way the micrographs are processed. Making the cell boundaries channel opaque prevents the signal from the inner tissues from being apparent, except in cross section. Perhaps the authors can make the cell boundary channel transparent or show a single longitudinal cross section to reveal expression along this axis more clearly. In general, the cross sections are far too small to evaluate the tissue or cell type specificity, especially for those HCR data showing expression in stele cells. Perhaps a magnified view of the stele can be shown instead of the whole root when this tissue is the primary focus.

Response: Thank you for this helpful suggestion. We have now added magnified views of markers specific to stele cell types in **Fig. 1d** to better depict the cell-type specificity, shown in **Resp. Fig. 12**.

We agree that longitudinal sections are particularly valuable for revealing expression patterns along the developmental axis, in some cases, more clearly distinguishing positive and negative cells, given the elongated shape of root cells. We have therefore preferentially used longitudinal sections both for pseudotime validation and for examining the expression of ERS-specific markers, shown in **Resp. Fig. 9a-c / Ext. Data Fig. 3a-c** and **Resp. Fig. 10 / Fig. 2e** and **Resp. Fig. 11f,g / Fig. 4f,g** and**Resp. Fig. 13 / Ext. Data Fig. 18**.



Resp. Fig. 12 / Fig. 1d *(part)*: Magnified views of HCR detection of markers specific to stele cell types to better depict the cell-type specificity. HCR signal of the transcripts shown in purple, and cell walls stained with Calcofluor-white.

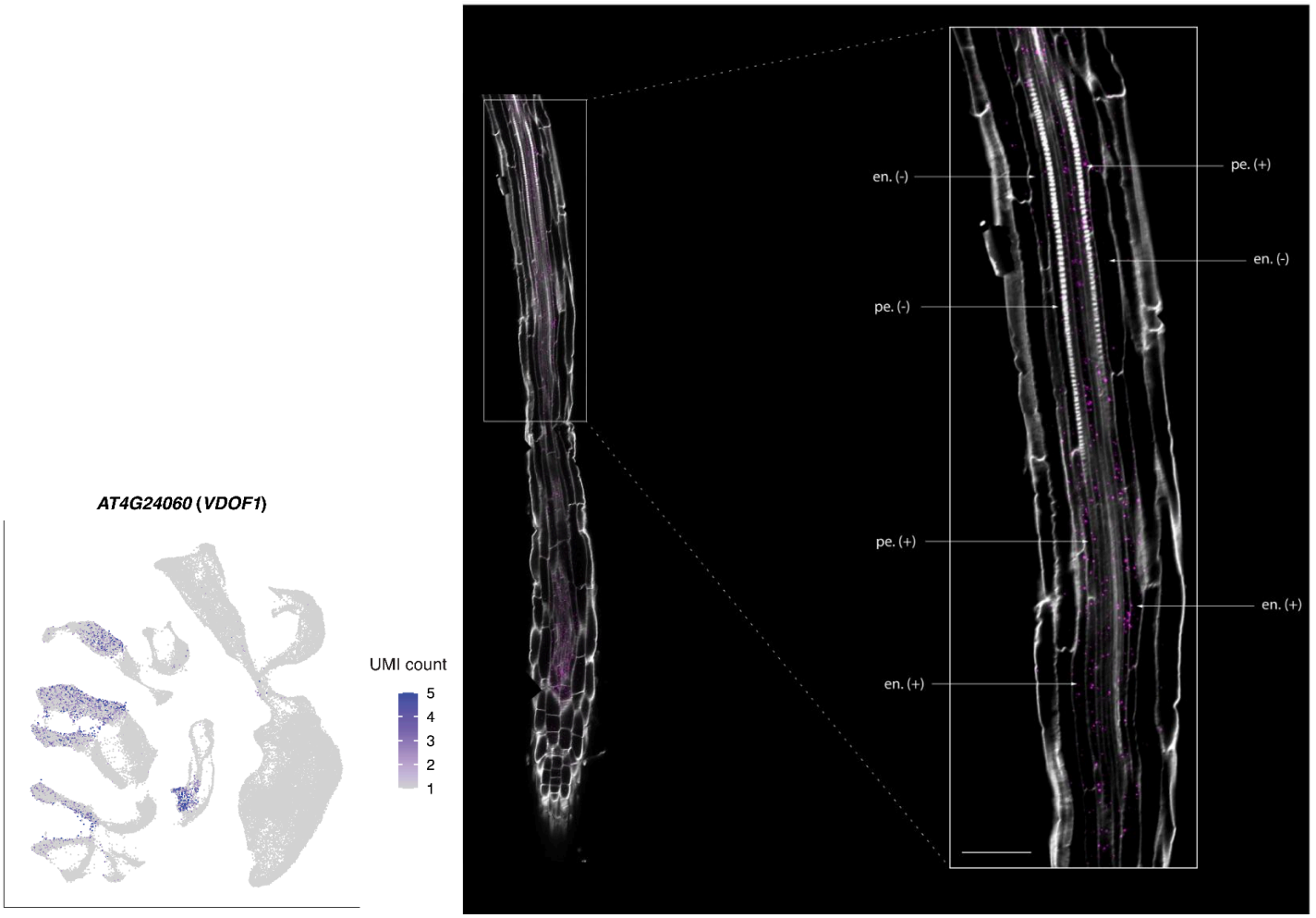
Referee 1 - point 9: Extended data 4d and e. CASP1 has been shown to be involved in deposition of Casparian Strip in all endodermal cells including the (future) passage cells. The passage cells only lack suberin lamellae. It is possible that the passage cells deposit Casparian strip at a later developmental stage compared to other endodermal cells. Are there any published data that would suggest these differences in Casparian Strip deposition between passage and non-passage endodermal cells? How does the PHO1;H3 expression differ along the root? Are key endodermal developmental regulators such as SCR present in both cell fate trajectory branches?

Response: We thank the referee for these suggestions. However, given the extensive new experiments and analyses that we have added to the revised manuscript, to improve the focus of the study we have decided to primarily focus on other aspects in the revised manuscript Therefore, we have elected to no longer include these previous analyses related to CASPs and passage cells in the revised manuscript.

Referee 1 - point 10: The authors identify genes that mark the ERS+ state in several cell lineages and use HCR to analyze the expression pattern of one marker for this state, VDOF1. However, the data are not convincing here. In Figure 2b, it would be important to show how the root is oriented. I believe that the cross sections represented at the top are closer to the root tip and the cross sections below are shoot ward? Is the expression of VDOF1 observed in the root apical meristem as is suggested by the scRNAseq data? To validate the ERS+ state, showing dual labeling of ERS+ and ERS- markers would be important to confirm that they are mutually exclusive states, as the data suggests. Again, the resolution of the images needs to allow for the discrimination of individual cells with and without expression of the marker gene.

Response: Thank you for pointing this out. It would indeed be ideal to simultaneously visualize markers for both ERS+ and ERS− populations to confirm the presence of these two states *in vivo*. However, we have not been able to identify genes specifically expressed in ERS− cells. Our interpretation is that all cells (ERS+ and ERS−) share a core set of lineage-defining, cell type–specific genes, but only a subset (ERS+) additionally expresses a distinct set of genes, while others (ERS−) do not. In other words, there are genes common to both ERS+ and ERS− cells, and additional genes uniquely expressed in ERS+ cells, but not the reverse. Thus, ERS− is not a mutually exclusive state relative to ERS+, but rather reflects the absence of the ERS. For this reason, dual labeling of ERS+ and ERS− markers is not feasible. We hypothesise that the acquisition of this ERS+ specific gene set may be necessary for a coordinated physiological function in a subset of cells. This is reinforced by the observation that the root adjusts the proportion of cells acquiring this state under drought conditions.

We agree, however, that the original *VDOF1* HCR analysis alone was not sufficient to convincingly demonstrate the ERS+ state *in vivo*. In the revised manuscript we have now significantly strengthened the independent experimental validation of the ERS state by: (1) increasing the number of ERS markers analyzed by HCR (1 in original manuscript, 5 in revised manuscript), (2) selecting markers with higher expression levels and better HCR detectability (more probe sets per transcript), and (3) focusing on markers expressed in outer root layers, which overcomes the challenges in visualising the very small diameter cells of the stele (see **Resp. Fig. 10 / Fig. 2**). In addition to the HCR of these four new ERS markers presented in **Fig. 2**, we also present new HCR views of *VDOF1* in **Resp. Fig. 13 / Ext. Data Fig. 18**.



Resp. Fig. 13 / Ext. Data Fig. 18: HCR validation of *VDOF1* expression. Left, UMAP plot showing the 'branch 1'-specific expression of *VDOF1 in silico*. **Right,** Longitudinal optical section showing the expression of *VDOF1* by whole-mount HCR. Cells enriched (+) or not (-) for VDOF1 transcripts *in vivo* in the endodermis (en.) or pericycle (pe.) are indicated by arrows. Scale bar = 50µm.

As suggested, we now provide full-view images of the roots for each example shown, with a clear delimitation of the analysed region to better localise and orient the area being described. To improve clarity, we have also added longitudinal sections corresponding to the same regions as the cross sections, shown in **Resp. Fig. 9a-c / Ext. Data Fig. 3a-c** and **Resp. Fig. 10 / Fig. 2e** and **Resp. Fig. 11f,g / Fig. 4f,g** and **Resp. Fig. 13 / Ext. Data Fig. 18**.

Together, we believe that the extensive new independent experimental ERS validation in the revised manuscript convincingly demonstrates the presence of this new layer of cell identity and transcriptional diversity amongst root cell lineages.

Referee 1 - point 11: It may be that there are multiple pathways that create heterogeneity in the transcriptional states of cell lineages including actual differences in cell identity (passage cell vs non-passage cells in the endodermis), or differences in signaling state due to heterogeneous microenvironments (see comment #3), or differences that arise from biological noise. The authors should more clearly define the potential sources of such variation and establish which variable patterns are the consequence of which phenomenon.

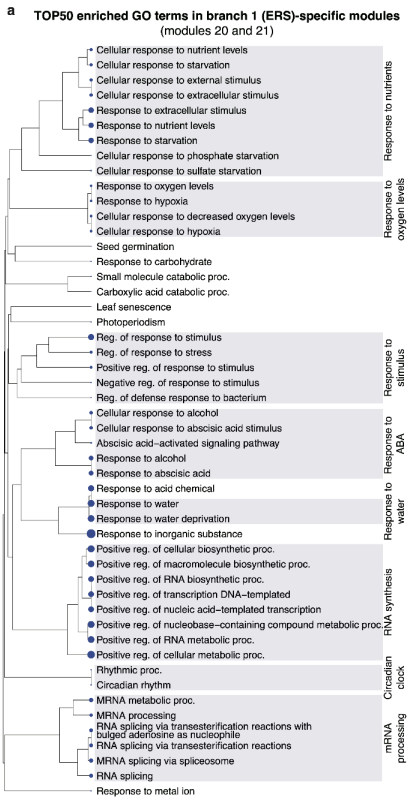
Response: There are certainly multiple potential sources of variation and heterogeneity in single-cell datasets, spanning technical (batch, growth condition, experimental set up) or biological (microenvironments, cell-cell

communication that can vary between individuals or growth conditions, or even stochasticity). However, the dichotomy that we describe and the genes that underlie it are very robust across genotypes, growth conditions, and even datasets (observed in all of the 30 scRNA-seq libraries of this study, as well as other studies, see response to **Referee 1 - point 2**). Globally, we see that if we project the ERS-specific modules across all these conditions, or if we assign cell identities to new datasets based on our atlas (**Resp. Fig. 1a / Ext. Data Fig. 13a**, **Resp. Fig. 2a / Ext. Data Fig. 12a**, **Resp. Fig. 3a / Ext. Data Fig. 14a**, **Resp. Fig. 4a-c / Ext. Data Fig. 8a-c**, **Resp. Fig. 15d / Fig. 4d**, **Resp. Fig. 18c / Fig. 3c**, **Resp. Fig. 19a-b / Ext. Data Fig. 20a-b**), we always detect the same two branches. Therefore, the presence of the phenomenon we describe does not seem to be strongly affected by these potential sources of variation, even though there may be small differences in what we assign as ERS+ across datasets, conditions, or even between cell types.

However, while the presence of these ERS+ and ERS− branches is highly robust, the proportion of cells that acquire the ERS can vary. This appears to depend on environmental conditions (for example, growth on a medium with low water potential) or when ABA signaling, either at the level of biosynthesis or perception, is perturbed. These observations suggest that such sources of variation influence the proportion of cells that acquire that state, rather than its existence *per se*.

Referee 1 - point 12: Extended data Fig 6a. It is unclear how the different cell types were assigned in this plot. What is defined as “stele” in this plot? How is this different from xylem and phloem?

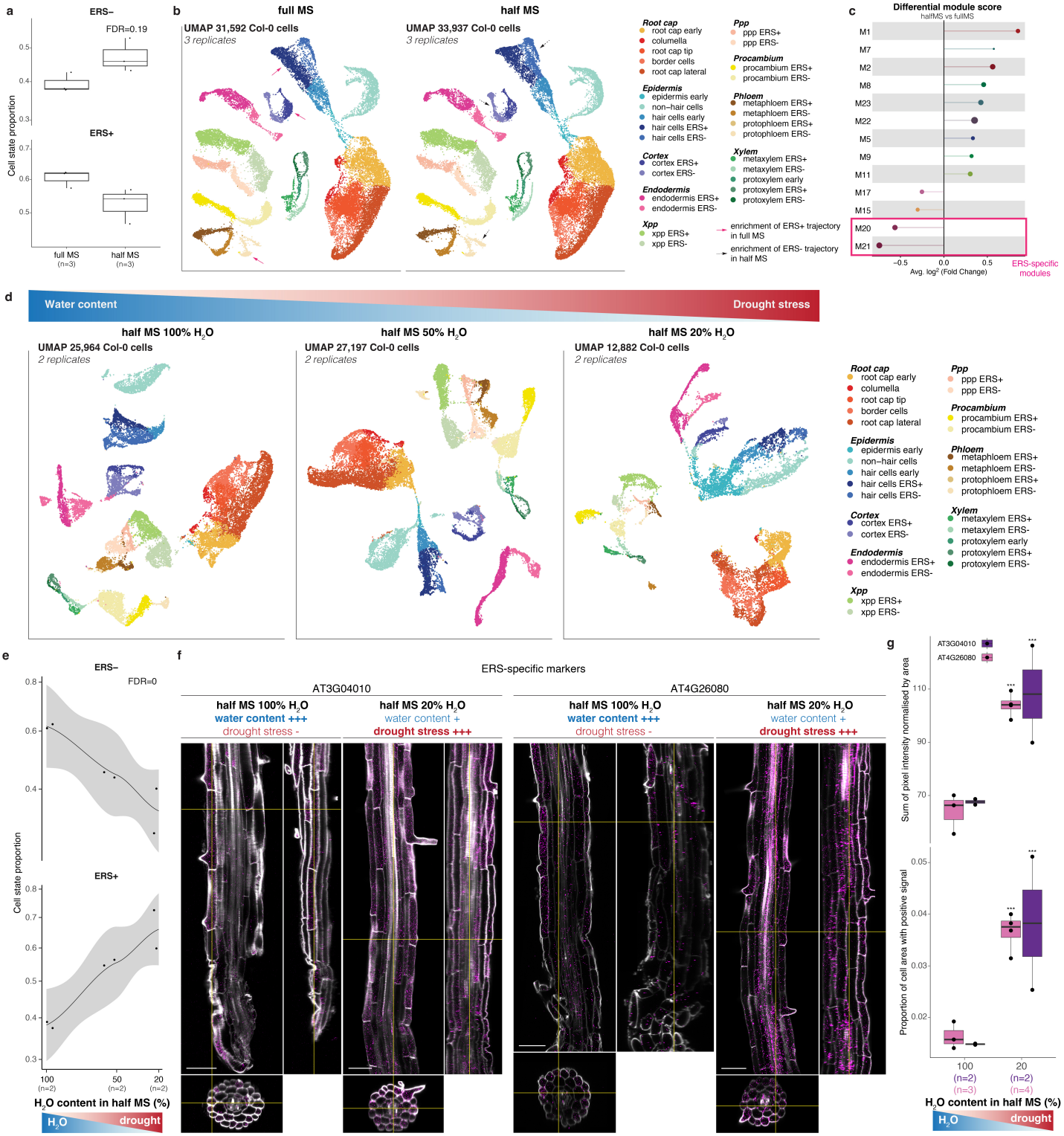
Response: These annotations were based on the expression patterns of co-expression modules shown in **Fig. 2**. However, to streamline the central focus of the study on the strong and specific activation of genes involved in the responses to various environmental stimuli in ERS+ cells, both this plot and the corresponding representation have been removed in the revised manuscript. In their place, the most salient point presented in this previous supplementary figure, the GO term functions enriched in ERS-specific gene co-expression modules, is now more clearly conveyed in revised **Resp. Fig. 14 / Fig. 3a**.



Resp. Fig. 14 / Fig. 3a: GO term functions enriched in ERS-specific gene co-expression modules.

Referee 1 - point 13: Page 10, Line 14: The branch 1 cells are classified as ERS+ based on the presence of genes related to ABA signaling pathway. It has been shown that ABA signaling is important for endodermal and xylem differentiation even under unstressed conditions. Perhaps, the role of basal levels of ABA in the development of these tissues is revealed by the branch 1 cellular state? What proportion of cells of a particular cell type are ERS+ vs ERS-?

Response: This is an interesting observation. While it would be difficult to directly test whether the ABA signaling required for establishing these specific cell types also influences the ERS+ state, multiple lines of evidence suggest that the phenomenon we describe is largely uncoupled from the developmental establishment of cell types: (1) we show that the proportion of ERS+ cells can be strongly modulated by environmental conditions, while all major cell types continue to be produced, suggesting that the ERS identity is not required or instructive for cell type specification (**Resp. Fig. 15 / Fig. 4**), and (2) in the *scr-3* mutant, where both endodermis, pericycle and xylem development are perturbed, we still detect both ERS+ and ERS− cells in proportions similar to wild type (see response to Referee 1 - point 19, **Resp. Fig. 19 / Ext. Data Fig. 20**). A boxplot showing the proportion of ERS+/− cells per cell type and per replicate in Col-0 and in the ABA mutants is presented in **Ext. Data Fig. 19**.



Resp. Fig. 15 / Fig. 4: The acquisition of the ERS is modulated under drought stress

a, Box plots showing the proportion of ERS+ (and consequently ERS–) cells in roots grown in full MS and half MS. **b,** UMAP plots showing cells from three replicates grown simultaneously in full MS or half MS, displayed separately. An enrichment of the ERS+ trajectory in full MS, and conversely of the ERS– trajectory in half MS, is indicated by arrows and is particularly visible in hair cells, cortex, endodermis, and protophloem. **c,** Lollipop plots showing differential expression of the modules identified in Fig. 2b (differential module score; Wilcoxon rank-sum test, adjusted P < 0.05 and |average log2 fold change| > 0.25). Modules are ranked by average log2 fold change. ERS-specific modules 20 and 21 are the most significantly downregulated in half MS and are highlighted by a pink rectangle. **d,** UMAP plots showing assigned cell identities in scRNA-seq profiling of roots grown under control conditions (half MS, 100% H_2O) and in the “hard agar” drought model, where water content in half MS medium was reduced to 50% (half MS, 50% H_2O) or 20% (half MS, 20% H_2O). **e,** Scatter plots showing a significant increase in ERS+ cell proportion with decreasing water content (sccomp, FDR < 0.001). **f,** Orthogonal confocal views of whole mount HCR detection of two ‘branch 1’-specific markers

described in Fig. 2e, *AT3G04010* and *AT4G26080* (*ABI1*), in roots grown in half MS (control, 100% H_2O) or under the harshest drought condition (half MS, 20% H_2O). Yellow lines indicate the relative positions of the three optical sections. Scale bar = 50µm. **g,** Box plots showing the increase in total signal intensity or in the area of positive signal (both normalised by total cytoplasmic area, see Methods) in the HCR detection in roots of the ERS-specific markers *AT3G04010* and *AT4G26080* (*ABI1*) under drought conditions (half MS, 20% H_2O) compared to control (half MS, 100% H_2O) (Wilcoxon rank-sum test, *** indicates a p-value < 0.001).

Referee 1 - point 14: The data provided in Supplementary Figure 6b is not very convincing. The enrichment of the expression programs for the ERS+ pathway seems limited. What is the statistical basis for determining whether a gene is biased towards one lineage or another? HCR would be useful to validate genes showing marginal enrichment like ABF1 and PYL8.

Response: The set of genes we consider enriched in ERS+ cells is defined based on their co-expression module scores, which show a clear enrichment in "branch 1" (ERS+) cells, as displayed in **Resp. Fig. 16 / Fig. 2b**, rather than on a statistical test of differential expression between branches. Within these ERS-specific modules, individual genes vary in expression level and in the degree of enrichment in ERS+ compared to ERS− cells. In the revised manuscript we have increased the number of ERS-specific genes profiled by HCR (now 5) to include markers spanning a range of expression levels and relative enrichment (see **Resp. Fig. 10 / Fig. 2e** and **Resp. Fig. 13 / Ext. Data Fig. 18**).

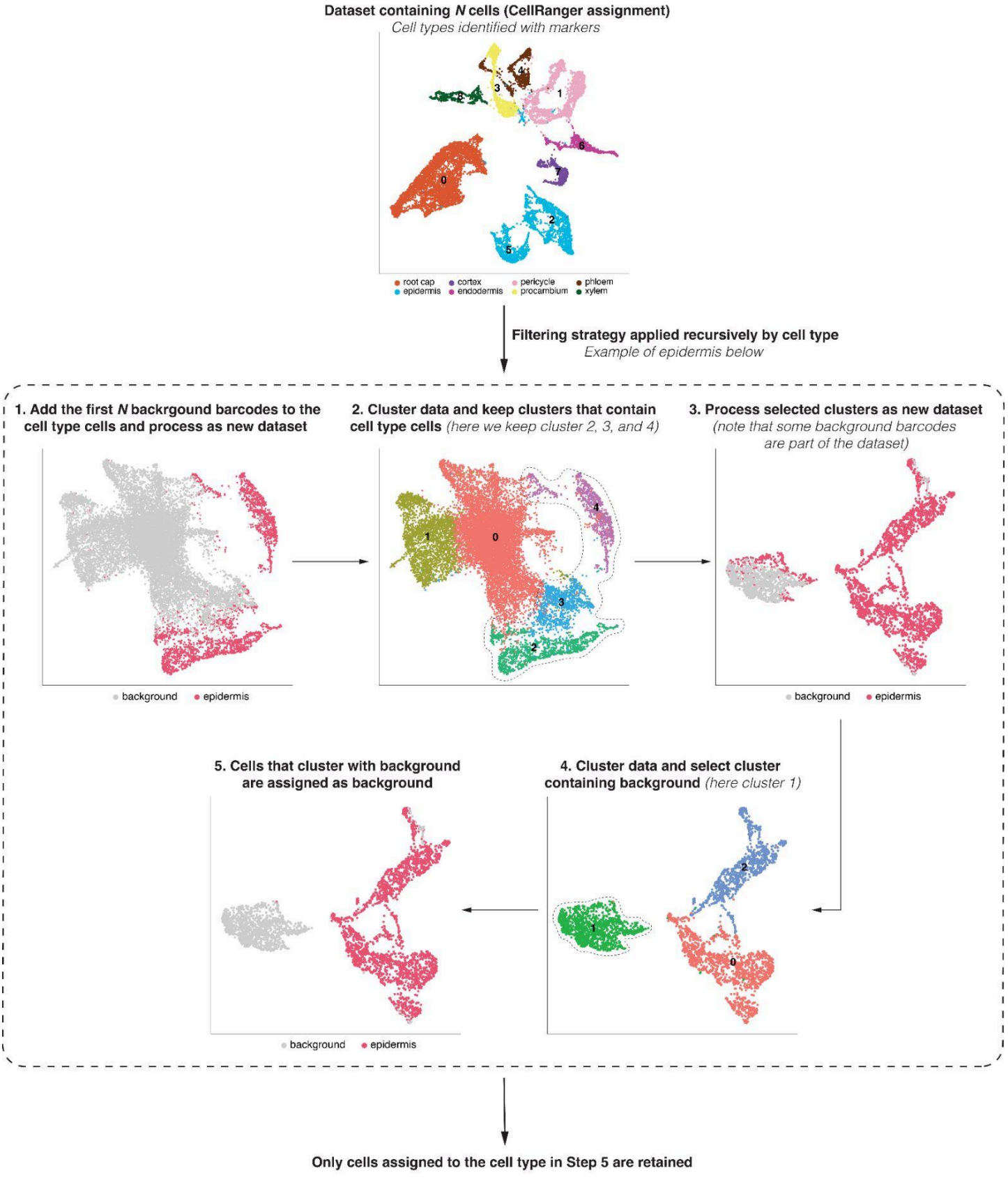


Resp. Fig. 16 / Fig. 2b: UMAP of root tip scRNA-seq atlas showing module score for Modules 20 and 21, which is highly enriched in "branch 1" (ERS+) cells.

Referee 1 - point 15: Does Ws-2 have a higher or lower tolerance to heat stress compared to the other accessions? What is the consequence of the DEGs in the ERS+ cells?

Response: Upon re-processing the data, we found that the Ws-2-specific genes originally described were in fact an artefact, resulting from ERS− cells being inadvertently filtered out as background in some Ws-2 samples. When we initially processed our datasets, the CellRanger pipeline used for cell calling applied stringent thresholds, leading to the exclusion of real cell populations with lower UMI content, such as root cap cells. To overcome this, we developed a method that used SNP counts from mixed ecotype samples to distinguish true cells from background.

However, this approach disproportionately filtered ERS- cells in the Ws-2 preparations, introducing a bias that appeared as accession-specific differences. Since then, we have completely redesigned our data pre-processing pipeline. CellRanger no longer filters out these populations by default, but instead tends to be too permissive. We have therefore implemented a custom filtering strategy to carefully exclude low-quality cells that pass CellRanger's thresholds, without discarding biologically relevant populations (outlined in **Resp. Fig. 17 / Ext. Data Fig. 1**). In the revised manuscript, using this improved pipeline, we no longer observe the previously reported differences for Ws-2.



Resp. Fig. 17 / Ext. Data Fig. 1 (part): Diagram illustrating the steps used to filter low-quality cells. See methods for further details.

Referee 1 - point 16: The authors observe a depletion of ERS+ cells in half MS compared to full MS. If half MS represents the nutrient limited conditions, wouldn't one expect the cellular states that express ABA signaling components to increase? For cell types in which there is no change in proportion of ERS- cells but ERS+ cells are depleted (metaxylem, phloem pole pericycle, procambium), do the authors observe fewer cells in the half MS grown plants compared to full MS? An alternate hypothesis is that in half MS condition, the cells

progress through differentiation faster (more/longer root hairs) and hence the ERS+ cell states are not resolved as in the full MS.

Response: These are important points to discuss. We will first discuss the sampling analyses and strategies for quantification of cell type frequencies, followed by the changes in cellular states that might be expected in the half MS and full MS conditions.

Regarding quantification of cell type frequencies, there can indeed be variation in the total number of cells of a specific type (regardless of ERS state), which can influence statistical tests. However, we believe these differences are due to subsampling effects rather than true biological variation. Each library profiles ~10,000 cells, and the number of cells captured for a given cell type can vary between replicates, especially for less abundant cell types that are more prone to sampling fluctuations. Importantly, the organisation of concentric root layers is not altered between full MS and half MS conditions, and given the physical constraints required to form these layers, it is very unlikely that there are major differences in absolute cell numbers between conditions. No differences in the abundance of these cell types were observed by microscopy for any of the conditions investigated.

However, the method we initially used to test for changes in cell population proportions was sensitive to this sampling bias and could potentially be misleading. For example, as noted by the referee, in some cell types we observed an apparent decrease in ERS+ cells without a corresponding increase in ERS− cells, or even a decrease in both, due to sampling noise.

In the revised manuscript we have now used a different strategy and applied an alternative statistical approach (sccomp[6]) that accounts for replicate variability, which was not the case in the initial submission. To specifically assess biological variation in ERS+ (and consequently ERS−) proportions, we restricted the analysis to cells at the developmental stage where the ERS+/− dichotomy occurs. In other words, we excluded earlier-stage cells and root cap cells. If cells are assigned an ERS+ or ERS− identity when projected onto our atlas and annotated, it means they have already reached this differentiation stage. For most lineages, only cells at the ERS+/− stage are captured, so no additional filtering was necessary. For the epidermal lineage, we excluded cells annotated as *"epidermis-meristem"*, *"hair cells early"*, or *"non-hair cells"*, where PAGA did not resolve two clear branches. Once only cells that had reached the stage where the branching occurs were considered (either within a lineage or across the whole root), we tested for variation in ERS+/ERS− proportions. In this refined analysis, the differences detected in ERS+ proportions cannot be explained by changes in differentiation rate.

Regarding the interpretation that the ERS decreases in response to nutrient-limited growth conditions, it is true that we originally referred to full and half MS as media differing in nutrient concentration, implying that half MS could represent an abiotic stress due to nutrient limitation. However, both media are widely used in plant research and typically do not induce overt stress during development. Conversely, and as briefly mentioned in the original discussion, these media can also be considered in terms of osmolarity. In this context, full MS may represent a slightly more stressful condition, as higher salt concentrations can make water uptake more difficult. From this perspective, the ERS might be engaged to help the root cope with such conditions and would require ABA, which is known to mediate responses to water and salt stress. This is the interpretation we have chosen to further explore in the revised manuscript, where we also show that under a more severe stress condition, using an agar-based drought model, the proportion of ERS+ cells increases markedly.

Referee 1 - point 17: Have the authors compared whole mount in situ HCR images of genes in ERS+ cell state in plants grown under full MS vs half MS?

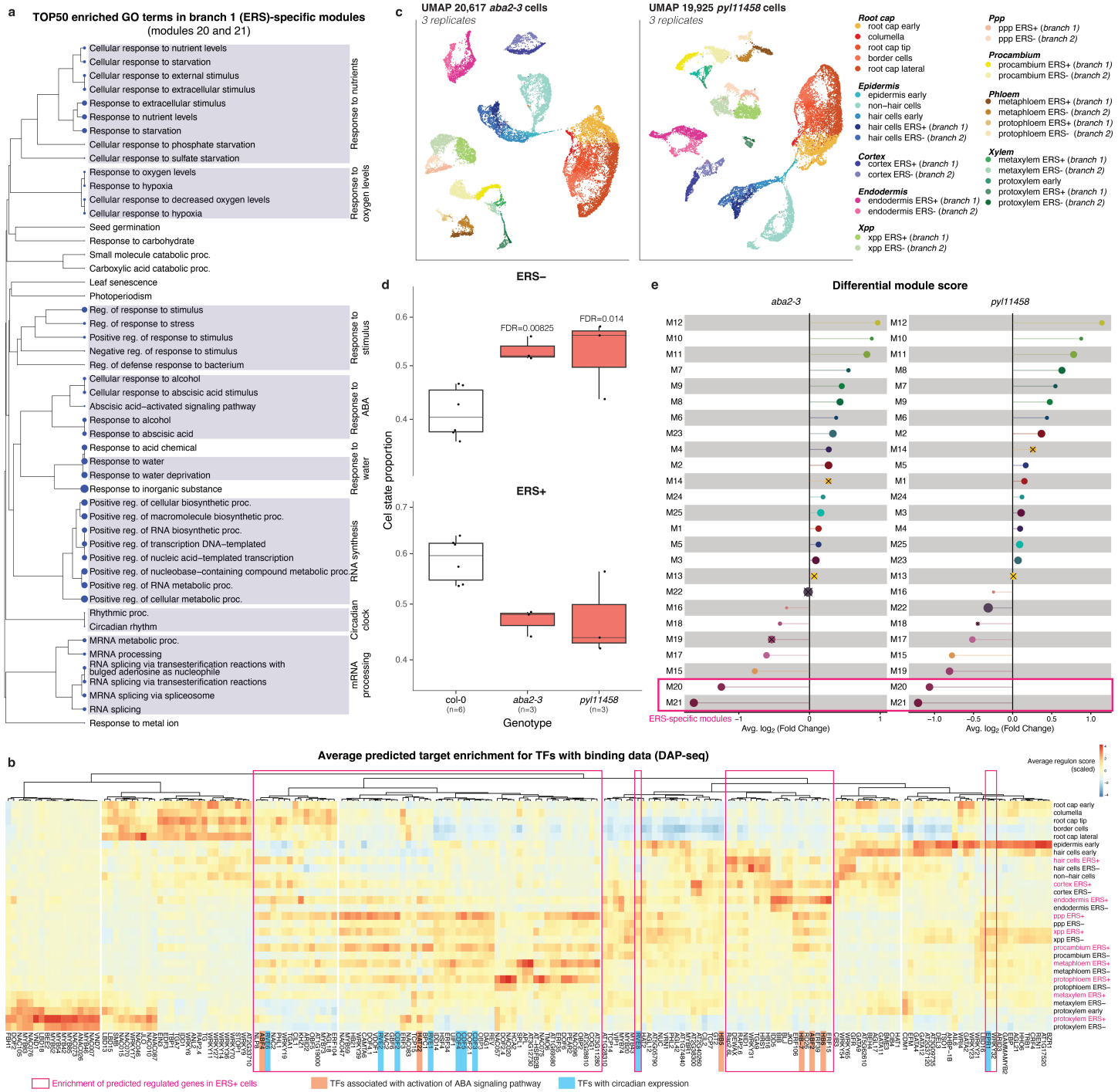
Response: This is indeed an important point to validate the modulation of ERS acquisition by environmental conditions. Rather than comparing ERS+ proportions by HCR in full MS versus half MS, we chose to perform this analysis in a "hard agar" low water potential model that accurately and efficiently mimics drought stress in a plate-based setup similar to Gonzalez *et al.*[7] 2024, which we find shows a much more pronounced change in ERS+ proportion in our new scRNA-seq analyses (**Resp. Fig. 15d,e / Fig. 4d,e**). Crucially, HCR imaging of multiple ERS+ marker genes in the roots of plants grown in these same conditions consistently showed a significant increase in the number of ERS+ cells under stress conditions compared to control (**Resp. Fig. 15f / Fig. 4f**), including by our new approach for quantitative analysis of HCR signal intensity and signal spread (**Resp. Fig. 15g / Fig. 4g**).

Referee 1 - point 18: The TF-binding analysis presented is correlative and should be followed up with a mutant analysis to test whether they show more or less ERS+/- cells or show differences in transcriptional state of the ERS cells.

Response: We now use the TF-binding analysis with a slightly different purpose than identifying candidate TFs for knockout experiments. When analysing the functions of ERS-specific genes, we found enrichment for

pathways involved in environmental responses, such as ABA signaling and the circadian clock (**Resp. Fig. 18a / Fig. 3a**). Screening all TFs expressed in the root for those for which we could define regulons (using DAP-seq binding data combined with scRNA-seq data) revealed that the majority of TFs acting as effectors in these pathways, for which we had available data, have regulons enriched in ERS+ cells (**Resp. Fig. 18b / Fig. 3b**). This further supports the idea that these pathways likely contribute to the establishment of the ERS identity.

However, mutating individual TFs to test their role in ERS establishment is unlikely to be conclusive, due to high functional redundancy among regulators. Therefore, instead of attempting to disrupt an entire pathway by targeting single TFs - which, except in rare cases of master regulators, often yields inconclusive results - we chose to modulate the input signals to one of these pathways and assess the functional consequences. Specifically, to test the role of ABA signaling in ERS establishment, we profiled an ABA biosynthesis mutant (*aba2-3*[8]) and a quintuple ABA receptor mutant (*pyl11458*[9]). In both genetic backgrounds, we observed a significant decrease in the proportion of ERS+ cells (**Resp. Fig. 18c-e / Fig. 3c-e**), confirming the involvement of ABA signaling in establishing this cellular state. Thus, perturbing signalling pathways that from the genomics data we predicted may regulate the ERS indeed validated their functional role.

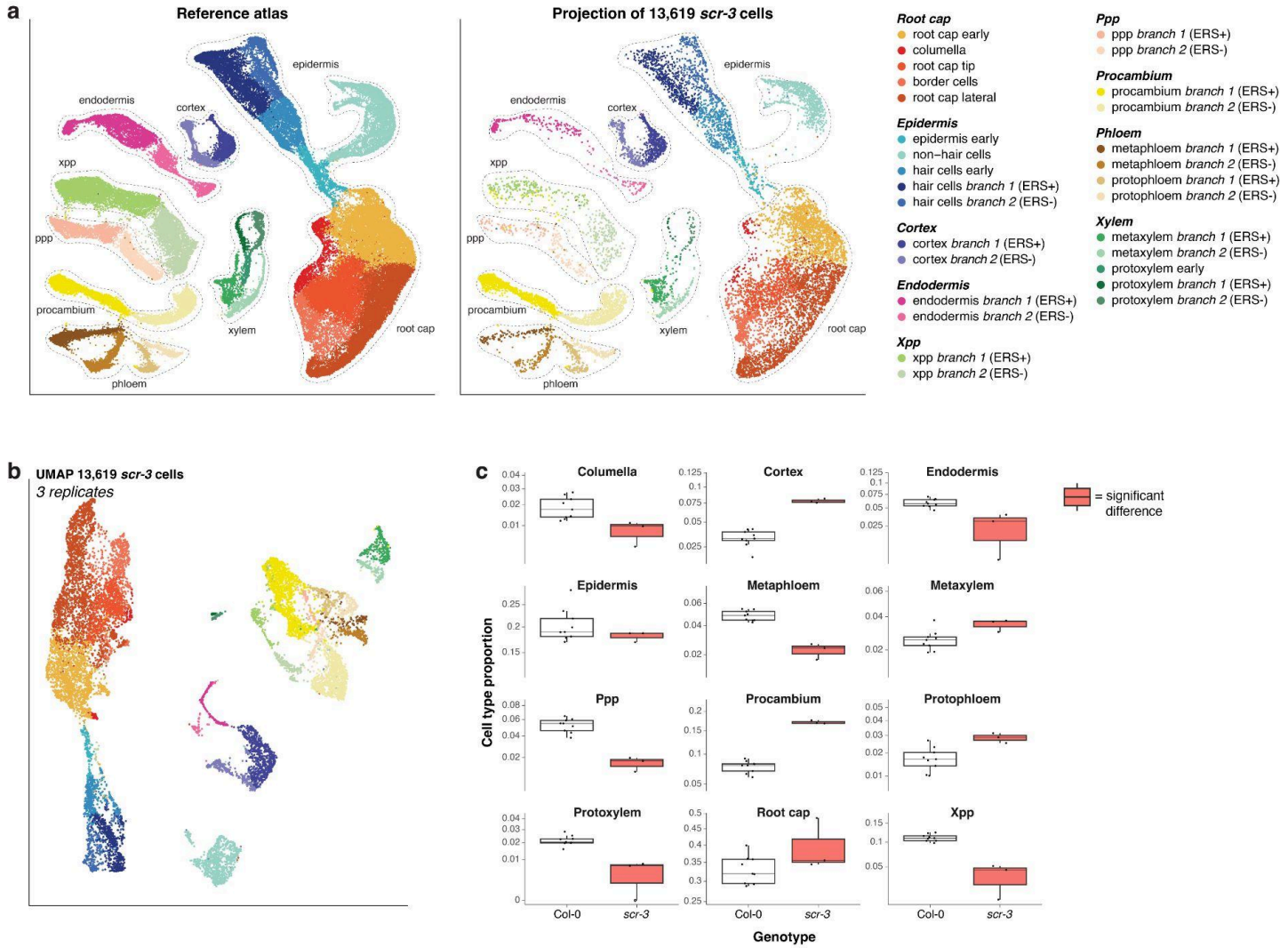


Resp. Fig. 18 / Fig. 3: The 'branch 1'-specific environmentally responsive state (ERS) is regulated by ABA

a, Hierarchical clustering tree showing the top 50 enriched GO terms (biological process) in the two 'branch 1'-specific modules 20 and 21 (FDR < 0.01; terms ranked by fold enrichment). Larger dots indicate more significant P-values. **b,** Heatmap showing regulon enrichment per cell identity for 186 transcription factors (TFs), based on DAP-seq, cis-BP2 binding similarity, and scRNA-seq data for the 6,649 genes assigned to a co-expression module. TF regulons enriched in ERS-positive (ERS+) cells of multiple cell types are highlighted by a pink rectangle. TFs known to be regulated by ABA or circadian clock pathways are highlighted in pink and blue, respectively. **c,** UMAP plots showing predicted cell identities using our reference atlas in the ABA-deficient mutant *aba2-3* and the ABA-insensitive mutant *pyl11458*. **d,** Box plots showing significant depletion of ERS+ cells (and a corresponding increase in ERS-negative (ERS–) cells in *aba2-3* and *pyl11458* (sccomp, FDR < 0.05). **e,** Lollipop plots showing differential expression of the 25 modules identified in Fig. 2b (differential module score, Wilcoxon rank-sum test, adjusted p-value < 0.05). Modules are ranked by average log2 fold change. Dot size indicates the number of genes in each module and an "X" marks modules that do not reach statistical significance. The ERS-specific modules 20 and 21 are the most significantly downregulated in the mutants and highlighted by a pink rectangle.

Referee 1 - point 19: The hypothesis that columella and pericycle cells have altered cell type identity in scr-3 should be validated using reporter genes, HCR or physiological assays.

Response: As the shifts in cell identity in *scr-3* are not a main focus of this study, we did not perform additional functional analyses (such as reporter lines, HCR, or physiological assays) to validate this hypothesis. However, we have increased the number of biological replicates profiled (3 in the revised manuscript versus 1 in the initial submission), which provides stronger support for a change in cell identity in pericycle cells (**Resp. Fig. 19 / Ext. Data Fig. 20**). In the revised manuscript we have focused less on root cap identities (i.e. columella), as they do not show the ERS+/− dichotomy.



Resp. Fig. 19 / Ext. Data Fig. 20: The ERS is maintained upon developmental perturbations of cell identities in *scr-3*

a, UMAP plots showing our reference atlas and the projection of cells from *scr-3* replicates into the atlas UMAP embedding, with cells coloured by their assigned identity based on our atlas. **b,** *scr-3* cells atlas shown in their atlas UMAP embedding **c,** Box plots showing the distribution of cell types in our reference Col-0 atlas and in the *scr-3* mutant. Each dot represents an individual replicate. Tests showing a significant difference in cell type proportion compared to Col-0 are highlighted in pink (sccomp, FDR<0.05).

Referee 1 - point 20: The authors state that 20% of DEGs responsive to MS concentration are in ERS+ cells. Is this greater than expected by chance?
Response: Rather than performing a DEG analysis between MS concentrations and intersecting the results with the module analysis, which lacks statistical power, we have now conducted a differential module expression analysis, statistically assessing changes in module scores using the hdWGCNA package[10]. We found that the two ERS-specific modules (Modules 20 and 21) are the most significantly downregulated in half MS compared to full MS (see **Resp. Fig. 15c / Fig. 4c**, above).

Minor comments:

Referee 1 - point 21: Results line 9: "Protoplasts were isolated from root tips of two different Arabidopsis ecotypes, mixed at the same concentration" Can authors explain why there is a large variation in the number of cells from the different ecotypes (Col: 31611 cells and Ler: 6356 cells) and how this could influence the comparison of differentially expressed genes between accessions?
Response: This variation is due to there being fewer replicates for some ecotypes than others. We made sure to include a minimum of three replicates for each (except for Col-0, which has six). There is also some variation in the number of cells obtained per replicate, as some preparations used our mixed ecotype approach and some did not, but this will not influence the comparison of differentially expressed genes between accessions since we used a pseudobulk approach for the differential expression analysis.

Referee 1 - point 22: Page 10 Line 21-22: The statement that the ERS signature is "independent from the developmental lineages" is not quite accurate since it seems to occur predominantly in the endodermis and stele.
Response: Thank you for pointing this out. By this statement, we mean that all lineages are affected by the dichotomy described in the trajectory analysis, and that 'branch 1' paths consistently share common genes with 'branch 1' paths in other cell types, a state we define as the ERS. This explains why we detect more genes and UMIs across all 'branch 1' paths.
We agree, however, that there is still cell type specificity within the ERS, as highlighted in our previous co-expression module analysis, where modules appeared more fragmented. To clarify this point, we now explicitly state in the Discussion: *"We note that while an ERS can be detected across all lineages, it is not entirely equivalent in all cell types. Some ERS-specific genes are enriched in all ERS+ cells, whereas others are restricted to specific lineages, suggesting a degree of cell type specificity in the acquisition of this transcriptional state, likely shaped by the underlying regulatory networks of each cell type."*

Referee 1 - point 23: Fig. 5b, Number of root hairs plotted instead of root hair density. The authors should also provide the root length data for the two conditions tested.
Response: For both cases, the same portion of root was analyzed, as described in the Methods section: "*A portion corresponding to approximately 2.5mm from the tip was selected for each root picture, and root hairs were counted manually using the Cell Counter plugin in Fiji*."

Referee 1 - point 24: Extended Data Fig. 10b. Do the authors observe a higher number of columella cells in half MS grown plants compared to full MS or does the change in relative proportion reflect a difference in protoplasting efficiency for this cell type?
Response: We could not detect a significant difference in root cap cells proportion between the two conditions.

Referee 1 - point 25: Page 18 Line 20, No data has been presented that involve changes to osmotic pressure. Are the authors referring to the difference in the concentration of salts from the MS media? What is the difference is osmotic pressure between ½ and full strength MS?
Response: We did not directly measure the osmotic potential of full-strength versus half-strength MS medium, however we expect the osmotic potential difference between 1xMS and 0.5xMS (both with 1% sucrose and 0.9% agar) to be about 0.12MPa, based on van't Hoff's equation. Instead, the manuscript now concentrates on

ERS acquisition in a drought model (following Gonzalez *et al.*), in which the available water in the ½ MS medium was reduced to ~20% of its original content. In that treatment both salts and agar become effectively more concentrated, so the medium's osmotic potential is expected to become substantially more negative compared with either standard 1× MS or 0.5× MS.

Referee 1 - point 26: The authors should state whether the cells for scRNAseq were collected at the same time of day.
Response: Yes, as mentioned in the Methods, seedlings used in all experiments were grown in the exact same conditions, and collected and processed at the same time of the day.

Referee 1 - point 27: Line 24, page 19: What is meant by "gatekeeper cells". This is suggestive of a regulatory role for ERS+ cells that has not been demonstrated.
Response: We agree that this statement was not cautious enough. It was intended to convey a potential role of ERS when we correlated it with a passage cell marker, assuming it could be a way of controlling access to the root interior, which was not demonstrated. We have now decided to focus on other aspects and no longer include the analysis related to CASPs and passage cells, and have therefore removed this statement.

Referee #2:

The single cell Arabidopsis root atlas reported in the manuscript is an informative reference data set, because it also includes information from Arabidopsis ecotypes. The observation of two developmental trajectories within the root lineages (branch I and II), caused in response to environmental stimuli such as nutrient availability, is indeed novel and promises an improvement in the quality and diversity of single cell datasets. The data and methodology are described in detail and presented clearly.

Major comments:

Referee 2 - point 1: However, a major concern of the results reported in this manuscript is the lack of in vivo validation and therefore the lack of evidence for the conclusions. Without additional in vivo validation, the novel aspect between environmental responses and cell identity is not efficiently proven, reducing the originality and significance of the findings reported in this manuscript.
Response: Thank you for this constructive feedback. As other referees also noted, the initial version of the manuscript lacked sufficient *in vivo* evidence to demonstrate that the transcriptional state we describe represents a true biological signal modulated by environmental changes. In the revised version, we have focused on addressing this major concern by:

(1) Ruling out potential experimental and analytical artefacts (see responses to points 2, 5, and 7).
(2) Increasing the number of ERS-specific genes profiled by whole-mount *in situ* imaging by Hybridization Chain Reaction (HCR) to validate their *in vivo* expression (see response to points 3, 4, 7, and 8).
(3) Demonstrating the role of the phytohormone ABA in establishing this state by profiling ABA-deficient and ABA-insensitive mutants and comparing them to wild-type samples (see response to point 8).
(4) Showing that the root markedly increases the proportion of cells acquiring this state in an agar plate-based drought model, validated both by scRNA-seq and whole-mount HCR (see response to point 8).

For detailed descriptions of all of these major improvements, please see the responses to specific comments below.

Referee 2 - point 2: One major concern with the observation of the two-branch-theory reported in this manuscript is that the cell content varies very dramatically between all cells in branch I compared to branch II (in all lineages where the effect was observed). This suggests that the branches are caused by cell quality, rather than biological significance. Although this problem has been mentioned and partly addressed by the authors through a sub-clustering approach according to the median UMI/cell (Extended Data Fig7); indicating that the overall cell cluster distribution is robust and that gene expression between branch I and II is not influenced by protoplast generation; this approach did not include a separate trajectory analysis which would be necessary to observe, if branch I and branch II are still detectable even in the remaining cells.
Response: Thank you for raising this important point. Differences in UMI content could indeed, in principle, reflect cell quality - low-UMI cells could simply represent "burst" versions of high-UMI cells and should then be discarded. In that scenario, the genes characterizing the ERS identity might merely correspond to lowly expressed genes that due to their abundance are no longer detectable (i.e. drop out) in burst cells. In the initial

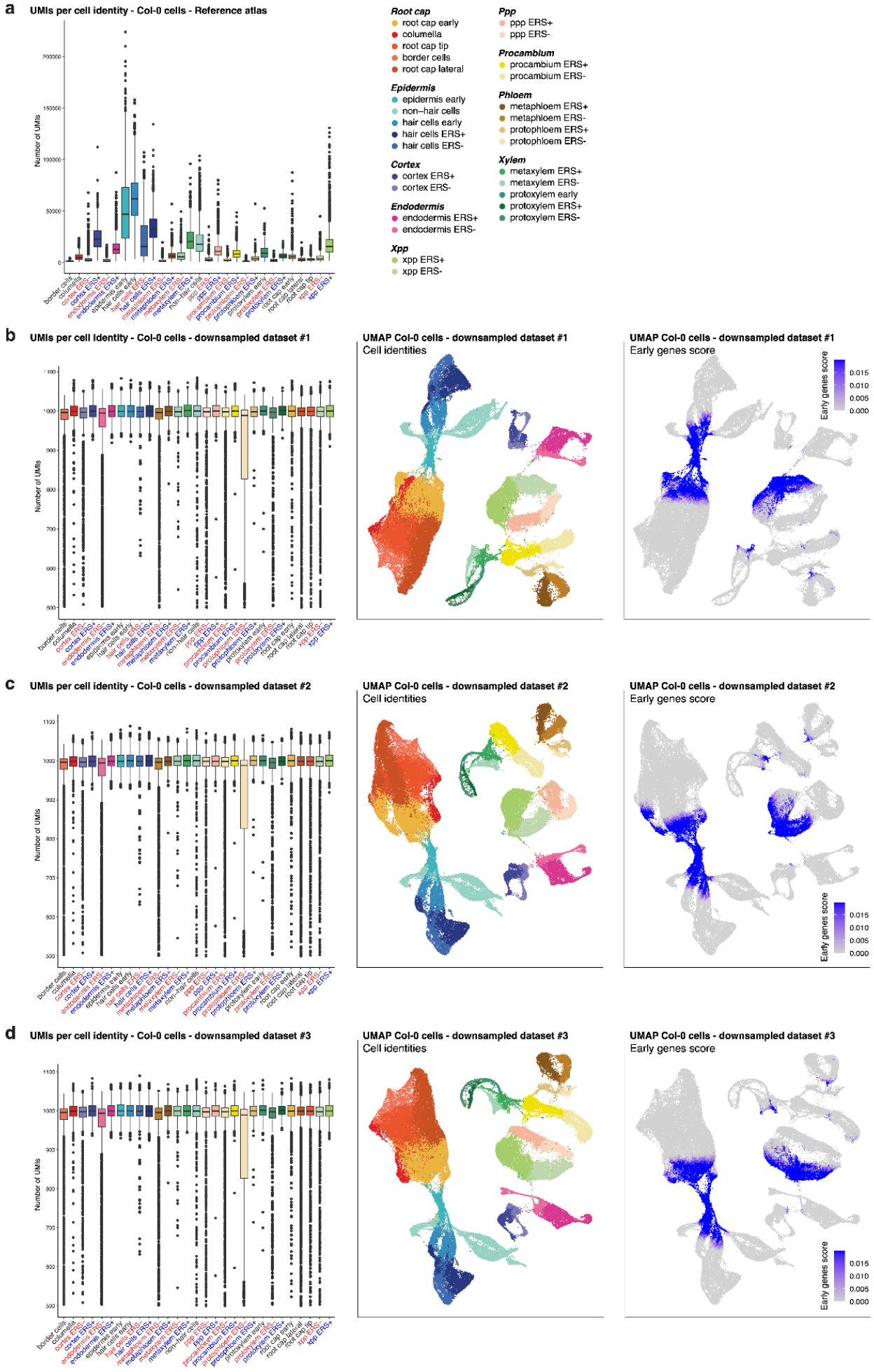
manuscript we addressed this concern using a downsampling approach, which showed that the branching event and the detection of ERS-specific genes cannot be explained by differences in sequencing depth between the two populations. Even in subsampled data, the ERS+ and ERS− cells still segregated clearly. As requested, we have now added separate trajectory analysis to the downsampled dataset, which still detects a clear t=0 between ERS− and ERS+ populations, confirming that the trajectories are not an artefact of sequencing depth (**Response Figure 20 / Extended Data Figure 11**).

We also provide additional evidence supporting the biological relevance of the ERS identity, and that the branches are not caused by cell quality:

(1) ERS-specific genes are not globally lowly expressed compared to genes expressed in both ERS+ and ERS− cells (**Resp. Fig. 21 / Ext. Data Fig. 10**).

(2) Both ERS+ and ERS− populations are also detected in independent single nucleus (sn) RNA-seq datasets, with the same co-expressed gene modules, further supporting that the differences between ERS+ and ERS− populations are not due to technical artefacts such as profiling whole cells versus nuclei (see response to point 5, below).

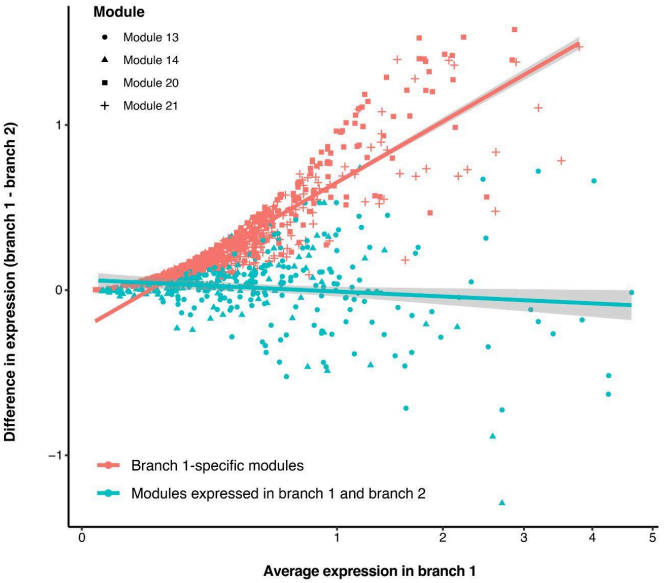
(3) Implementation of an additional method to detect and remove any low-quality cells that may have passed CellRanger's initial filtering, described in more detail in response to point 7, below.



Resp. Fig. 20 / Ext. Data Fig. 11: Branching events are not an artefact caused by coverage

a, Box plots showing the UMI content distribution per cell identity in our reference atlas. **b–d,** UMAP plots and box plots showing the distribution of UMI content per cell, along with cell identities and 'meristematic score' used for pseudotime assignment, after three independent UMI downsampling computations (see Methods). Branches are detected in all three downsampling replicates, with the cells with the highest 'meristematic score' per lineage consistently positioned between branches, as in the reference atlas.

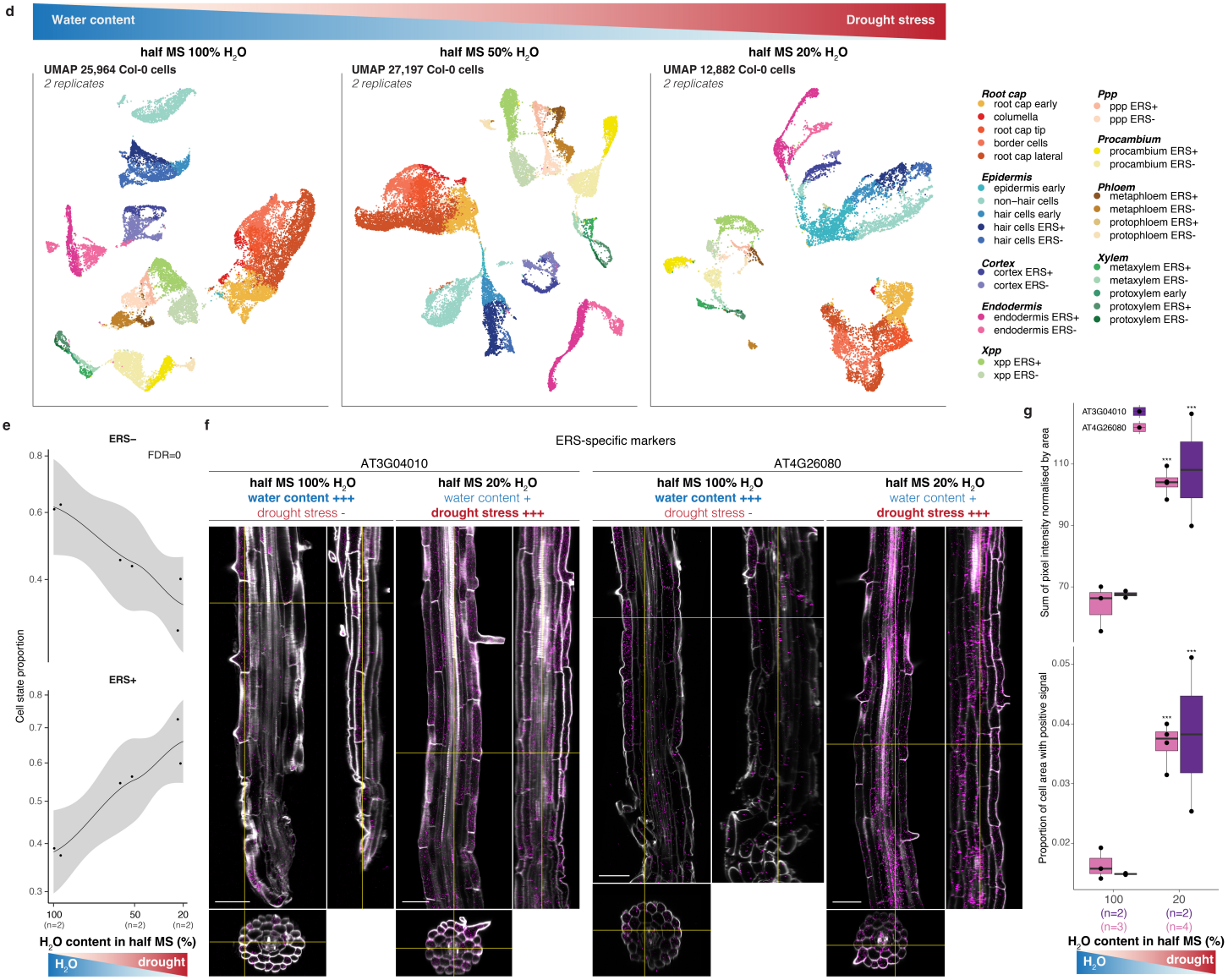
Resp. Fig. 21 / Ext. Data Fig. 10: Differences in gene content between 'branch 1' and 'branch 2' are not driven by lowly expressed genes.
Scatter plot showing, for the xylem pole pericycle, the average expression in 'branch 1' versus the difference in average expression between 'branch 1' and 'branch 2' for genes from two categories of co-expression modules: those that are 'branch 1'-specific (Modules 20 and 21) and those symmetrically expressed in both branches (Modules 13 and 14). Both categories of co-expressed genes span the same range of expression levels, indicating that the 'branch 1'-specific genes are not simply lowly expressed genes detected in cells with higher UMI content.



Other points that should be addressed to provide solid evidence for the robustness of the two developmental branches and the stated conclusions:

Referee 2 - point 3: 1) Validation of branch I and branch II-specific genes were provided by VDOF1 and AT3G47510 (branch I) and CASP1 (branch II). While the provided HCR pictures indeed indicate a spatial expression pattern along a developmental trajectory, it only proofs that their expression is more enriched in mature cells. There is no direct evidence provided that would connect an environmental response (observed for branch I cells) influences the pattern. Because the novelty of this manuscript lays in the report of an environmental influence on cell developmental stages, it is crucial to provide in vivo validation.
The authors further characterize the branches into environmentally responsive state ERS+ cells (=branch I) and ERS- cells (=branch II), indicating an ERS+-specific enrichment of ABA-responsive genes and genes related to a function in circadian rhythm. Again, no in vivo validation is provided that would support the observed transcription patters. Further, it would also be informative, if the spatial expression pattern of VDOF1 (or others) is indeed associated with ABA or circadian rhythm.
Response: Although some of these genes were also used to illustrate variation in expression along the longitudinal axis (and thereby along pseudotime), the primary reason for selecting them was to show that they are expressed only in a subset of cells within a given cell type. This confirms the presence of two distinct populations - one expressing these genes and one not - consistent with the existence of ERS+ and ERS− populations *in vivo*. In the revised manuscript, we have increased the number of profiled ERS-specific genes analysed by HCR and provided improved image views, which we hope make this clearer.

Regarding differential environmental conditions influencing the developmental branching of cell lineages into ERS+ and ERS− populations, we now provide direct *in vivo* evidence for this. We used a "hard agar" low water potential model that accurately and efficiently mimics drought stress in a plate-based setup[7]. Under low water potential conditions, we observe a significant and pronounced increase in the proportion of ERS+ cells both by scRNA-seq and by HCR (**Resp. Fig. 22d,e / Fig. 4d,e**). Imaging of multiple ERS+ marker genes in the roots of plants grown in control or low water potential conditions consistently showed a significant increase in the number of ERS+ cells under drought conditions compared to control (**Resp. Fig. 22f / Fig. 4f**), including by our new approach for quantitative analysis of HCR signal intensity and signal spread (**Resp. Fig. 22g / Fig. 4g**). Notably, this includes an increased number of cells expressing two ERS-specific marker genes (**Resp. Fig. 22f / Fig. 4f**), *ABI1* (*AT4G26080*) and *MIZ1* (AT2G41660), both of which are transcriptionally induced by ABA and involved in ABA signal transduction[11–13]. Thus, we now experimentally validate *in vivo* the presence of both ERS+ and ERS− populations and clearly demonstrate the modulation of ERS acquisition by environmental conditions, including ABA-responsive genes.



Resp. Fig. 22 / Fig. 4 *(part)*: The acquisition of the ERS is modulated under drought stress

d, UMAP plots showing assigned cell identities in scRNA-seq profiling of roots grown under control conditions (half MS, 100% H_2O) and in the "hard agar" drought model, where water content in half MS medium was reduced to 50% (half MS, 50% H_2O) or 20% (half MS, 20% H_2O). **e,** Scatter plots showing a significant increase in ERS+ cell proportion with decreasing water content (sccomp, FDR < 0.001). **f,** Orthogonal confocal views of whole mount HCR detection of two 'branch 1'-specific markers described in Fig. 2e, *AT3G04010* and *AT4G26080* (*ABI1*), in roots grown in half MS (control, 100% H_2O) or under the harshest drought condition (half MS, 20% H_2O). Yellow lines indicate the relative positions of the three optical sections. Scale bar = 50µm. **g,** Box plots showing the increase in total signal intensity or in the area of positive signal (both normalised by total cytoplasmic area, see Methods) in the HCR detection in roots of the ERS-specific markers *AT3G04010* and *AT4G26080* (*ABI1*) under drought conditions (half MS, 20% H_2O) compared to control (half MS, 100% H_2O) (Wilcoxon rank-sum test, *** indicates a p-value < 0.001).

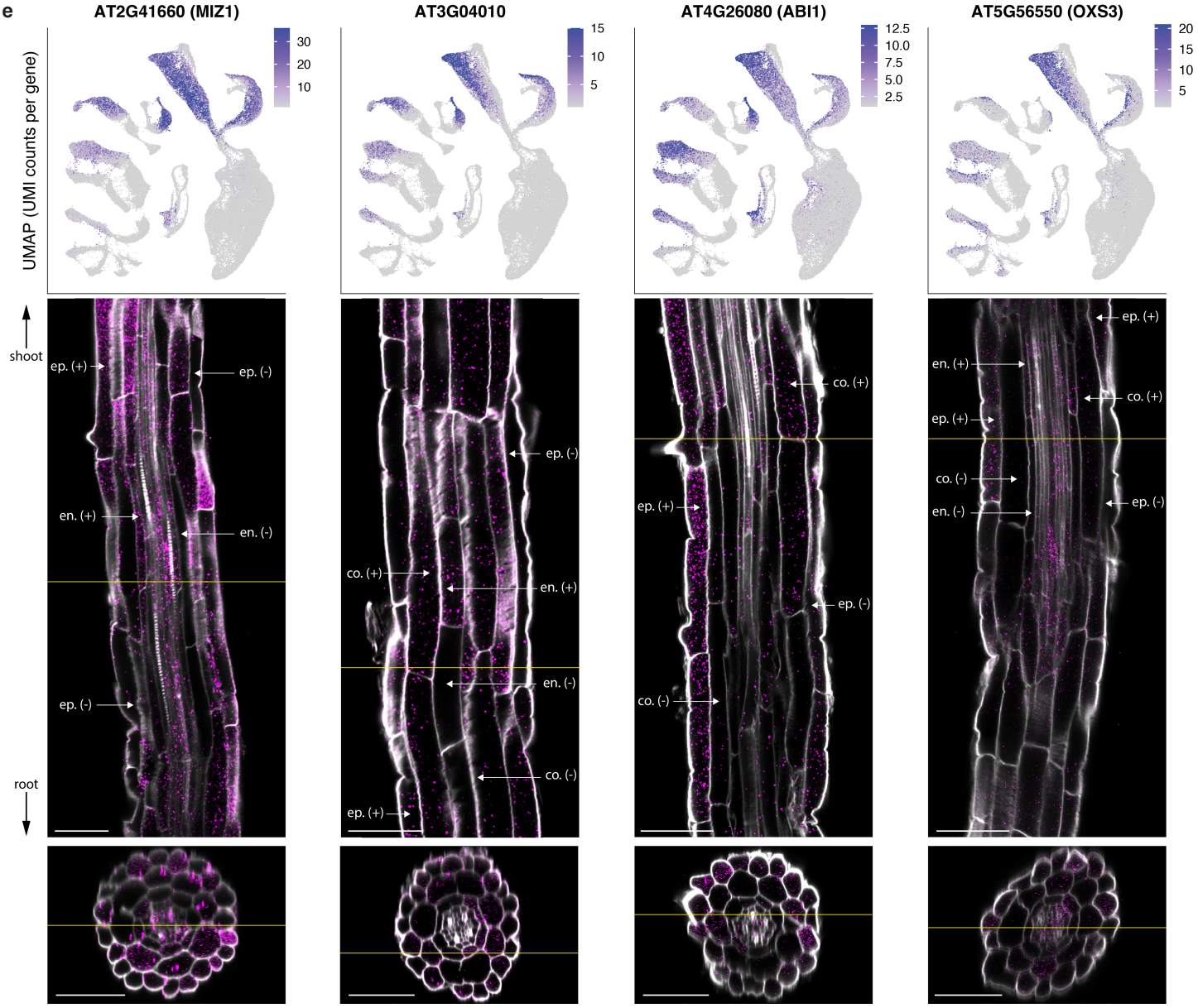
Referee 2 - point 4: 2) Figure 2b: There is no quantification of signal intensity given from the HCR pictures. A quantification of gene expression is important, because the reported expression pattern of e.g. VDOF1 (similar to XTH21 and other genes shown in Extended Fig.4) follows a sensitive gradient and is not completely absent vs. strongly induced.

Response: The main quantification we intended to provide with these images in the initial manuscript was the clear distinction between individual cells that express the marker genes of the ERS+ cell identity and those that do not, including within the same root cross section (i.e. same developmental stage), rather than capturing a gradient of expression along the longitudinal axis. Ideally, this could be addressed by 3D segmentation of plant cells and per-cell quantification, but such approaches remain technically challenging, particularly for the very thin and elongated inner root cells. While our optimisation of the plant whole-mount *in situ* imaging by Hybridization Chain Reaction (HCR) method has enabled extensive independent validation of the scRNA-seq

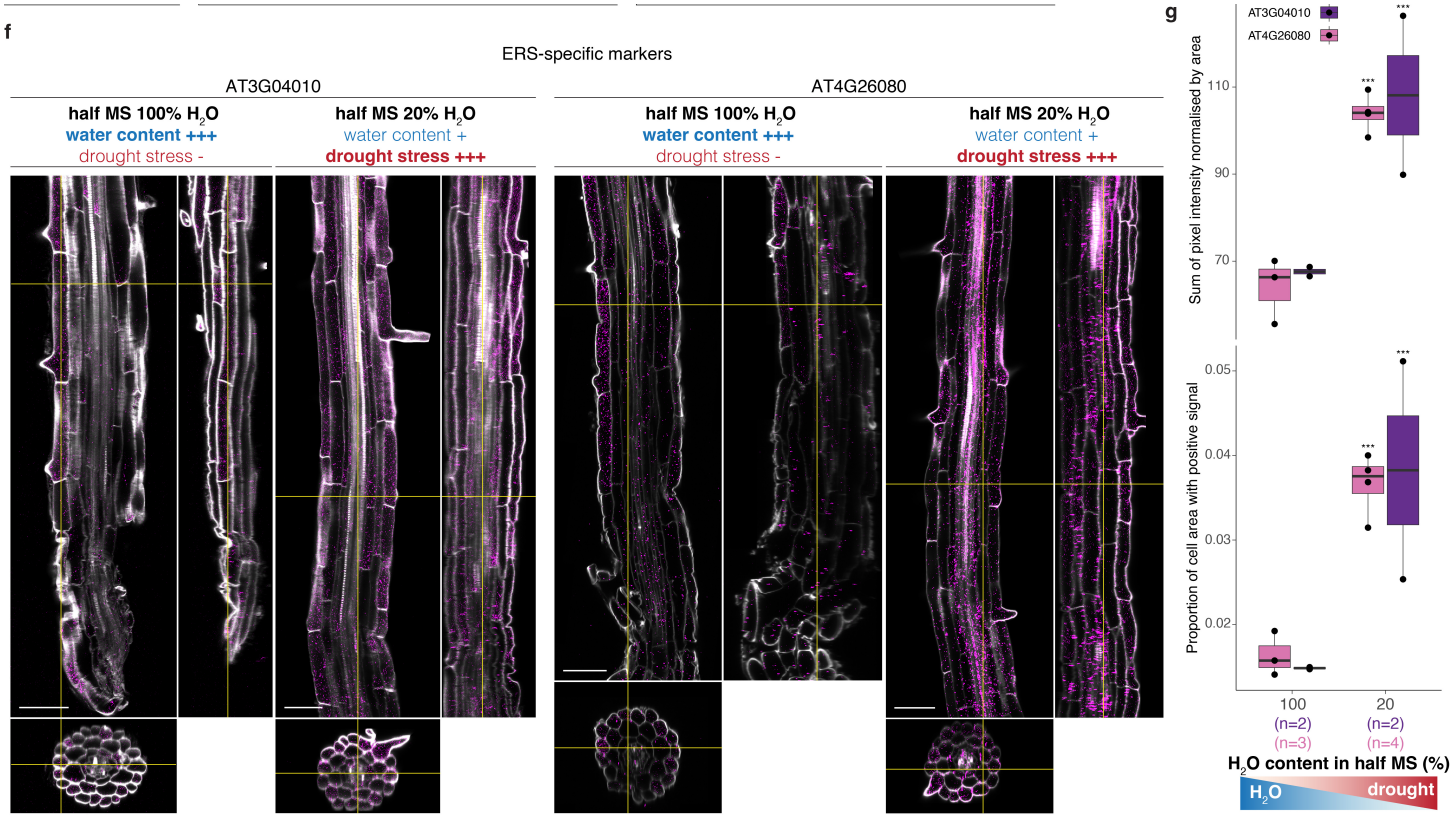
data in this study, accurate per-cell quantification of gene expression remains challenging due to the difficulty of reliably segmenting the thin, elongated stele cells in which these markers are expressed.

To improve the visualisation of ERS+ versus ERS− cells, we have now increased the number of profiled marker genes, including selecting markers with higher expression levels and better HCR detectability (more probe sets per transcript), and focused on markers expressed in the larger outer root layers, where individual positive and negative cells can be more easily distinguished (**Resp. Fig. 23 / Fig. 2e**).

Because the heterogeneity of ERS+ and ERS− cells is now clearly visible in the new HCR images, demonstrating subsets of cells of multiple lineages that acquire the ERS identity, we have not performed per-cell quantification. However, when assessing environmental modulation of the proportion of cells that acquire the ERS identity under drought-like conditions, we have now added quantitative analyses of HCR image signals. In these new analyses, we quantify the HCR signal by measuring both the overall signal intensity (sum of all pixel intensities normalised by the measured area) and the spread of the signal (area containing positive signal normalised by the measured area), which clearly shows the pronounced increase in the transcript abundance of multiple ERS+ marker genes in response to drought-like conditions *in vivo* (**Resp. Fig. 24f,g / Fig. 4f,g**).



Resp. Fig. 23 / Fig. 2e: HCR validation of expression of multiple ERS-specific genes only in a subset of cells, of multiple cell types. UMAP plots and optical sections showing transcript detection of four 'branch 1' markers, *AT2G41660* (*MIZ1*), *AT3G04010*, *AT4G26080* (*ABI1*), and *AT5G56550* (*OXS3*), both *in silico* from scRNA-seq data and *in vivo* by whole-mount *in situ* Hybridization Chain Reaction (HCR). Yellow lines indicate the relative positions of longitudinal and transverse cross sections. Example cells that are positive (+) or negative (–) for each transcript in the epidermis (ep.), cortex (co.), or endodermis (en.) are indicated with arrows. Scale bar = 50µm. Also see **Ext. Data. Fig. 17** that shows the position of these longitudinal images in the context of the root tip.



Resp. Fig. 24 / Fig. 4 *(part)*: HCR validation of the increased proportion of ERS+ cells in response to drought stress. f, Orthogonal confocal views of whole mount HCR detection of two 'branch 1'-specific markers described in Fig. 2e, *AT3G04010* and *AT4G26080* (*ABI1*), in roots grown in half MS (control, 100% H_2O) or under the harshest drought condition (half MS, 20% H_2O). Yellow lines indicate the relative positions of the three optical sections. Scale bar = 50µm. **g,** Box plots showing the increase in total signal intensity or in the area of positive signal (both normalised by total cytoplasmic area, see Methods) in the HCR detection in roots of the ERS-specific markers *AT3G04010* and *AT4G26080* (*ABI1*) under drought conditions (half MS, 20% H_2O) compared to control (half MS, 100% H_2O) (Wilcoxon rank-sum test, *** indicates a p-value < 0.001).

Referee 2 - point 5: 3) Comparing the cell content between cells of both branches in previously published datasets (Ext. Fig. 5) suggests less difference of median UMI content between branch I and II in some references (pointed out in Ext. Fig 5; e.g. Wendrich et al.; Shulse et al.; Denyer et al.;) compared to this study. A statistical analysis would help in determining the significance of the difference in UMI content. Why is there no difference in cell content observed between both branches in some datasets, even though the sequencing depth or overall UMI count in all those studies are like the here reported dataset? How many cells were identified in each branch? Are the here observed transcriptional responses (e.g. enrichment of ABA-responsive genes) also observed in branch I cells found in the other references?

Response: Thank you for pointing this out. In the revised manuscript we have now refined our comparative analysis by focusing on fewer previously published datasets (Shahan *et al.* integrates data from Denyer *et al.* and Ryu *et al.*, and our analysis spans both snRNA-seq and scRNA-seq) but providing a more detailed examination of each. Specifically, we compared our data to the following:

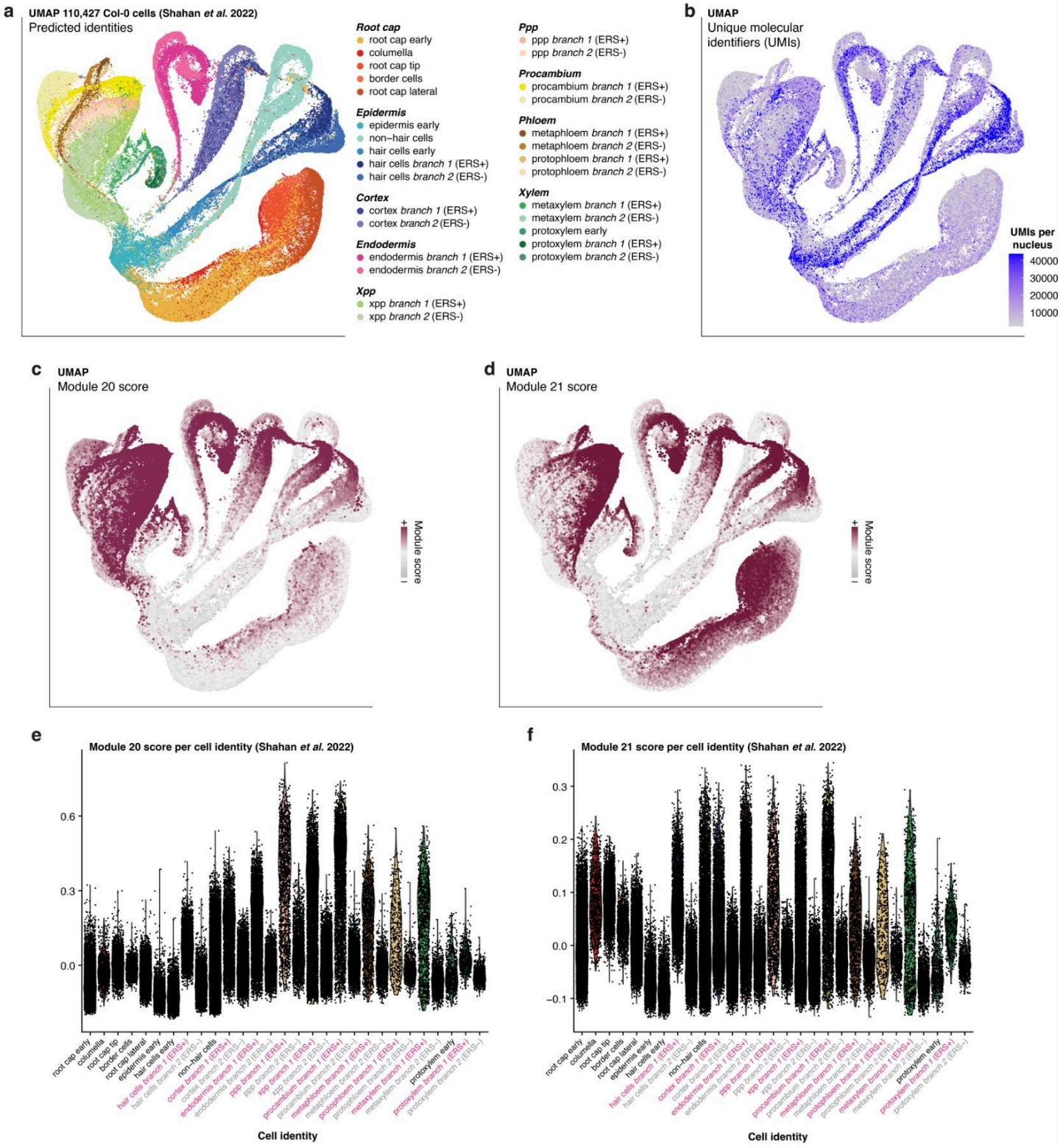
- Shahan *et al.*[3], the largest published scRNA-seq atlas of the Arabidopsis root tip to date. In their study, a similar developmental bifurcation of multiple cell lineages is clearly visible in their UMAP, which correspond to the ERS+ and ERS− cell lineages that we define, and which showed very similar differences in UMI content between these two branches in their study (**Resp. Fig. 25 / Ext. Data Fig. 14**) as we observe between branches in our data.
- Wendrich *et al.*[1], which, as noted, showed a smaller difference in UMI content between cells identified as ERS+ and ERS− using our atlas. It is important to note that in the Wendrich *et al.* study, the authors applied a hard UMI threshold to distinguish cells from damaged cells or background, discarding all data below this cutoff. This was common practice in earlier scRNA-seq studies and has been applied in other datasets as well, however arbitrary and overly stringent thresholds could lead to the loss of biologically relevant information. Indeed, we show that their approach indeed removed a substantial proportion of ERS− cells, making it more difficult to detect the presence of two populations, and

explaining why smaller differences in UMI content were previously observed when using their processed data. When we re-processed the Wendrich *et al.* dataset from fastq files using our pipeline, which more carefully discriminates real cells from damaged cells or background (see response to point 7 below), we recovered many more cells and were able to identify both ERS− and ERS+ cells across lineages (**Resp. Fig. 26 / Ext. Data Fig. 13**).

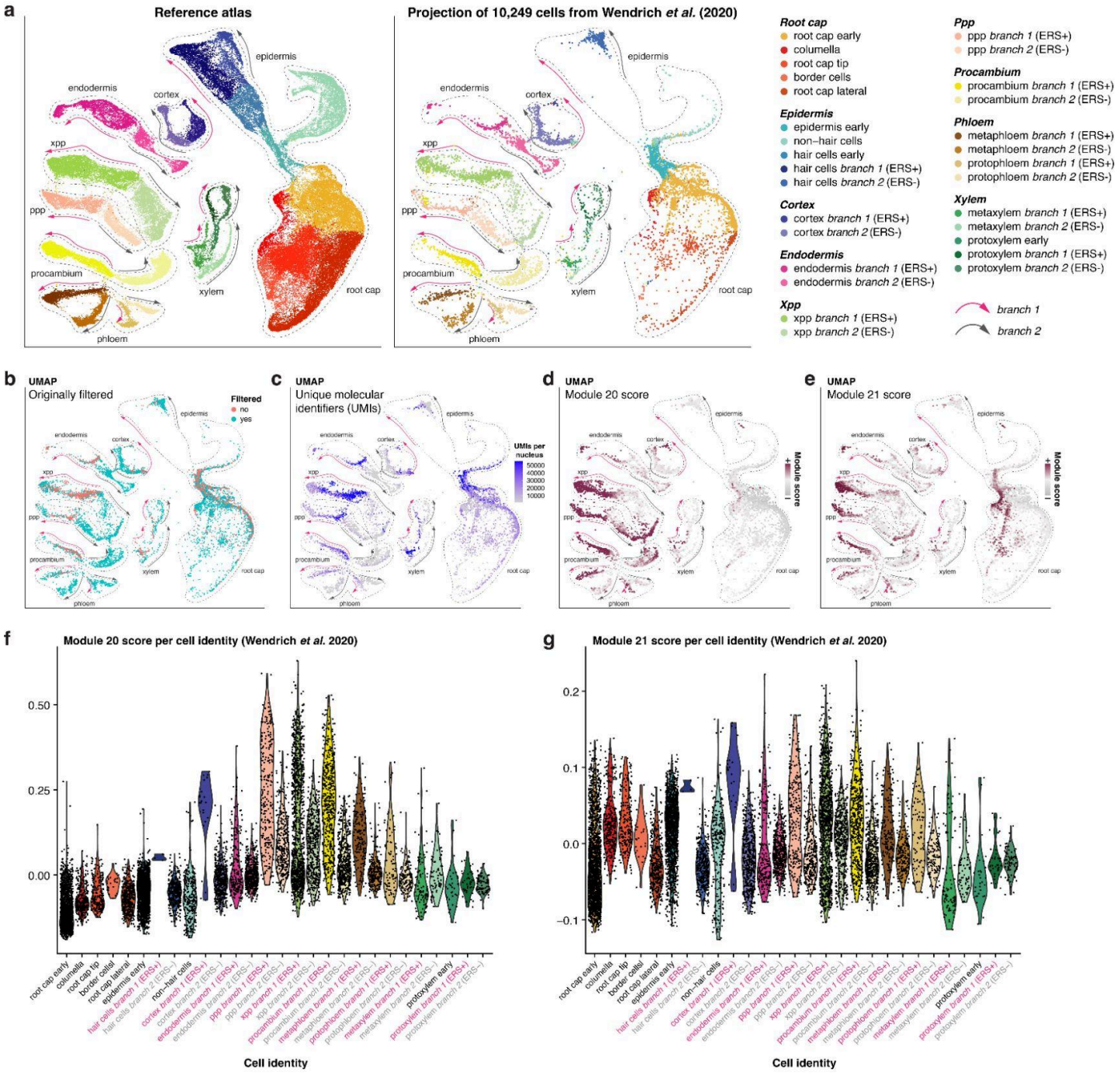
- Farmer *et al*.[2], which is based on nuclei rather than whole cells. Our new analyses of their single nucleus (sn) RNA-seq data in the revised manuscript clearly identifies both ERS+ and ERS− states across many lineages (**Resp. Fig. 27 / Ext. Data Fig. 12**), demonstrating it is also clearly evident in single nuclei datasets.

Importantly, in all three comparative analyses, we show that we recover both ERS+ and ERS− populations across the same cell types as in our study, that they consistently display a difference in UMI content between both branches for the vast majority of bifurcating lineages (even in single nuclei data), and that the cells identified as ERS+ are specifically enriched in the same co-expression modules (Modules 20 and 21), which include ABA-responsive genes. Therefore, these branching lineages and their key features that we have discovered are consistently reproduced, though not explored, in the datasets from these other studies.

Regarding how many cells were identified in each branch, in the revised manuscript we now detail the proportion of cells identified in each branch of the bifurcating lineages for all replicates in our study, shown in the in **Ext. Data Fig. 15, Ext. Data Fig. 19, Ext. Data Fig. 22, and Ext. Data Fig. 24**.

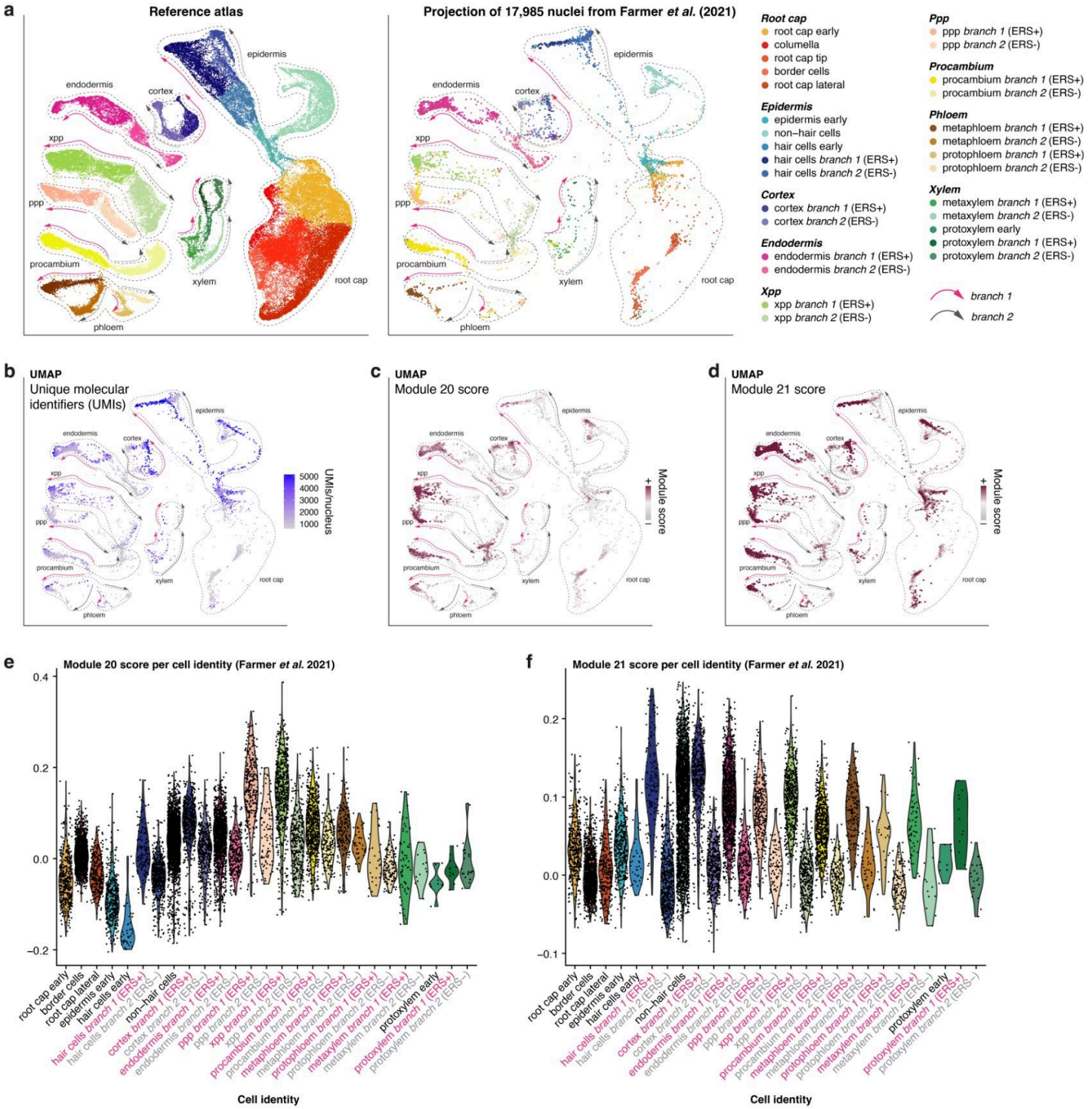


Resp. Fig. 25 / Ext. Data Fig. 14: Branches and their associated ERS-specific genes are detected in the scRNA-seq dataset from Shahan *et al.* a, UMAPs showing the reference atlas embedding from Shahan *et al.*, with cells coloured by their assigned identity based on our atlas. **b,** UMAP showing the distribution of UMI content per cell in their atlas. **c–f,** UMAPs (c,d) and violin plots (e,f) showing the distribution of expression scores for our 'branch 1'-specific modules 20 and 21 in their scRNA-seq data.



Resp. Fig. 26 / Ext. Data Fig. 13: Branches and their associated 'branch 1'-specific genes are detected in the scRNA-seq dataset from Wendrich *et al.*

a, UMAPs showing our reference atlas and the projection of cells from Wendrich *et al.* into the atlas UMAP embedding, with cells coloured by their assigned identity based on our atlas. **b,** UMAP showing whether cells were originally discarded ('filtered') in their study based on UMI content. **c,** UMAP showing the distribution of UMI content per cell. **d–g,** UMAPs (d, e) and violin plots (f, g) showing the distribution of expression scores for our 'branch 1'-specific modules 20 and 21 in their scRNA-seq data.



Resp. Fig. 27 / Ext. Data Fig. 12: Branches and their associated ERS-specific genes are detected in the snRNA-seq dataset from Farmer *et al.*

a, UMAPs showing our reference atlas and the projection of nuclei from Farmer *et al.* into the atlas UMAP embedding, with cells coloured by their assigned identity based on our atlas. **b,** UMAP showing the distribution of UMI content per nucleus in Farmer *et al*. **c–f,** UMAPs (c, d) and violin plots (e, f) showing the distribution of expression scores for our 'branch 1'-specific modules 20 and 21 in the snRNA-seq data.

Referee 2 - point 6: 4) What would be the biological relevance of having different transcriptome content in cells of the same cell identity? The authors have not provided any evidence that would answer why cells that respond to an environmental stimulus would show lower transcript abundance?

Response: We think it is rather the opposite: in response to an environmental stimulus, some cells acquire expression of an additional set of genes, likely linked to a specific physiological function, rather than cells that are responding to an environmental stimulus showing lower transcript abundance. While we haven't fully determined the exact biological function of this state, we show that the root can, and probably needs to, adjust the number of cells acquiring it under stress conditions, suggesting an adaptive role.

To clarify, regarding the specific question about *"why cells that respond to an environmental stimulus would show lower transcript abundance"*, this relates to our observation that there is a higher proportion of ERS+ cells in "full MS" compared to "half MS". We recognise that the way the initial manuscript framed the experimental design may have led to confusion, and we have revised the text to improve clarity. Initially, we described the difference between full MS (1x) and half MS (0.5x) in terms of nutrient availability, which might suggest that half MS constitutes a stress condition due to reduced nutrient content. However, both full and half MS are widely used for Arabidopsis seedling growth and do not typically induce any overt developmental stress phenotypes.

As mentioned briefly in the original discussion, an alternative and more relevant interpretation is based on osmotic strength: the higher salt concentrations in full MS may impose a mild osmotic challenge by making water uptake slightly more difficult. From this perspective, the increased proportion of ERS+ cells in full MS could reflect an adaptive response to this condition.

We propose that the additional gene expression program observed in ERS+ cells reflects a coordinated physiological response across multiple, but not all, cell types. While roots are anatomically organised into concentric layers of well-defined cell identities, functional communication occurs between these layers and likely varies spatially depending on physiological demands. For instance, the flux of water and nutrients from the outer root layers (which sense and absorb these resources) to the vasculature (which distributes them systemically) may not necessarily involve all cells equally and may shift in response to external conditions. The coordinated response may therefore require certain cells to express specific genes, or conversely, this expression may result from intercellular signalling underlying that coordination.

Referee 2 - point 7: 5) Cell filters: the material and methods sections did not suggest that the enrichment of mitochondrial or chloroplast gene expression was used as filter for low quality/dying cells. This is a standard filter, generally accepted in animal and plant scRNA-Seq reports, to improve data quality and removal of stressed cells.
Have chloroplast genes (which are in generally strongly suppressed in roots) been included in the cluster analysis and developmental trajectories? Were there any other criteria (in addition to the mentioned criterial on page 29 line 7-8: "Cells with <200 expressed genes were removed, and genes expressed in <3 cells were removed") set that could help in the identification of low-quality cells caused by stress and/or cell death?
Response: We have undertaken several steps to ensure that the ERS+ and ERS− populations we describe are not artefacts caused by low-quality or dying cells:

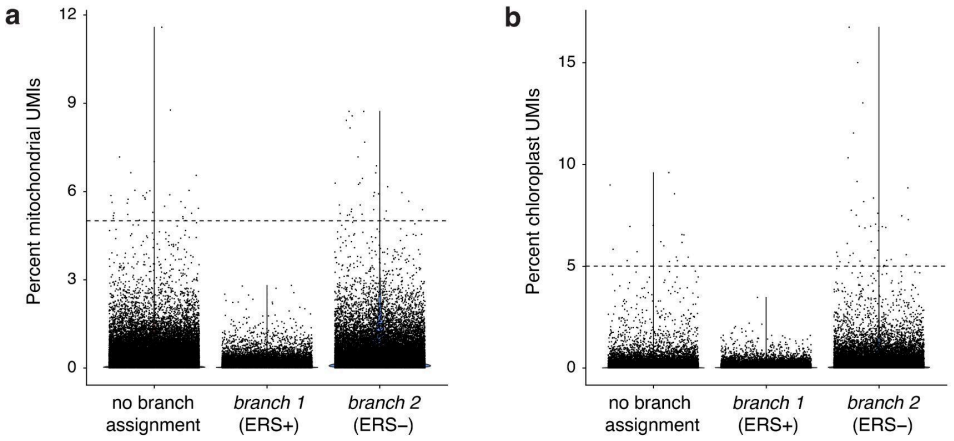
(1) As suggested, we have now excluded all mitochondrial and chloroplast genes from the variable features used for dimensionality reduction and all downstream analyses, including clustering and trajectory analysis. This did not alter the results or our interpretation of the data. We also show that although ERS+ cells tend to have a lower proportion of mitochondrial and chloroplastic transcripts, likely due to their much higher total UMI content, ERS− cells do not exhibit elevated levels of these organellar transcripts compared to other no-ERS cells (i.e. those from earlier developmental stages closer to the meristem or the root cap). Most importantly, nearly all cells in our dataset fall well below the commonly accepted threshold of 5% mitochondrial or chloroplastic transcripts (**Resp. Fig. 28 / Ext. Data Fig. 9**).

(2) We now show that both ERS+ and ERS− populations are detected in single nucleus (sn) RNA-seq data from Farmer *et al*.[2](see **response to point 5** and **Resp. Fig. 27 / Ext. Data Fig. 12** above), confirming that the observed differences cannot simply be explained by intact versus burst cells.

(3) In the revised manuscript we have now also implemented an additional method to detect low-quality cells that may have passed CellRanger's initial filtering, as follows. For each preparation with N identified cells, we processed the subsequent N barcodes classified as "background" by CellRanger together with the identified cell barcodes (see Methods and **Resp. Fig. 29 / Ext. Data Fig. 1**). This allowed us to identify and remove cells whose profiles clustered with background rather than reflecting true biological signal. Depending on the preparation quality, 8–35% of cells were removed using this approach. We believe this method is better suited to detect low-quality cells in a preparation-specific and careful manner, rather than relying on an arbitrary global threshold, thereby more carefully discriminating real cells from damaged cells or background.

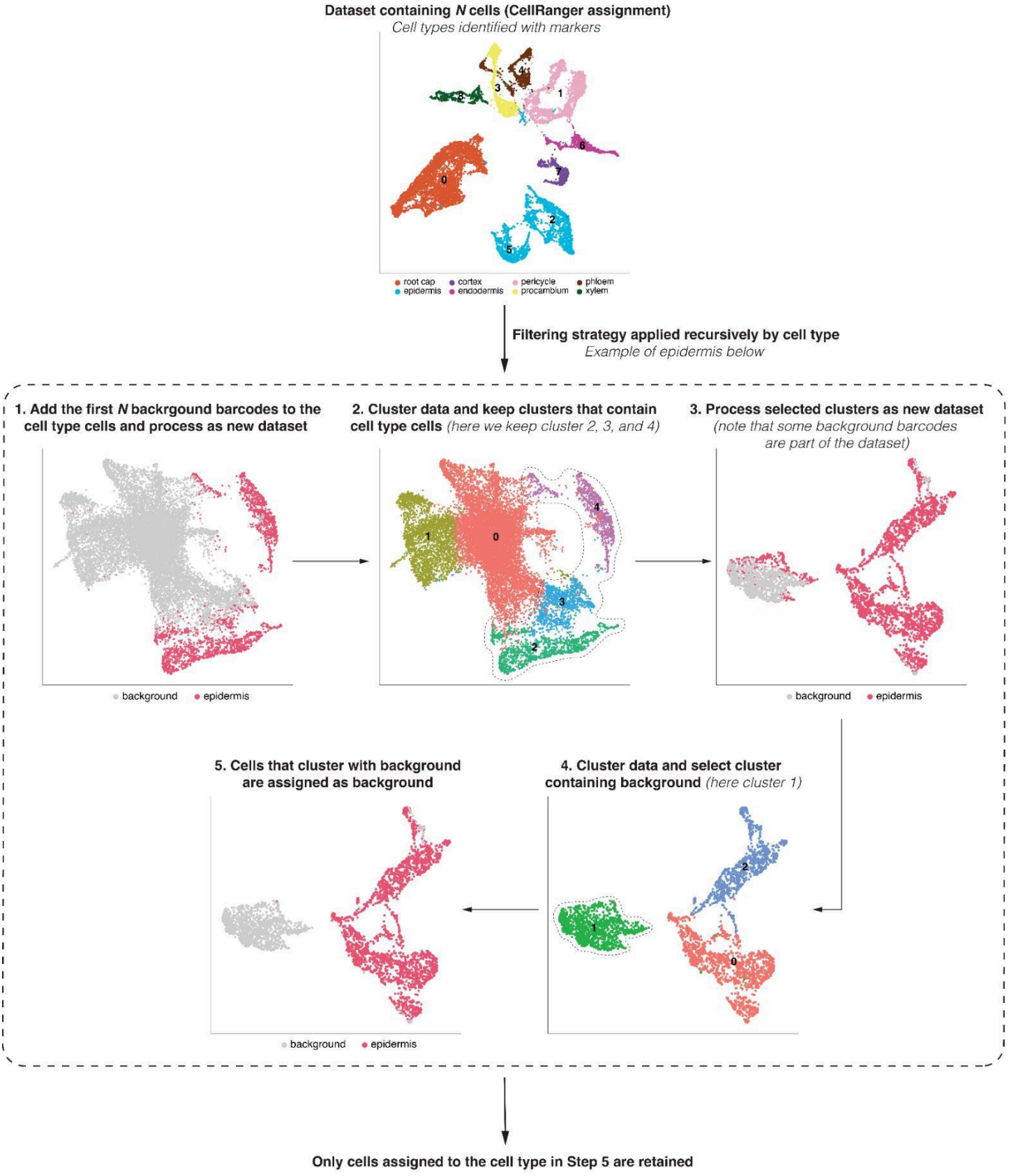
(4) Ultimately, our HCR experiments prove that both ERS+ and ERS− populations are not artefactual, as individual cells (across various cell lineages) expressing ERS-specific genes are clearly and reproducibly detected *in vivo*, while other individual cells in the same image show no signal (ERS−). This provides conclusive *in vivo* validation that within many distinct cell types of the root, a subset of the cells of each type become ERS+ cells and express marker genes of this state, while another subset of cells remain in the ERS− state. The HCR experiments show that these ERS+ and ERS− cells appear to be stochastically distributed, with individual neighboring cells within and between cell types exhibiting

the ERS+ or ERS− identities. Moreover, we show that the proportion of ERS+ cells changes under drought (see **response to point 3** and **Resp. Fig. 22d-g / Fig. 4d-g**) conditions in both scRNA-seq and HCR experiments, while the experimental and computational procedures remain unchanged. The *in vivo* HCR validation experiments are significantly expanded in the revised manuscript, and presented in **Resp. Fig. 9 / Ext. Data Fig. 3** and **Resp. Fig. 13 / Ext. Data Fig. 18** and **Resp. Fig. 23 / Fig. 2e** and **Resp. Fig. 24 / Fig. 4f**.



Resp. Fig. 28 / Ext. Data Fig. 9: 'Branch 2' cells do not show mitochondrial or chloroplast markers of low-quality cells.

Distribution of the percentage of mitochondrial and chloroplast genes per category: cells from the branch 1 (ERS+) trajectories, cells from the branch 2 (ERS–) trajectories, and cells not assigned to these branches (root cap and early stages before branching). Only a few dozen cells in our reference atlas of >87,000 cells exceed 5%, a commonly used threshold for low-quality cells. Moreover, 'branch 2' cells, which have lower UMI content, display mitochondrial and chloroplast gene proportions comparable to root cap and early cells, suggesting they are unlikely to represent damaged cells.



Resp. Fig. 29 / Ext. Data Fig. 1 (part): Diagram illustrating the steps used to filter low-quality cells. See methods for further details.

Referee 2 - point 8: 6) No in vivo validation is provided for the observation of the ERS+-specific ABA signaling or circadian clock-related gene expression on the developmental trajectories (Fig. 2); branch I/ERS+-enriched DEGs in Arabidopsis accessions (Fig. 4) or the nutrient availability single cell dataset (Fig. 5). The authors only provide in silico analyses that provide candidates involved in the potential molecular mechanism (Extended Data Figure 8). As stated already in point 1), in vivo validation is required to support the findings and their originality.

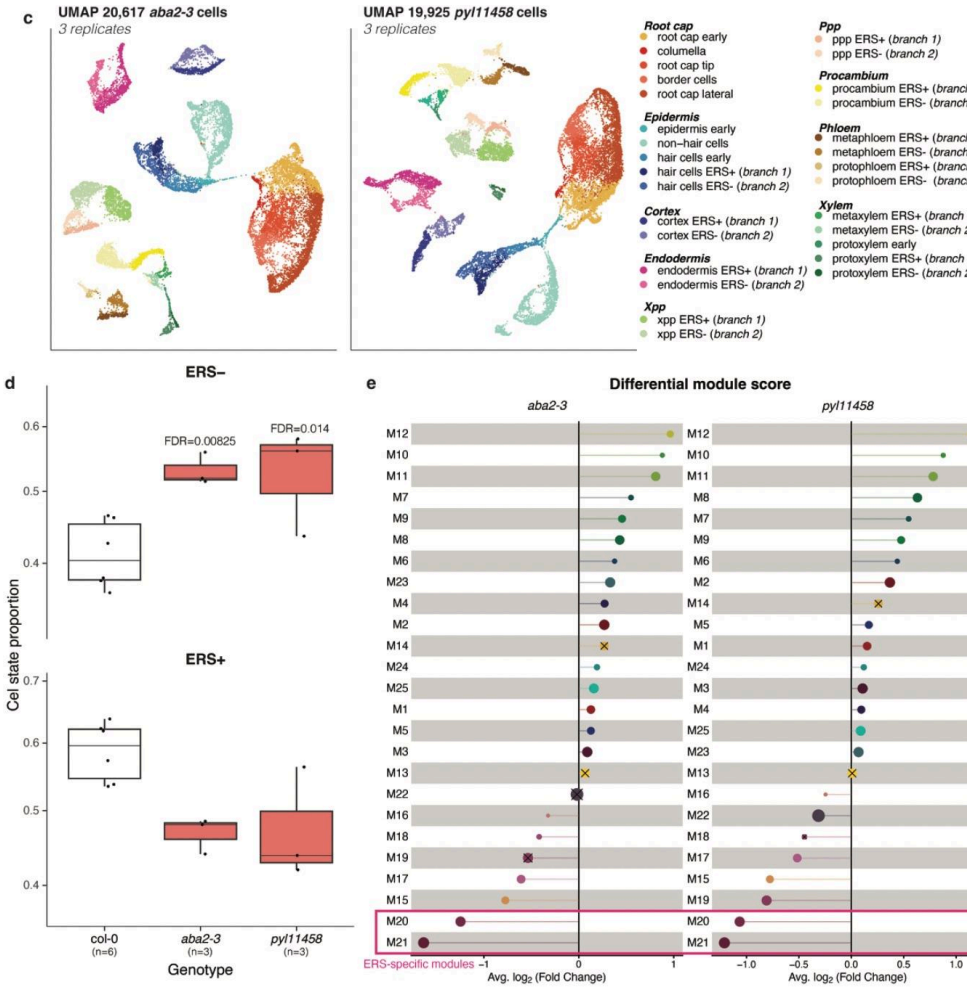
Response: As also detailed in responses above, we have significantly strengthened this aspect of the manuscript to provide independent experimental support of our claims and *in silico* analyses. Specifically, we extended our experimental validation of ERS-specific genes to include two genes transcriptionally upregulated by ABA and involved in ABA signal transduction: *MIZ1* (*AT2G41660*)[13] and *ABI1* (*AT4G26080*)[11,12], presented in **Resp. Fig. 23 / Fig. 2e** (above). This provides independent support for our observation of the ERS-specific ABA signaling gene expression signatures in the developmental trajectories (originally in Fig. 2C, now in **Fig. 3a**, see **Resp. Fig. 14**). The selection of genes for HCR validation is constrained by several factors, including expression levels and the number of probe sets that can be designed across the transcript, both crucial for ensuring sufficient signal for reliable detection and interpretation. Circadian clock genes were not optimal candidates for HCR, and since the revised manuscript focuses on the role of ABA, we concentrated on this pathway.

We have also validated changes in ERS proportion in response to growth conditions by HCR. Rather than comparing full vs half MS, which showed moderate variation in ERS+ proportion, we have now performed HCR on roots grown under the “hard agar” drought model, where the proportion of ERS+ cells are drastically increased, consistent with scRNA-seq data (**Resp. Fig. 22 / Fig. 4d-g**).

Furthermore, as detailed in response to Referee 1 - point 15, we have revised the ecotype analysis and now find only minor gene expression differences across ecotypes, with no longer any Ws-2-specific ERS signature detected. Therefore, we did not perform additional HCR experiments to further explore gene expression differences across ecotypes, as they were minor and not the central focus of our study.

Finally, to address the comment that we *“only provide in silico analyses that provide candidates involved in the potential molecular mechanism”*, in the revised manuscript we specifically test the role of ABA signaling in ERS establishment, by profiling an ABA biosynthesis mutant (*aba2-3*[8]) and a quintuple ABA receptor mutant (*pyl11458*[9]). In both genetic backgrounds, we observed a significant decrease in the proportion of ERS+ cells (**Resp. Fig. 30 / Fig. 3c-e**), confirming the involvement of ABA signaling in establishing this cellular state. Thus, by perturbing signalling pathways that through our *in silico* analyses we predicted may regulate the ERS, we validated their functional role and provide new insights into molecular mechanisms that regulate their formation and function.

Thus, through these additions in the revised manuscript we now provide extensive new *in vivo* validation, functional exploration, and mechanistic insights into the major novel discoveries of our study.



Resp. Fig. 30 / Fig. 3 *(part)*: ABA is required for the acquisition of the ERS+ identity. c, UMAP plots showing predicted cell identities using our reference atlas in the ABA-deficient mutant *aba2-3* and the ABA-insensitive mutant *pyl11458*. **d,** Box plots showing significant depletion of ERS+ cells (and a corresponding increase in ERS-negative (ERS–) cells in *aba2-3* and *pyl11458* (sccomp, FDR < 0.05). **e,** Lollipop plots showing differential expression of the 25 modules identified in Fig. 2b (differential module score, Wilcoxon rank-sum test, adjusted p-value < 0.05). Modules are ranked by average log2 fold change. Dot size indicates the number of genes in each module and an "X" marks modules that do not reach statistical significance. The ERS-specific modules 20 and 21 are the most significantly downregulated in the mutants and highlighted by a pink rectangle.

Additional comments/suggestions for improvement:

Referee 2 - point 9: - Some references (e.g. 13) are not up to date regarding their publication status.
Response: Thank you for pointing it out. The reference has now been updated.

Referee 2 - point 10: - Only two biological replicates are provided for the nutrient scRNA-Seq. A third replicate could provide more robustness to the observations.
Also for this dataset a validation of the observations would be required, e.g. including trichoblast markers or identified DEGs enriched in ERS+ cells. Without in vivo validation, e.g. including cell counting of ERS cells etc., there is no evidence given for the statement made on page 18 line 6-7: "This suggest that the proportion of cells affected by this superimposed state could be modulated under different nutrient availability concentrations."
Response: We have ensured that all experimental conditions now include at least three biological replicates, except for the drought experiment, where two replicates already show very similar and highly significant changes (**Resp. Fig. 22 / Fig. 4d,e**). We have also included *in vivo* HCR validation of the changes in ERS+ cell proportion in this experimental setup, using two ERS-specific genes, which show changes consistent with the scRNA-seq observations (**Resp. Fig. 22 / Fig. 4f,g**).

Referee 2 - point 11: - Page 2; Line 5-6: What evidence is available that shows that cell fate is influence by environment? There are indeed multiple reports on cell number/function/chemical or physical properties that are influenced by environmental stimuli, but it would be good to add references that show changes of cell fate.
Response: Numerous studies have shown that biotic and abiotic stresses can influence root development, both through growth responses (e.g., halotropism, gravitropism) and through adjustments in development (e.g., root hair density under different nutrient conditions, Casparian strip formation)[14]. We expect these changes to be reflected at the level of root cell identities, meaning either a shift between existing types or the acquisition of new characteristics under changing conditions. We agree with the referee that the term "fate" may not have been the most appropriate, as it implies a fixed developmental trajectory. Accordingly, the sentence in Line 5–6 has been revised to: *"This plasticity suggests that, at the cellular level, environmental inputs have a substantial impact on cell function and activity,"* which we believe more accurately conveys our intended meaning.

Referee 2 - point 12: - Page 3; Line 16-18: This statement is somehow contradictory to the description the authors provide in the material and method section Page 36; line 3-6. While the authors claim that other studies "[…] overlooked potential layers of gene regulation that do not depend on the developmental origin of the cell", the material section specifies that subsets (8) were generated based on developmental lineages (epidermis-root cap; cortex; endodermis; PPP; XPP; procambium, phloem, xylem) and that each lineage was processed independently further. In my understanding, the approach used by the authors follows the same principle as used in previous publications (e.g. Denyer et al.; Wendrich et al.; Shahan et al.). Could you please clarify?
Response: The trajectory analysis has indeed been done on each lineage independently to improve the accuracy of the computed trajectories. In previous studies, genes have generally been analyzed along these defined trajectories, which allows detailed characterization within each lineage. In contrast, our gene co-expression module analysis was performed on the whole root dataset rather than on each lineage separately. This approach allowed us to identify both lineage-specific patterns and commonalities between trajectories, which would have been harder to detect by examining gene expression along pseudotime independently in each cell type, and was crucial for the characterization of the ERS.

Referee 2 - point 13: - Page 3; Line 19-21: "[…] providing the most precise root cell atlas to date, […]" This statement is somehow confusing, given that there are already published scRNA-Seq data from Arabidopsis

root with much more cells (Shahan et al., 2022) and that also all annotated cell clusters have been identified and confirmed in previous reports.
Response: We thank the referee for pointing this out. We were wrongfully referring to the preprint version of the article, in which xylem pole pericycle and phloem pole pericycle were not discriminated. We have therefore removed this statement from the manuscript.

Referee 2 - point 14: - Page 19 line 22-24: Which cells exactly should function as "gatekeeper cells"? This is a very strong statement that has too many layers that were not addressed in this manuscript. For example: What would this function include? Can you pinpoint to the exact cells that you identified in vivo with such function?
Response: We agree that this statement was not cautious enough. It was intended to convey a potential role of ERS when we correlated it with a passage cell marker, assuming it could be a way of controlling access to the root interior, which was not demonstrated. We have now decided to focus on other aspects and no longer include the analysis related to CASPs and passage cells, and have therefore removed this statement.

Referee #3:

Summary:
Oliva et al. present a comprehensive single-cell transcriptome atlas, together with spatial mapping, to deeply investigate the developmental lineages of Arabidopsis roots. Impressively, the authors identified two separated trajectories (termed "branch 1" & "branch 2") for most of the lineages, which potentially correspond to the environmental responsive state (ERS). So far as we know, this is a novel finding. However, given that this is a quite large claim, further evidence and validations are necessary to make the findings more convincing.

Major comments:

Referee 3 - point 1: The authors claim that 1) different developmental branches exist and 2) these branches correspond to +/- ERS. Neither of the two claims are fully convincing: the former depends on single-cell clustering and embedding on a single dataset without validations on any independent datasets and the latter highly depends on identification of differentially expressed genes followed by GO analysis. I think more direct evidence is necessary, ideally orthogonal to the way in which these patterns were discovered, given that these are major claims – some suggestions include:
Response: In addition to addressing the revferee's helpful suggestions in the responses below, we would first like to summarise the extensive new set of experiments and analyses, orthogonal to the dataset used to identify them, that we have added to the revised manuscript. These provide independent validation of our claims that: (1) different developmental branches exist, and (2) environmental conditions influence the acquisition of the ERS+ and ERS- branches, as follows:

- We have extensively analysed three published Arabidopsis root single-cell datasets where the branching events were not previously described or fully explored. In all three cases, we show that: (1) cells can be reliably assigned as ERS+ or ERS- using our reference atlas; (2) they exhibit the same differences in UMI/gene content; and (3) they display the same gene modules that characterise the ERS+ populations. This includes:
    - The dataset from Shahan *et al.*[3], which also incorporates data from Denyer *et al.*[4] and Ryu *et al.*[5], where the authors had identified a branching event occurring across all lineages using a computational method different from the one we used. This supports that the branching we observe is not specific to the PAGA method, as also confirmed by our Monocle-based analysis (see response to Referee 3 - point 5). We further show that the branches they distinguish correspond to the ERS+/ERS- dichotomy described in our manuscript.
    - A dataset from Wendrich *et al.*[1], in which both ERS+ and ERS− populations were recovered upon reanalysis, after the original study had discarded ERS− cells due to their lower UMI content.
    - A snRNA-seq dataset (Farmer *et al.*[2]), which demonstrates that the ERS+/ERS- distinction is unlikely to be an artefact of damaged cells (i.e. nuclei) versus intact cells, or a result of protoplasting, as the same signal is observed in nuclei isolated by tissue chopping (see response to Referee 3 - suggestion 1).
- We also analysed the snATAC-seq dataset accompanying the Farmer *et al.*[2] snRNA-seq data. Using chromatin accessibility, we independently identified populations corresponding to ERS+ and ERS- in all lineages. As expected, ERS+ cells show signs of increased transcriptional activity, independently of RNA data, such as higher fractions of reads in peaks and increased transcription start site enrichment.

Importantly, this increase is observed specifically in the genes identified as part of ERS-specific modules, confirming with independent data and methods that the same pathways and genes define this state (see response to Referee 3 - suggestion 1).

- To further validate the involvement of the ABA signalling pathway, which is the only signaling pathway in our enrichment analysis, we profiled two mutant lines in which this pathway is perturbed: *aba2-3*, an ABA biosynthetic mutant[8], and the ABA receptor quintuple mutant *pyl11458*[9]. In both mutants, we observed a significant decrease in ERS+ cells across all root lineages, along with a strong downregulation of the gene modules we identified as ERS-specific, providing functional support for the role of this pathway in establishing the ERS (see response to Referee 3 - suggestion 3 (A), below).
- Regarding the environmentally responsive nature of the ERS, we further explored the initial differences we observed between full MS and half MS, now interpreting them in terms of osmolarity rather than nutrient stress (see *subpoint* of response to Referee 3 - point 3). We went a step further by comparing control conditions with a “hard agar” plate-based low water potential model that effectively mimics drought stress similar to Gonzalez *et al.*[7]. In roots grown under this more stressful condition, we observed a significant increase in ERS+ cells and an upregulation of ERS-specific modules (see response to Referee 3 - suggestion 3 (B)).
- Using whole-mount HCR to detect transcripts in 3D in intact roots, we validated *in vivo* the increase in ERS+ cell proportion under low water potential. We observed a significant increase in both overall signal and the number of cells expressing two ERS-specific genes, including *ABI1*, which is known to be directly regulated by the ABA signalling pathway (see response to Referee 3 - suggestion 3 (C)).
- We also expanded the set of ERS-specific genes used to support the presence of two distinct populations (ERS+ and ERS-) from one gene to five (see response to Referee 3 - point 2). All five genes showed a mix of positive and negative cells within the same image, reinforcing the idea that only a subset of cells express this gene set. Among them, two genes - *MIZ1 (AT2G41660)* and *ABI1 (AT4G26080)* - are direct ABA targets[11–13], and one - *OXS3 (AT5G56550)* - is linked to responses to water deprivation[15].

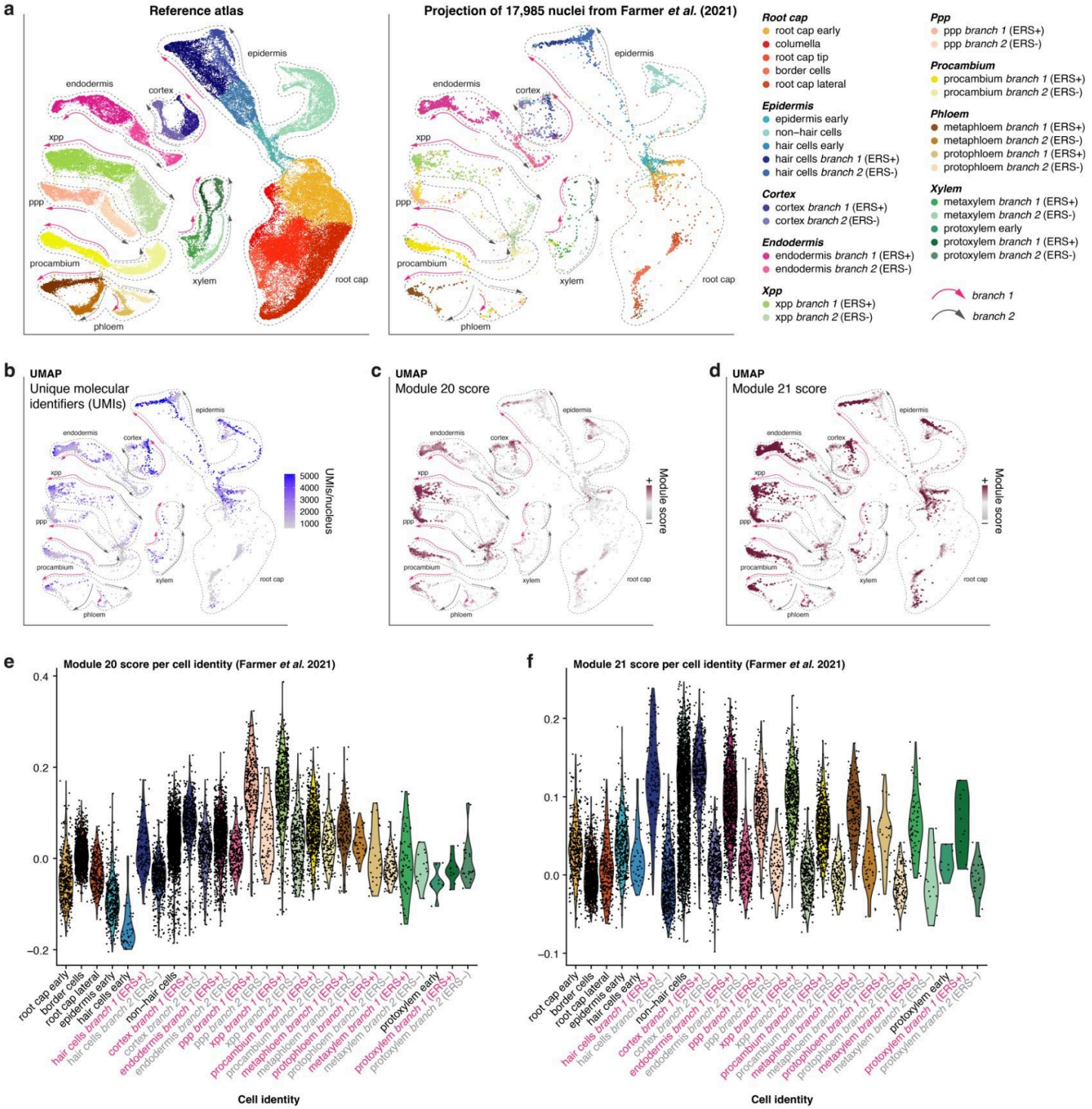
Taken together, we believe this extensive set of new data addresses all major points raised by the referee. It strongly supports the biological relevance of the ERS+/ERS- dichotomy, validates the differences in transcriptional activity between the two states, confirms the accuracy of our identified gene modules and pathways through functional evidence, and demonstrates the environmental regulation of the ERS through changes in ERS+ cell proportions in a drought stress model.

Referee 3 - suggestion 1 - *It would be better to provide some epigenetic-level evidence, for example, chromatin accessibility or methylation difference between ERS +/- states. I think the epigenetics regulations would be more sensitive to environmental responses.*

Response: Thank you for this suggestion. We agree that having an orthogonal level of evidence that some cells are more transcriptionally active than others increases confidence in the biological relevance of the ERS+ state. In particular, we support the idea that epigenetic regulation could underlie environmentally responsive states such as ERS+, and that chromatin-level differences are highly relevant in this context.

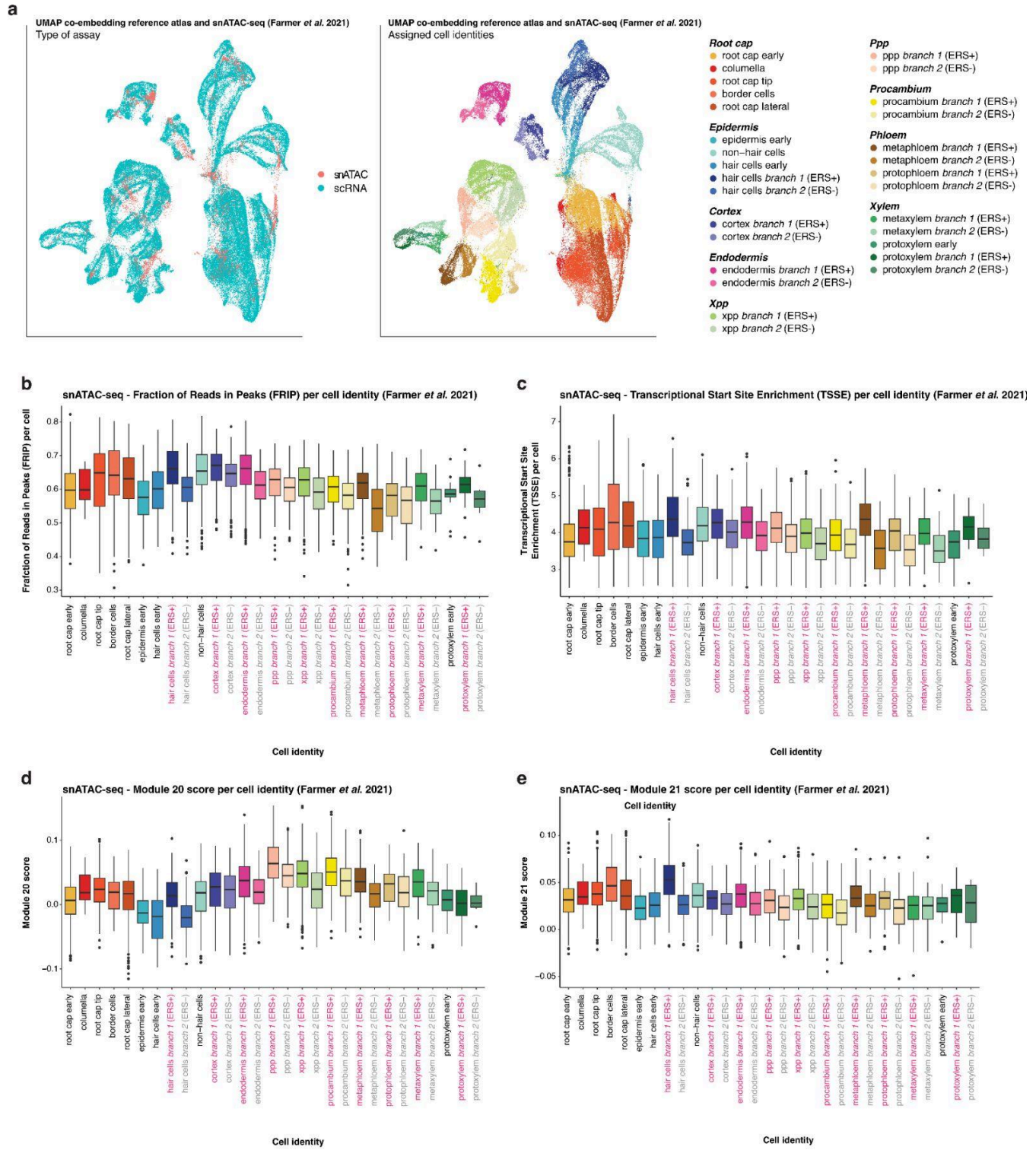
To address this point, we took advantage of a published dataset that includes both snRNA-seq and snATAC-seq data from a similar portion and developmental stage of the root[2]. We show that ERS+ and ERS− populations can be detected in the snRNA-seq data (**Response Figure 31 / Extended Data Figure 12**) and that, using the snATAC-seq chromatin accessibility data and estimating gene activities, we can also independently identify ERS+ and ERS− populations (**Resp. Fig. 32 / Ext. Data Fig. 16**). As expected, ERS+ cells display signatures of increased transcriptional activity, including a higher fraction of reads in peaks and greater transcription start site enrichment (**Resp. Fig. 32b-c / Ext. Data Fig. 16b-c**). Importantly, the genes with increased chromatin accessibility in ERS+ cells include those from the ERS-specific co-expression modules described in our study (**Resp. Fig. 32d,e / Ext. Data Fig. 16d,e**).

These results provide chromatin-level evidence supporting the distinction between ERS+ and ERS− populations and reinforce the view that ERS+ represents a biologically meaningful transcriptional state.



Resp. Fig. 31 / Ext. Data Fig. 12: Branches and their associated 'branch 1'-specific genes are detected in the snRNA-seq dataset from Farmer *et al.*

a, UMAPs showing our reference atlas and the projection of nuclei from Farmer *et al.* into the atlas UMAP embedding, with cells coloured by their assigned identity based on our atlas. **b,** UMAP showing the distribution of UMI content per nucleus in Farmer *et al*. **c–f,** UMAPs (c, d) and violin plots (e, f) showing the distribution of expression scores for our 'branch 1'-specific modules 20 and 21 in the snRNA-seq data.



Resp. Fig. 32 / Ext. Data Fig. 16: The ERS-specific high transcriptional activity is also supported in snATAC-seq data from Farmer *et al.*

a, UMAP plot showing the co-embedding of our scRNA-seq reference atlas and the snATAC-seq data from Farmer *et al.*, obtained by imputing RNA expression for each nucleus. Cells/nuclei are coloured by assay type or imputed cell identity. **b,** Box plots showing the distribution of the fraction of reads in peaks (FRIP) per assigned identity for snATAC-seq nuclei. **c,** Box plots showing the distribution of transcription start site enrichment (TSSE) per assigned identity for snATAC-seq nuclei. **d-e,** Box plots showing the expression score for the two 'branch 1'-specific modules 20 (d) and 21 (e) in snATAC-seq nuclei, per assigned identity.

Referee 3 - suggestion 2 - *Could the two branches be identified for the other four ecotypes by repeating the same analysis on them? Also, the current findings are way unexpected, it would be better to repeat the same analysis on an independent dataset, either published or newly generated.*

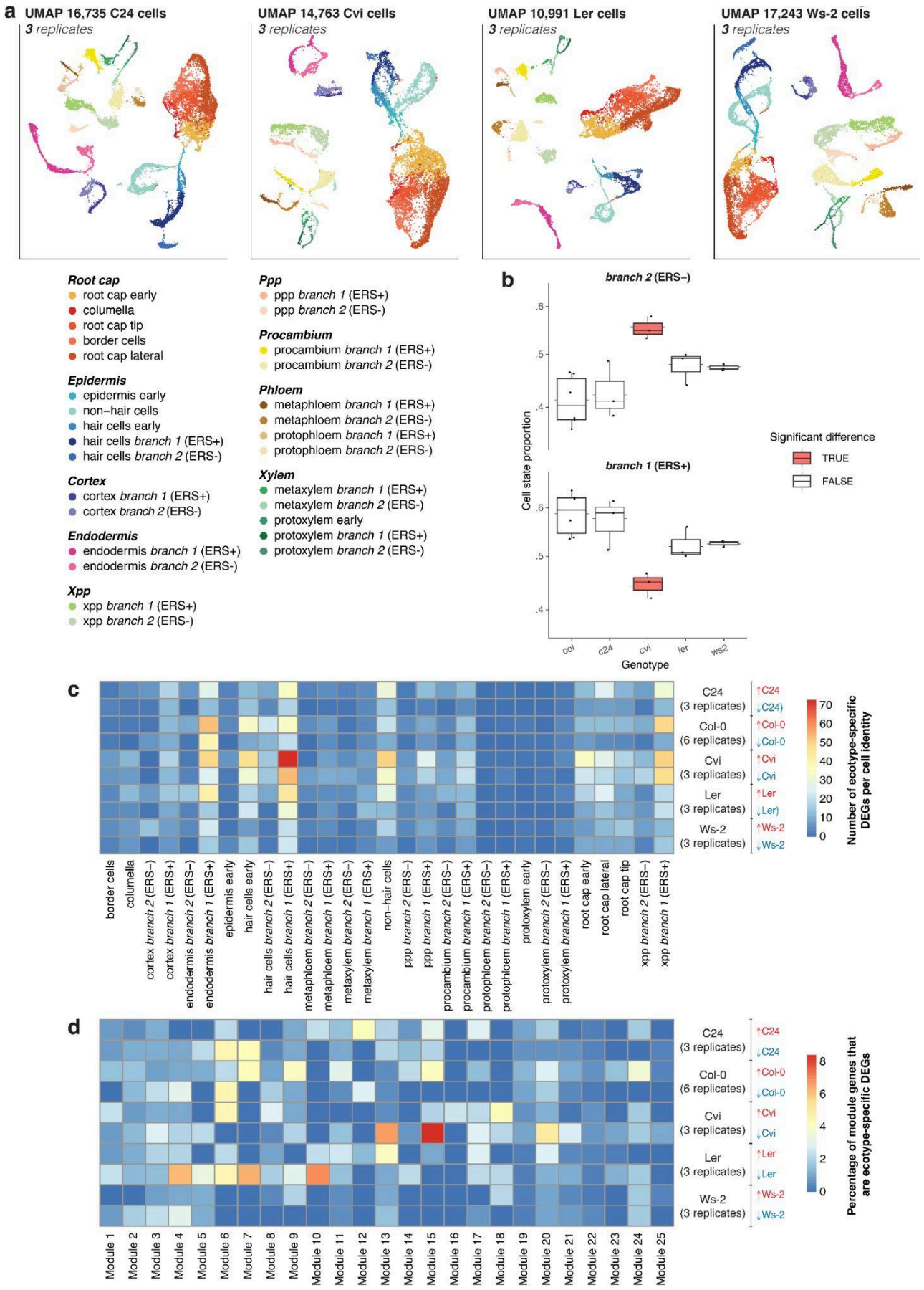
Response: Both ERS+ and ERS− populations are detected in the triplicate scRNA-seq profiling that we have conducted on the four other ecotypes (C24, Cvi, Ler, Ws-2) when projected onto our Col-0 atlas and annotated

(**Resp. Fig. 33a / Ext. Data Fig. 15a**). We show that expression of very few genes change between ecotypes across all cell identities (considering the ERS+ and ERS− populations of each cell type), now presented in **Resp. Fig. 33c,d / Ext. Data Fig. 15c,d**, demonstrating that the presence of these two distinct populations in each lineage is robust across ecotypes.

We have also added a new comparative analysis with three previously published datasets (see response to **Referee 3 - point 4**). In all of them, we show that:

(1) both ERS+ and ERS− populations are detected across the same cell lineages,
(2) ERS+ populations consistently express more genes and have higher UMI counts than the corresponding ERS− populations, and
(3) ERS+ populations are enriched for the same ERS-specific co-expression modules (Modules 20 and 21) identified in our study.

The branching is also clearly visible in the UMAP from Shahan *et al*.[3] and briefly mentioned in the main text and a supplementary figure in their study, but not deeply explored. Overall, the data from these independent studies demonstrates that independent scRNA-seq datasets also exhibit the lineage branching features.

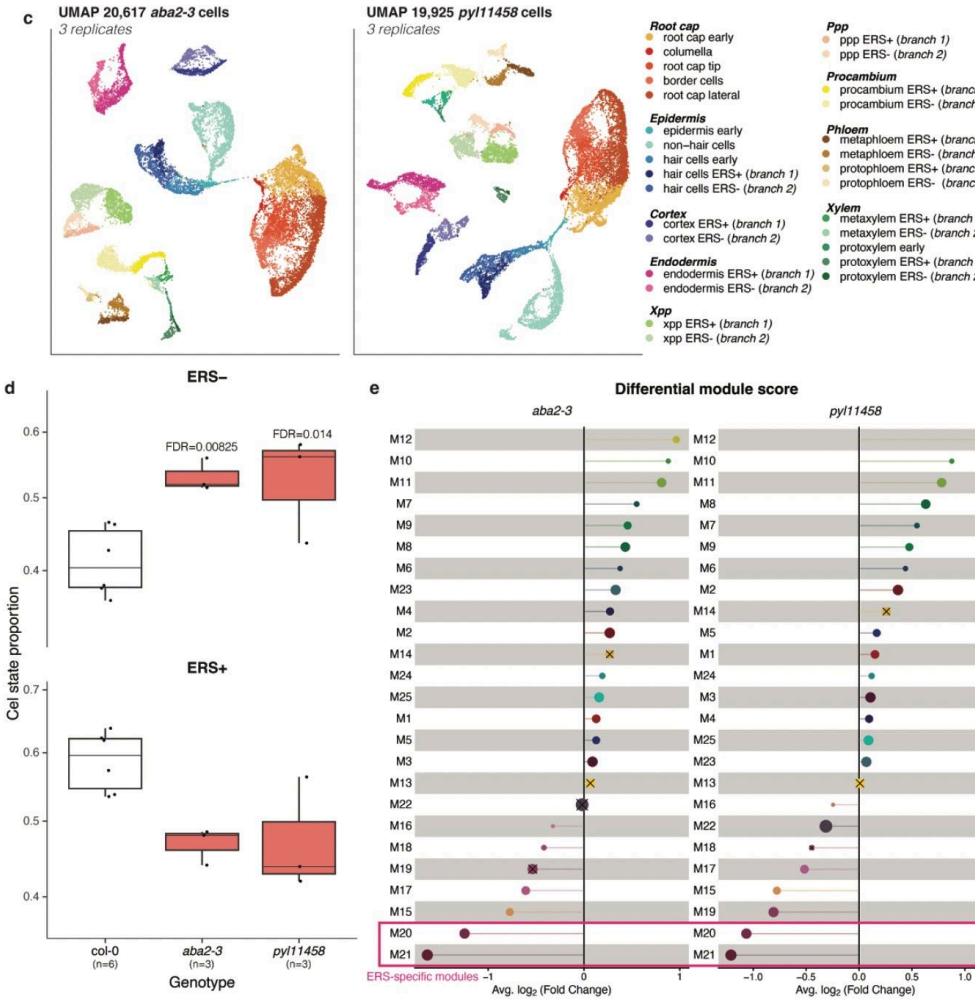


Resp. Fig. 33 / Ext. Data Fig. 15: Branches are robustly detected in all five tested Arabidopsis ecotypes **a,** UMAP plots showing the atlases from C24, Cvi-0, Ler, and Ws-2 cells, coloured by identity assigned using our reference atlas. **b,** Box plots showing the proportion of 'branch 1' (ERS+) and consequently 'branch 2' (ERS-) cells in atlases from all five tested ecotypes. Colours indicate branch proportions significantly different from Col-0. **c,** Heatmap showing the number of ecotype-specific up- or down-regulated genes. **d,** Heatmap showing the percentage of ecotype-specific up- or down-regulated genes per gene co-expression module.

Referee 3 - suggestion 3 - *Experimental validation would be very helpful, for example, knocking down a key gene, blocking the corresponding pathway, or adjusting the environmental conditions, which will potentially make the additional branch disappear. Otherwise, the current differentially expressed genes & GO analysis is not sufficient. The nutrient depletion experiment shown in the last section (Fig.5) is quite interesting, but I think such analysis based on the cell composition changes might need more replicates. HCR validation that fewer ESR+ cells exist in nutrient depleted conditions would also be nice.*

Response: We agree that, as noted by all referees, one of the main limitations of the initial version of the manuscript was the lack of *in vivo* and functional validation of our *in silico* findings and predictions. We have therefore made this a major focus of the revised version, through the following additions:

A. As suggested, we tested the role of ABA in ERS establishment using mutants. This hormone emerged as a key pathway from our GO enrichment analysis and is well known for its involvement in environmental responses and stress. Given the high redundancy between transcription factors that would have been candidates in this pathway, mutating a single TF would likely not abolish the ERS. Instead, In the revised manuscript we specifically test the role of ABA signaling in ERS establishment, by profiling an ABA biosynthesis mutant (*aba2-3*)[8] and a quintuple ABA receptor mutant (*pyl11458*)[9]. In both genetic backgrounds, where the ABA signal is reduced, we observe a significant decrease in the proportion of ERS+ cells (**Resp. Fig. 34 / Fig. 3c,d**), confirming the involvement of ABA signaling in establishing this cellular state.

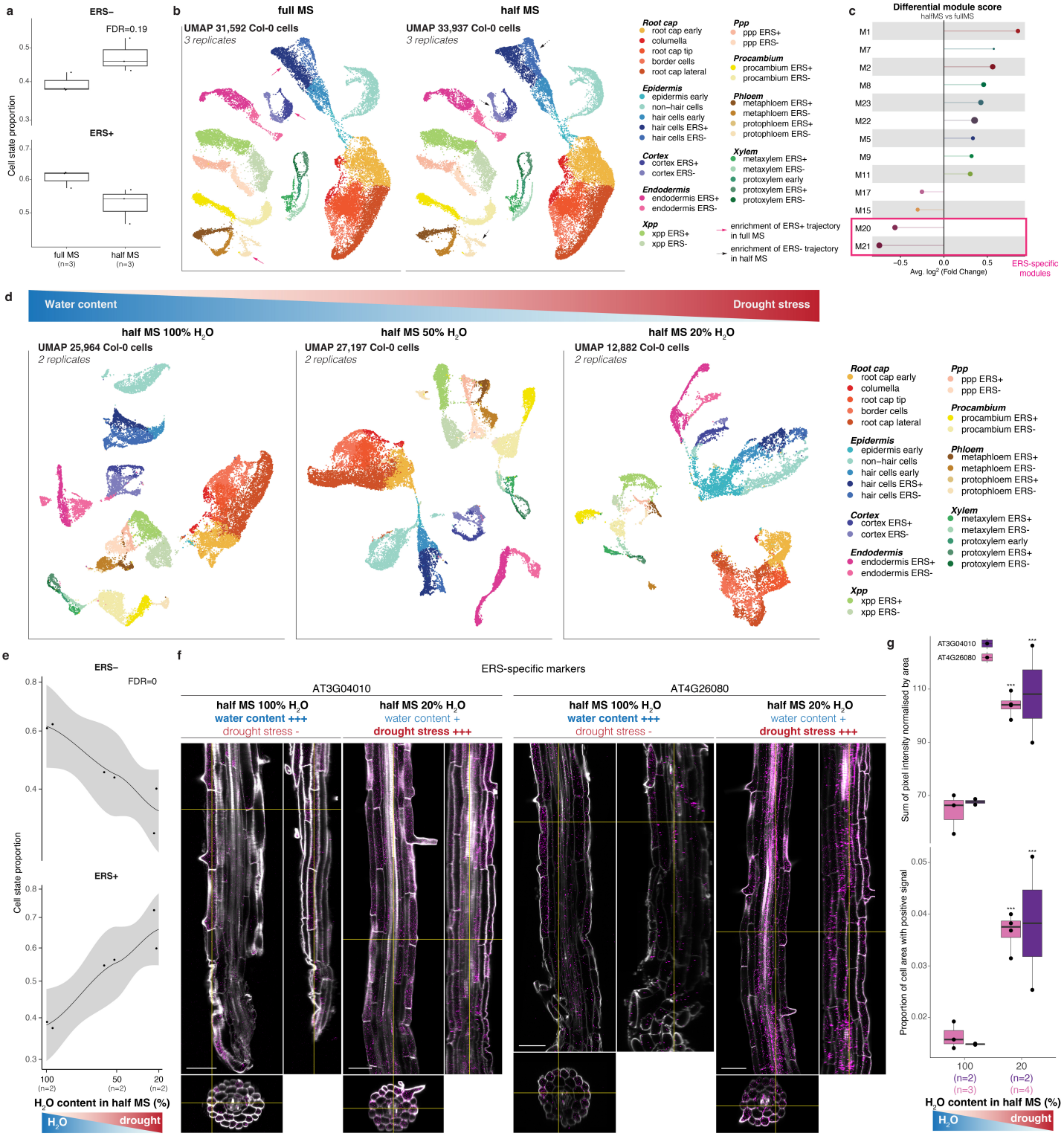


Resp. Fig. 34 / Fig. 3 *(part)*: ABA is required for the acquisition of the ERS+ identity. c, UMAP plots showing predicted cell identities using our reference atlas in the ABA-deficient mutant *aba2-3* and the ABA-insensitive mutant *pyl11458*. **d,** Box plots showing significant depletion of ERS+ cells (and a corresponding increase in ERS-negative (ERS–) cells in *aba2-3* and *pyl11458* (sccomp, FDR < 0.05). **e,** Lollipop plots showing differential expression of the 25 modules identified in Fig. 2b (differential module score, Wilcoxon rank-sum test, adjusted p-value < 0.05). Modules are ranked by average log2 fold change. Dot size indicates the number of genes in each module and an "X" marks modules that do not reach statistical significance. The ERS-specific modules 20 and 21 are the most significantly downregulated in the mutants and highlighted by a pink rectangle.

B. We have now added a third replicate to the MS/nutrient experiment. This replicate showed very similar results to the first two, with a clear tendency towards reduced ERS+ proportions (**Resp. Fig. 35a,b / Fig. 4a,b**). Using the new statistical test to assess ERS+ cell proportions, the changes were significant in only a subset of all cell types (hair cells, cortex, and protophloem; **Resp. Fig. 36 / Ext. Data Fig. 22**), despite the consistent trend and pronounced downregulation of the ERS-specific gene expression modules (**Resp. Fig. 35a,c / Fig. 4a,c**). While additional replicates could be added, one possible explanation is that switching between full (1x) and half (0.5x) MS, both of which are widely used standard conditions for growing seedlings, may not trigger a strong enough physiological adaptation.

While this setup can indeed be interpreted as a change in nutrient availability, as initially discussed in the paper, we also considered that it represents a change in water potential due to altered osmolarity of the medium. Given the well-established role of ABA in water-related stresses, we decided to explore this hypothesis further using the "hard agar" model of drought similar to Gonzalez *et al*. 2024[7]. In this setup, the water content of the MS medium is reduced to 50% or 20% of its normal value, which also concentrates agar and nutrients and thereby lowers the water potential. This model has been shown to trigger gene expression changes in the root similar to those observed under drought conditions on soil[7]. Through scRNA-seq analysis of the root tips of plants grown in normal (100%), 50%, and 20% water content conditions, we observed a significant and pronounced increase in the proportion of ERS+ cells in drought conditions compared to control, with multiple replicates showing very similar and robust changes (**Resp. Fig. 35d,e / Fig. 4d,e**).

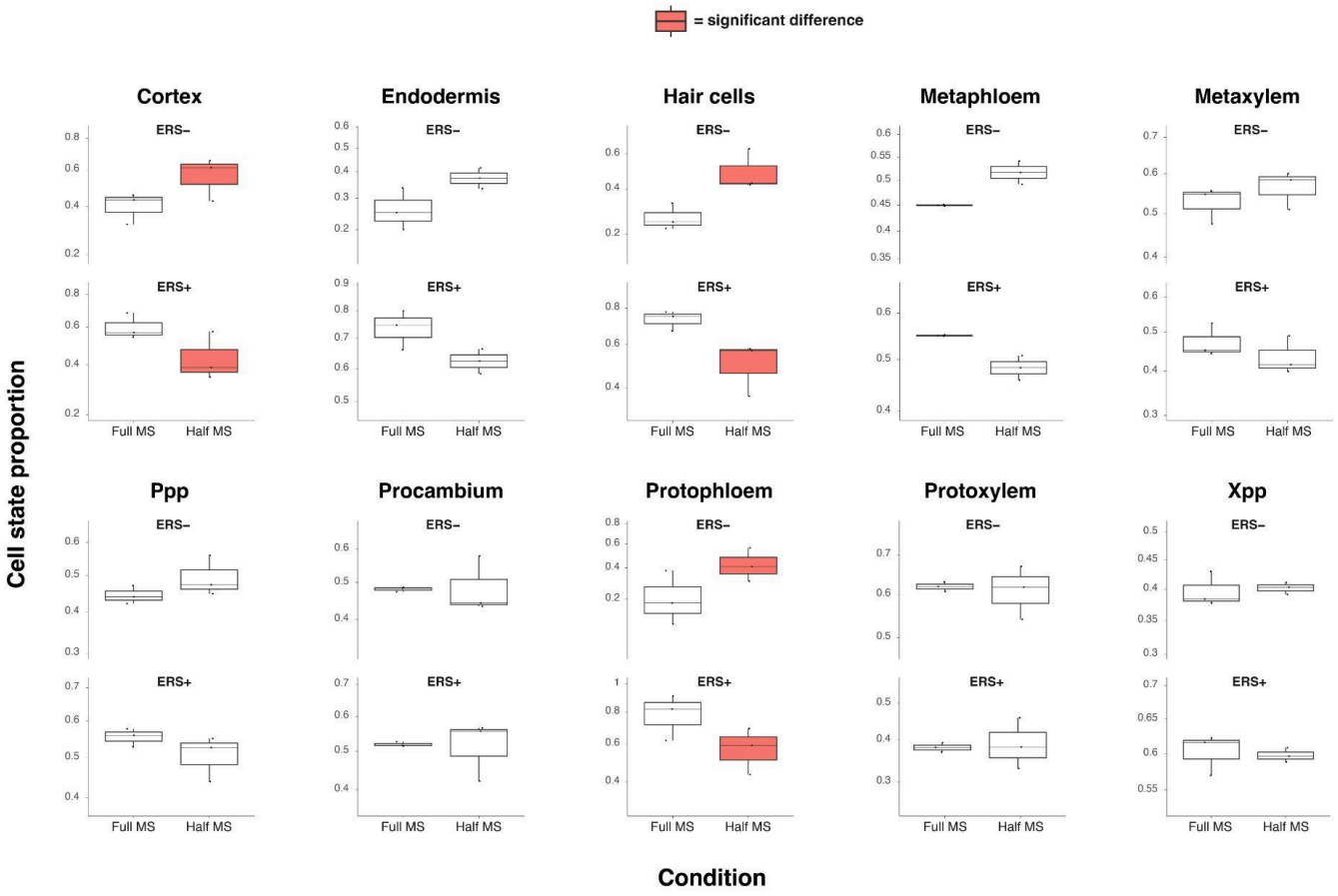
C. In addition to the scRNA-seq showing elevation of ERS+ cell frequency in response to drought-like conditions, we validated these changes *in vivo* using HCR. Imaging of multiple ERS+ marker genes in the roots of plants grown in either normal or low water potential conditions consistently showed a significant increase in the number of ERS+ cells under the drought conditions compared to control, both visually (**Resp. Fig. 35f / Fig. 4f**) and through quantitative analysis of HCR signal intensity and signal spread (**Resp. Fig. 35g / Fig. 4g**). Notably, this showed an increased number of cells expressing two ERS-specific marker genes (**Resp. Fig. 35f,g / Fig. 4f,g**), *ABI1* (*AT4G26080*) and *MIZ1* (*AT2G41660*), both of which are transcriptionally induced by ABA and involved in ABA signal transduction[11–13].



Resp. Fig. 35 / Fig. 4: Acquisition of the ERS is modulated under drought stress

a, Box plots showing the proportion of ERS+ (and consequently ERS–) cells in roots grown in full MS and half MS. **b,** UMAP plots showing cells from three replicates grown simultaneously in full MS or half MS, displayed separately. An enrichment of the ERS+ trajectory in full MS, and conversely of the ERS– trajectory in half MS, is indicated by arrows and is particularly visible in hair cells, cortex, endodermis, and protophloem. **c,** Lollipop plots showing differential expression of the modules identified in Fig. 2b (differential module score; Wilcoxon rank-sum test, adjusted P < 0.05 and |average log2 fold change| > 0.25). Modules are ranked by average log2 fold change. ERS-specific modules 20 and 21 are the most significantly downregulated in half MS and are highlighted by a pink rectangle. **d,** UMAP plots showing assigned cell identities in scRNA-seq profiling of roots grown under control conditions (half MS, 100% H_2O) and in the "hard agar" drought model, where water content in half MS medium was reduced to 50% (half MS, 50% H_2O) or 20% (half MS, 20% H_2O). **e,** Scatter plots showing a significant increase in ERS+ cell proportion with decreasing water content (sccomp, FDR < 0.001). **f,** Orthogonal confocal views of whole mount HCR detection of two 'branch 1'-specific markers

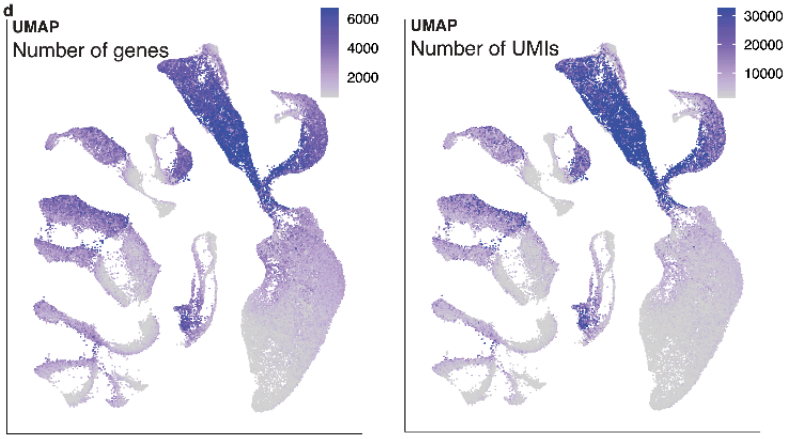
described in Fig. 2e, *AT3G04010* and *AT4G26080* (*ABI1*), in roots grown in half MS (control, 100% H_2O) or under the harshest drought condition (half MS, 20% H_2O). Yellow lines indicate the relative positions of the three optical sections. Scale bar = 50µm. **g,** Box plots showing the increase in total signal intensity or in the area of positive signal (both normalised by total cytoplasmic area, see Methods) in the HCR detection in roots of the ERS-specific markers *AT3G04010* and *AT4G26080* (*ABI1*) under drought conditions (half MS, 20% H_2O) compared to control (half MS, 100% H_2O) (Wilcoxon rank-sum test, *** indicates a p-value < 0.001).



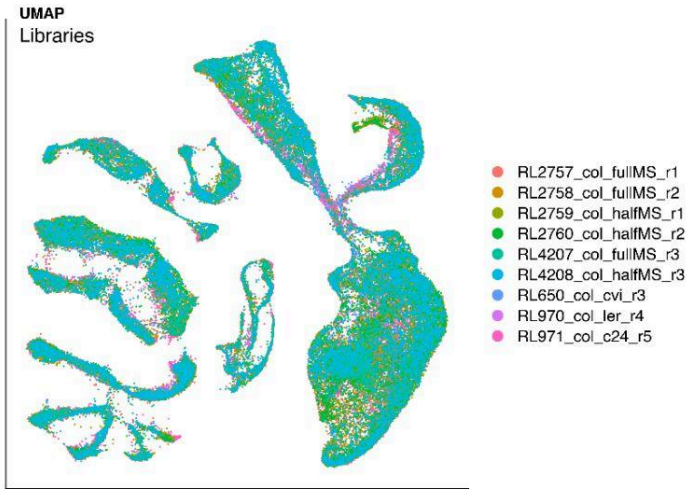
Resp. Fig. 36 / Ext. Data Fig. 22: Reduction of ERS+ proportion in half MS compared to full MS
Box plots showing the distribution of ERS+ cells, and consequently ERS– cells, per cell type affected by branching, in three replicates grown simultaneously on either full MS or half MS. Tests showing a significant difference in cell state proportion between conditions are highlighted in pink (sccomp, FDR<0.05).

Referee 3 - point 2: More evidence is necessary to eliminate the possibility that these results are due to artifacts. Some suggestions:
- Coloring the UMAP in Fig.1b by 1) UMI count, 2) gene count, 3) ecotype identity, 4) sequencing or experimental batches (if available);
Response: We have included UMAPs coloured by UMI count and gene count per cell (originally in Ext. Data Fig. 5, now shown in **Resp. Fig. 37 / Fig. 2d**), and a UMAP coloured by experimental batches is provided in **Resp. Fig. 38 / Ext. Data Fig. 6d**. The ecotypes are no longer integrated with Col-0 in a single atlas, as we mainly focus on the Col-0 dataset in the revised study. Nonetheless, as mentioned in the response to Referee 3 - suggestion 2 above, the presence of these two distinct branches/populations in each lineage is robustly detected in all ecotypes (**Resp. Fig. 33a / Ext. Data Fig. 15a**).

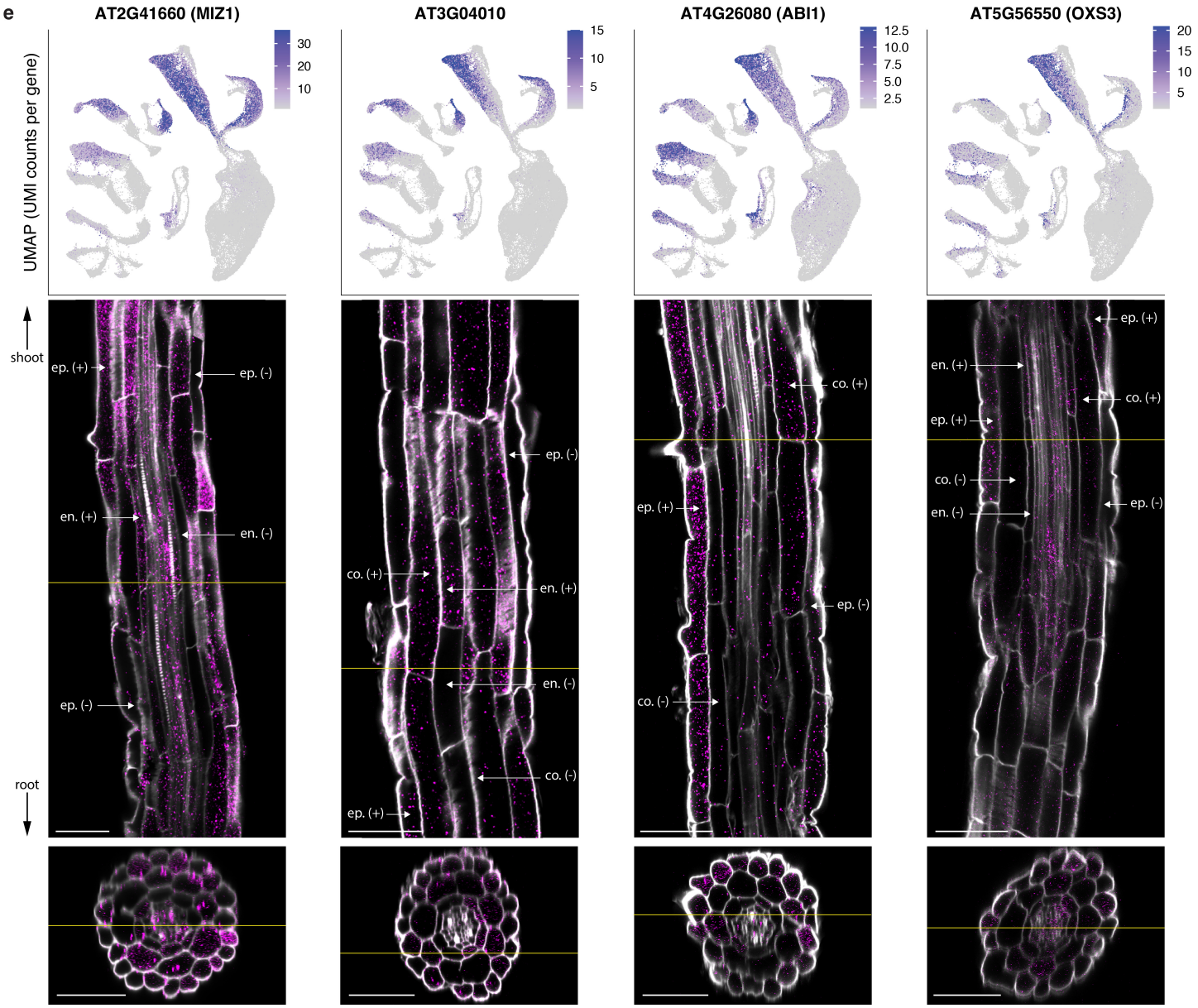
Although differences in UMI and gene count could, in principle, represent confounding factors, we are confident that they reflect true biological variation in transcriptional activity rather than artifacts. We show that these differences cannot be explained by cell quality (see responses to **Referee 2 - point 2** and **Referee 3 - point 3**), that both ERS+ and ERS− populations can be robustly identified across diverse cell types *in vivo* by HCR using multiple markers (**Resp. Fig. 39 / Fig. 2e** and **Resp. Fig. 40 / Ext. Data Fig. 18**), and in independent root tip scRNA,single nucleus (sn) RNA-seq, and snATAC-seq datasets (see response below and **Resp. Figs. 31, 32, 42, 43 / Ext. Data Fig. 12-14, 16**), and, importantly, that the proportion of cells acquiring this highly transcriptionally active state varies with growth conditions (**Resp. Fig. 35 / Fig. 4**). Crucially, this environmental modulation was further validated experimentally by HCR (**Resp. Fig. 35f,g / Fig. 4f,g**).



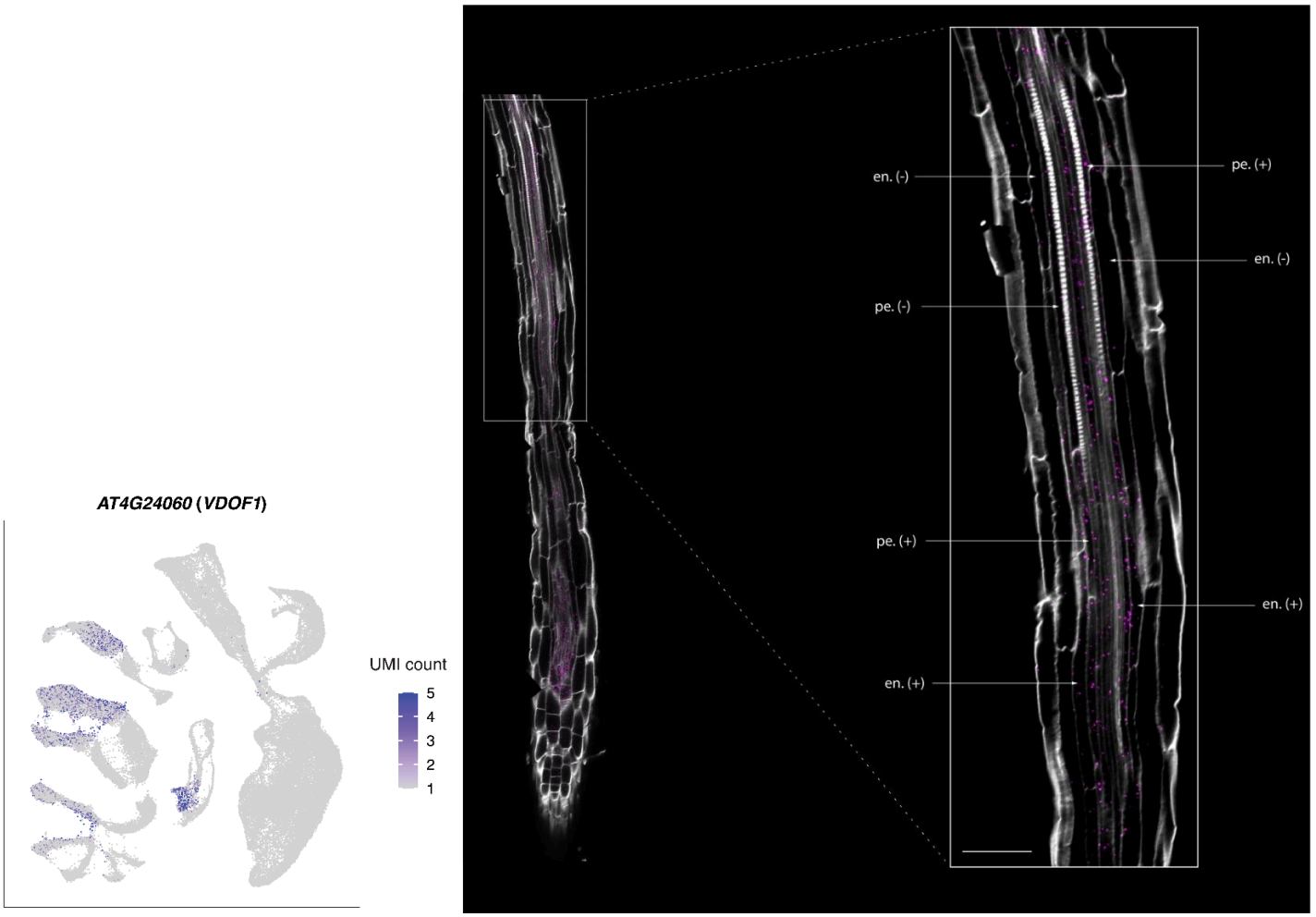
Resp. Fig. 37 / Fig. 2d: Col-0 root tip scRNA-seq reference atlas UMAPs coloured by UMI count and gene count per cell.



Resp. Fig. 38 / Ext. Data Fig. 6d: Col-0 root tip scRNA-seq reference atlas UMAPs coloured by experimental batch.



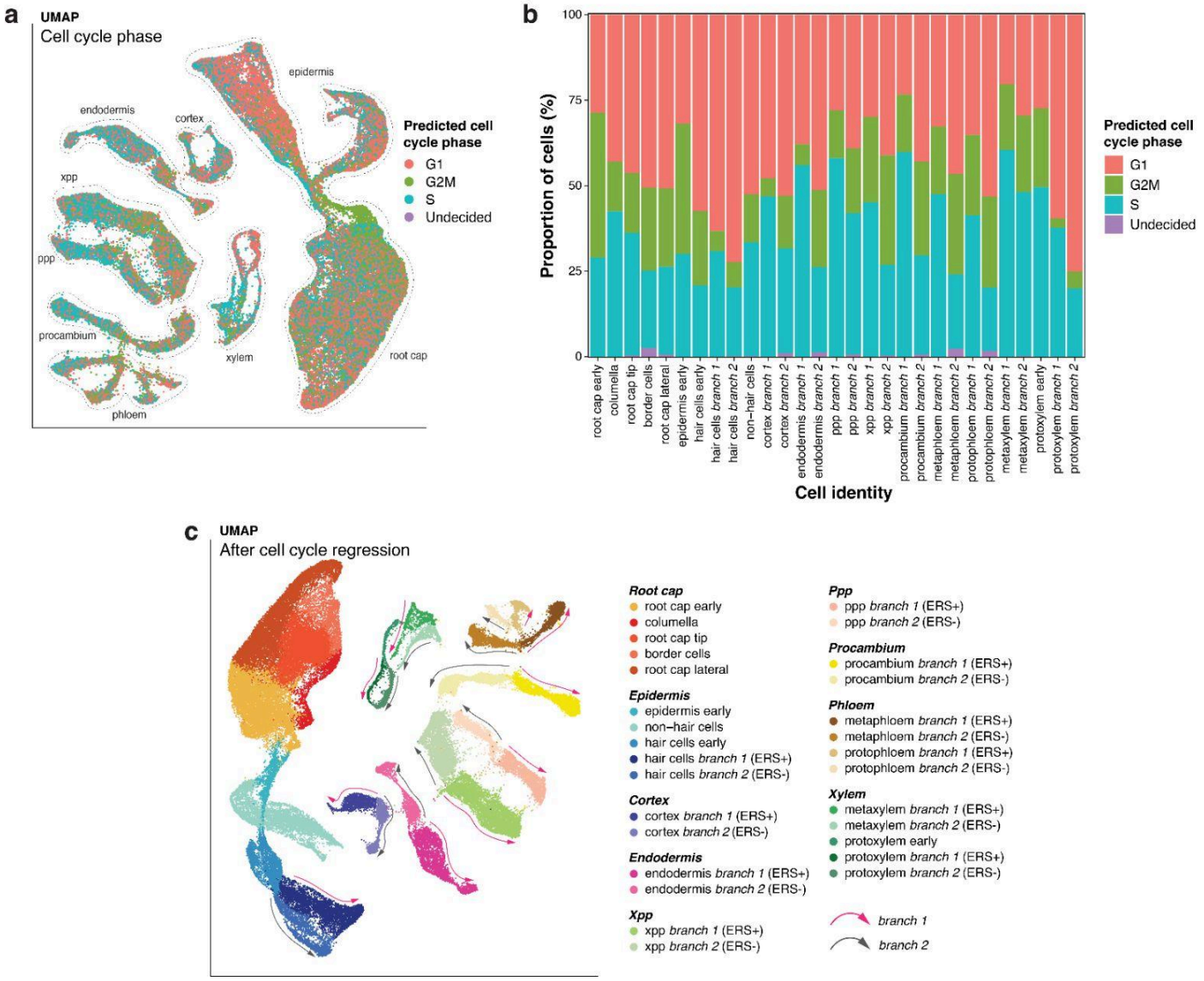
Resp. Fig. 39 / Fig. 2e: HCR validation of expression of multiple ERS-specific genes only in a subset of cells, of multiple cell types. UMAP plots and optical sections showing transcript detection of four 'branch 1' markers, *AT2G41660* (*MIZ1*), *AT3G04010*, *AT4G26080* (*ABI1*), and *AT5G56550* (*OXS3*), both *in silico* from scRNA-seq data and *in vivo* by whole-mount *in situ* Hybridization Chain Reaction (HCR). Yellow lines indicate the relative positions of longitudinal and transverse cross sections. Example cells that are positive (+) or negative (–) for each transcript in the epidermis (ep.), cortex (co.), or endodermis (en.) are indicated with arrows. Scale bar = 50µm. Also see **Ext. Data. Fig. 17** that shows the position of these longitudinal images in the context of the root tip.



Resp. Fig. 40 / Ext. Data Fig. 18: HCR validation of *VDOF1* expression. Left, UMAP plot showing the 'branch 1'-specific expression of *VDOF1 in silico*. **Right,** Longitudinal optical section showing the expression of *VDOF1* by whole-mount HCR. Cells enriched (+) or not (-) for VDOF1 transcripts *in vivo* in the endodermis (en.) or pericycle (pe.) are indicated by arrows. Scale bar = 50µm.

- Coloring the UMAP in Fig.1d by the same features above plus cell cycle phases.

Response: Cell cycle phase analysis and colouring of the UMAP was originally presented in Fig. S3 and is now in **Resp. Fig. 41 / Ext. Data Fig. 6**. We show that the ERS+ and ERS- populations contain approximately the same proportion of cells in each cell cycle phase. Importantly, we demonstrate that the two populations remain clearly distinguished and separated after cell cycle regression (**Resp. Fig. 41c / Ext. Data Fig. 6c**), indicating that they are not artefacts of cell cycle progression. Additionally, the branching event occurs at a developmental stage where root cells have already differentiated and are not expected to undergo further cell division.



Resp. Fig. 41 / Ext. Data Fig. 6: Branches in the trajectories are not driven by the cell cycle.
a, UMAP plot showing the predicted cell cycle stage of cells in the reference atlas from this study. **b,** Bar plot showing the proportion of cells in each stage per cluster. **c,** UMAP plot of the reference atlas after regressing out cell cycle effects, showing that the branches are maintained.

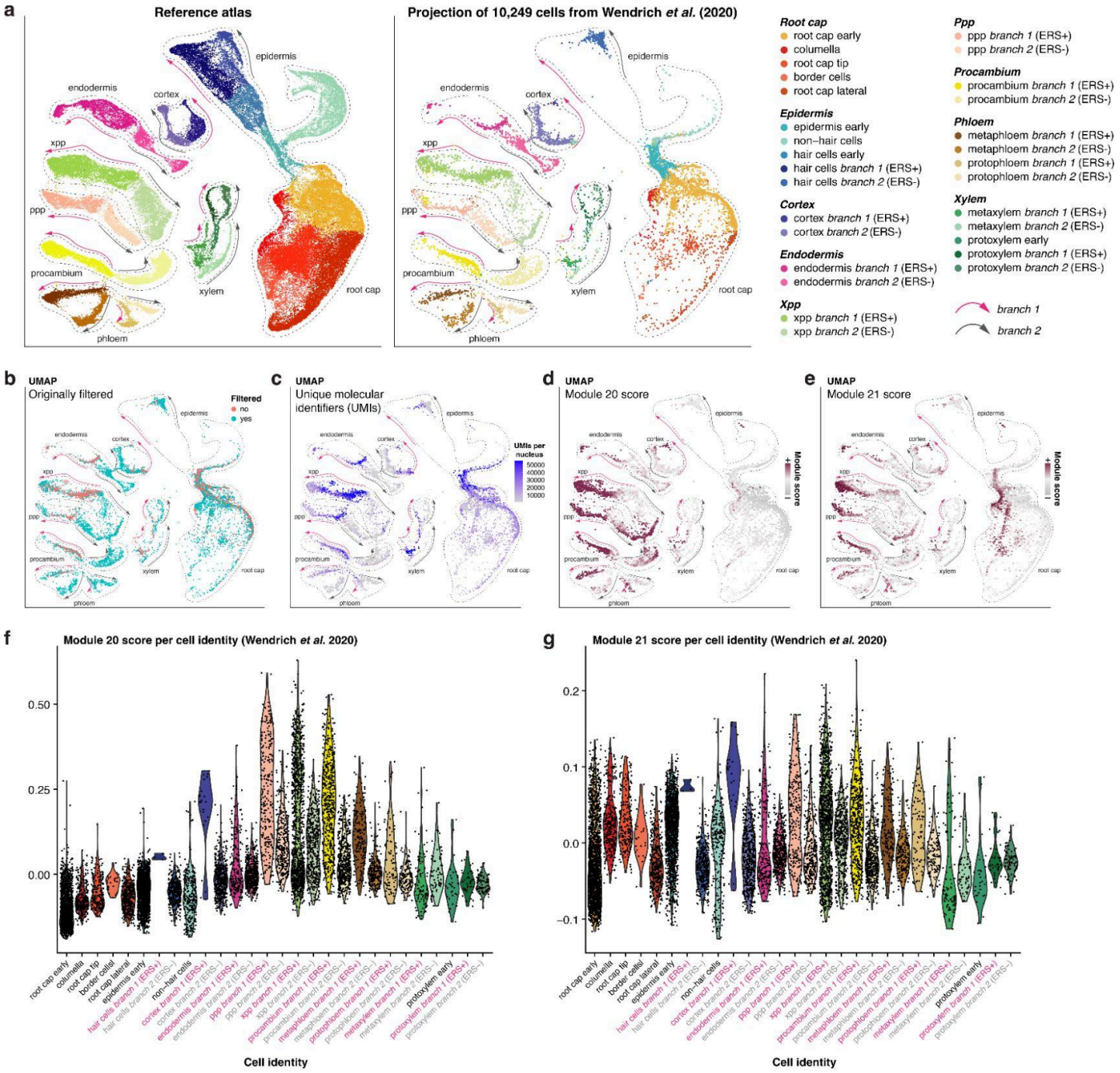
- Instead of presenting the cell label transferring result, could the authors provide the integration results (i.e. UMAP with co-embedding) of the current study with the published datasets? Can the published datasets be completely analyzed separately with the author's analysis pipeline to show that the two branches can be identified in published datasets?

Response: Thank you for the suggestion. We have refined our comparison to other studies by focusing on three representative datasets and performing a more detailed analysis. This is also detailed in our responses to **Referee 1 - point 2** and **Referee 2 - point 5**, above. Our new analyses now include co-embedding their data into our UMAP, except for Shahan *et al*., which is a very large dataset where the branching is already clearly visible in their published UMAP, although it was not explored deeply in their study. In all three comparative analyses (Farmer *et al*: **Resp. Fig. 31 / Ext. Data Fig. 12**; Wendrich *et al*: **Resp. Fig. 42 / Ext. Data Fig. 13**; Shahan *et al*: **Resp. Fig. 43 / Ext. Data Fig. 14**), we show that:

(1) Both ERS+ and ERS− populations/branches are identified across the multiple cell types,
(2) The ERS+ and ERS− consistently display differences in UMI content (even in the single nucleus RNA-seq datasets in Farmer *et al*.[2], and
(3) ERS+ cells are enriched in the same co-expression modules (Modules 20 and 21), which include ABA-responsive genes.

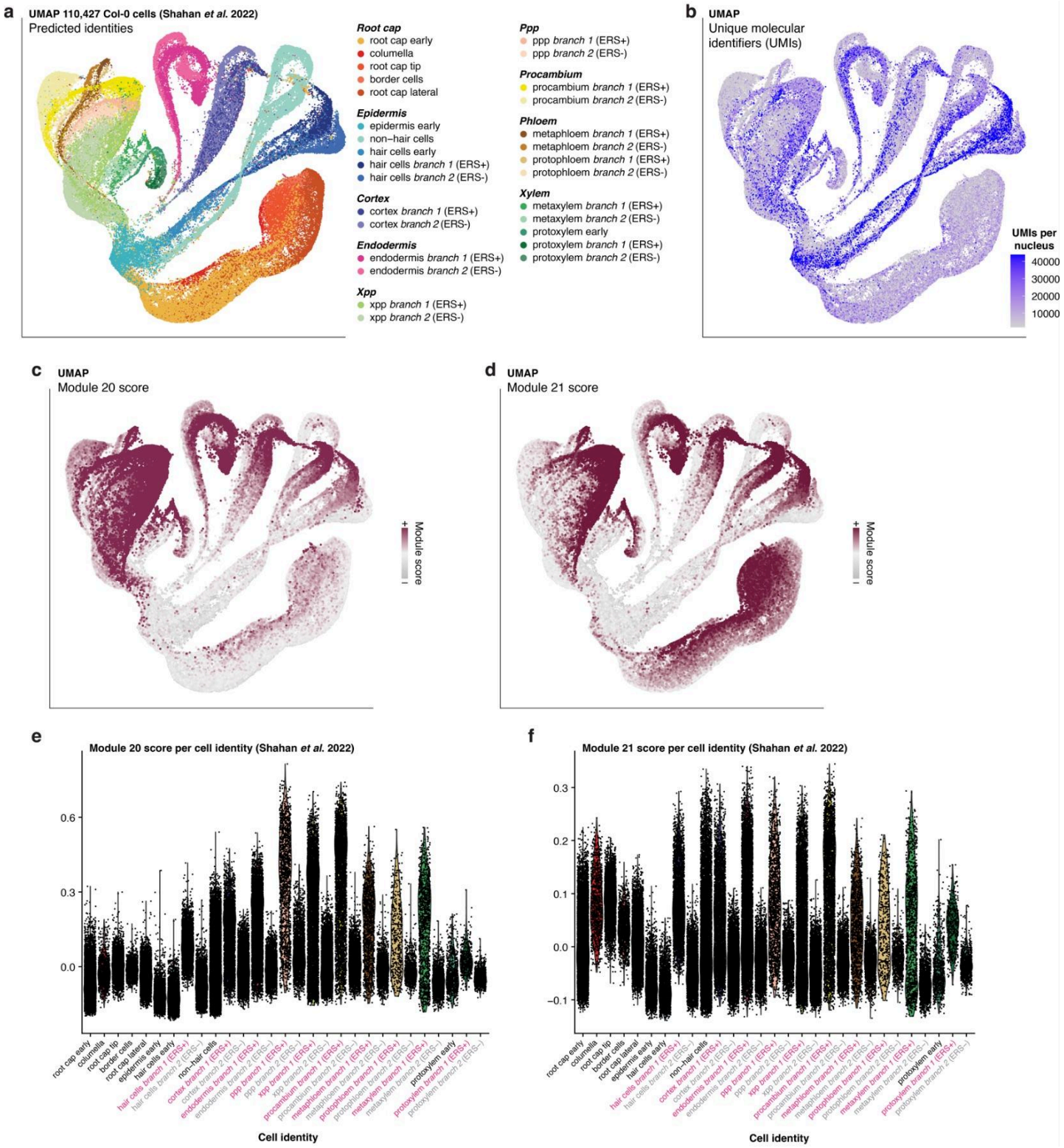
It is important to note that in the Wendrich *et al*. study[1], the authors applied a hard UMI threshold to filter out cells classified as damaged or background, which was common practice in the field at the time. However, we show that this filtering step discarded many ERS− cells, making it difficult to detect the two populations. When we re-processed the raw data using our pipeline, which more carefully discriminates real cells from damaged

cells or background (see response to **Referee 2 - point 7**), we recovered a much larger number of cells and were able to identify both ERS− and ERS+ populations across lineages.



Resp. Fig. 42 / Ext. Data Fig. 13: Branches and their associated ERS-specific genes are detected in the scRNA-seq dataset from Wendrich *et al.*

a, UMAPs showing our reference atlas and the projection of cells from Wendrich *et al.* into the atlas UMAP embedding, with cells coloured by their assigned identity based on our atlas. **b,** UMAP showing whether cells were originally discarded ('filtered') in their study based on UMI content. **c,** UMAP showing the distribution of UMI content per cell. **d–g,** UMAPs (d, e) and violin plots (f, g) showing the distribution of expression scores for our 'branch 1'-specific modules 20 and 21 in their scRNA-seq data.



Resp. Fig. 43 / Ext. Data Fig. 14: Branches and their associated 'branch 1'-specific genes are detected in the scRNA-seq dataset from Shahan *et al.*

a, UMAPs showing the reference atlas embedding from Shahan *et al.*, with cells coloured by their assigned identity based on our atlas. **b,** UMAP showing the distribution of UMI content per cell in their atlas. **c–f,** UMAPs (c,d) and violin plots (e,f) showing the distribution of expression scores for our 'branch 1'-specific modules 20 and 21 in their scRNA-seq data.

Referee 3 - point 3: Similarly, it seems like the paper primarily focuses on "branch 1" (the ERS+ branch). Since "branch 2" (ERS- branch) seems like it could potentially be artefactual, the paper would be more convincing if branch 2 was explored in more depth throughout the paper.

- For example, when validating the branches in XPP cells using whole-mount HCR (Extended Data Figure 4C), the authors look at two marker genes, an earlier XPP marker gene that should be expressed in all XPP cells,

and a marker gene that should be specific to branch 1 at a later time point. As expected the earlier time point gene is expressed in all XPP cells while the branch 1 later time point gene is not expressed in all cells, which the authors conclude "(...) confirms the branching nature of these trajectories over development, which reveals two previously unidentified in vivo alternate cellular states". While this seems to confirm an earlier and a later cell state (which I believe was previously known), it doesn't seem to compelling prove there are two branches (ERS+ and ERS-). Showing (via HCR if possible) that a subset of cells express a branch 2 marker gene that is not expressed in cells that express a branch 1 marker gene would be much more convincing.

Response: It would indeed be ideal to visualize markers for both ERS+ and ERS− populations simultaneously to validate the existence of these two populations *in vivo*. However, we have not been able to identify genes that are specific to the ERS− population. Our interpretation of these two states is that all cells (ERS+ and ERS−) share a core set of cell type–specific genes defining their lineage, but only a subset of cells additionally express a specific set of genes (ERS+), while others do not (ERS−). Thus, there are genes common to both ERS+ and ERS− cells, and additional genes expressed exclusively in ERS+ cells, but not the reverse.

Therefore, since we cannot rely on complementary markers to label both populations, we have used other strategies to rule out the possibility that the ERS− population is artifactual:

(1) We have expanded the number of ERS-specific genes profiled by HCR (5 marker genes total). In all cases, we clearly observe cells expressing these genes and other cells not expressing them, consistent with the existence of two distinct populations (see **Resp. Fig. 39 / Fig. 2e** and **Resp. Fig. 40 / Ext. Data. Fig. 18**).

(2) It could be hypothesized that ERS− cells are low-quality cells that burst during processing, leading to lower UMI content and dropout of lowly expressed genes. To address this, in the revised manuscript we show that:

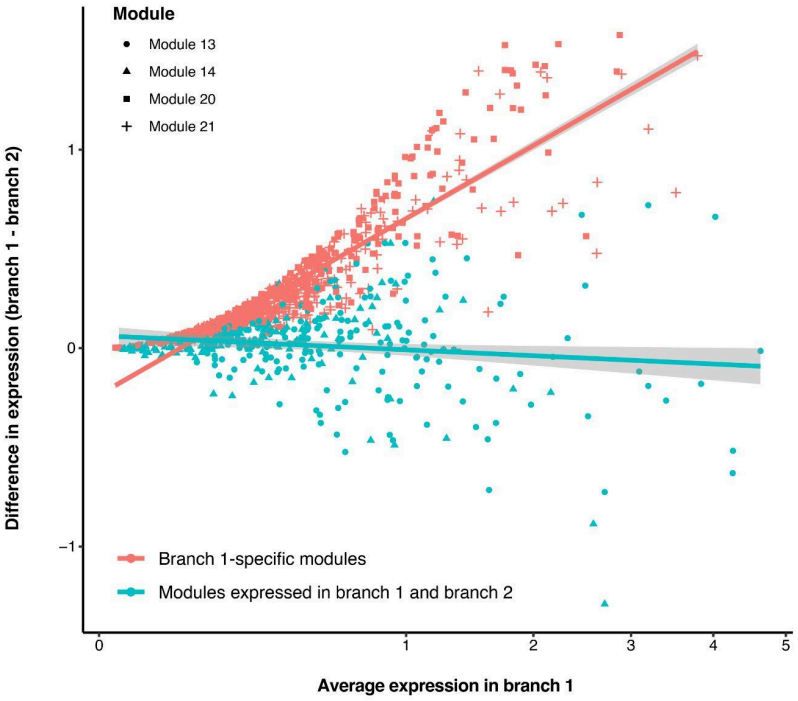
  (a) ERS-specific genes are not globally lowly expressed compared to genes expressed in both ERS+ and ERS− cells (**Resp. Fig. 44 / Ext. Data Fig. 10**).

  (b) Both ERS+ and ERS− populations are also detected in independent snRNA-seq datasets[2], displaying the same co-expression modules and inter-branch differences in UMI abundance, which rules out artefacts related to profiling whole cells versus nuclei (see response to **Referee 3 - suggestion 1**, and **Resp. Fig. 31 / Ext. Data Fig. 12**).

(3) Finally, the proportion of ERS+ (and consequently ERS−) cells varies significantly and reproducibly in response to perturbation of ABA signalling pathways (**Resp. Fig. 34 / Fig. 3**) and changes in growth conditions (**Resp. Fig. 35 / Fig. 4**). These environment-associated changes are now also validated experimentally *in vivo* by HCR using ERS-specific genes, while all processing steps remain identical, further supporting the biological significance of both ERS+ and ERS− populations (**Resp. Fig. 35f,g / Fig. 4f,g**).

Resp. Fig. 44 / Ext. Data Fig. 10: Differences in gene content between 'branch 1' and 'branch 2' are not driven by lowly expressed genes.

Scatter plot showing, for the xylem pole pericycle, the average expression in 'branch 1' versus the difference in average expression between 'branch 1' and 'branch 2' for genes from two categories of co-expression modules: those that are 'branch 1'-specific (Modules 20 and 21) and those symmetrically expressed in both branches (Modules 13 and 14). Both categories of co-expressed genes span the same range of expression levels, indicating that the 'branch 1'-specific genes are not simply lowly expressed genes detected in cells with higher UMI content.



- Are there ERS- marker genes, and if so, what are the GO-terms associated with those?

Response: There are no genes specifically enriched in the ERS− population compared to ERS+. Our interpretation is that all cells (ERS+ and ERS−) share a core set of lineage-defining, cell type–specific genes, but only a subset (ERS+) additionally expresses a distinct set of genes, while others (ERS−) do not. In other words, ERS− cells do not exhibit a unique transcriptional program relative to ERS+ cells; rather, ERS+ cells express an additional gene module not found in ERS− cells.

We propose that this may reflect a coordinated root response involving multiple cell types, but not all cells within each type. Although root tissues are typically organised into concentric layers of uniform cell types, these layers communicate with one another, and this communication likely varies locally depending on physiological needs. For example, the flux of water and nutrients between the outer sensing and uptake layers and the central vasculature involves multiple cell types, but probably does not engage all cells equally, and is likely modulated by environmental conditions such as water or nutrient availability.

The expression of ERS genes in only a subset of cells - and their modulation in response to environmental cues - could therefore either be required in specific cells involved in such processes or, alternatively, result from intercellular signalling triggered by local physiological demands.

- The authors show that there are some DEGs between the two nutrient conditions tested (figure 5), and that 20% of those DEGs are enriched in branch 1 (ERS+) cells – are any DEGs specific to branch 2 and not branch 1 (hard to tell by just looking at extended data figure 10C)? More broadly put, apart from changes in the ratio of ERS+ to ERS- cells, are ERS- cells truly unresponsive to changes in the environment?

Response: Instead of performing a DEG analysis between conditions and intersecting it with other components of the study (such as gene modules), in the revised manuscript we now assess changes in differential module scores to capture global variations across the 25 expression modules detailed in **Fig. 2**, in order to directly examine the co-regulated groups of genes we identified as most interesting. We observe that the most significantly affected modules are the ERS-specific Modules 20 and 21, consistent with the decrease in the ERS+ population in the half MS condition (**Resp. Fig. 35a,c / Fig. 4a,c**). However, we also detected changes in other modules that are expressed in both branches, indicating that ERS− cells can also undergo transcriptional changes in response to environmental cues. ERS− does not mean that these cells are unresponsive to the environment; rather, it means they have not acquired the specific transcriptional state that we define as “environmentally responsive” (ERS+), which is a shared gene expression signature detected in a subset of cells from multiple distinct cell types.

- I could be misunderstanding (or naive on my part), but it seems perhaps slightly counterintuitive that the cells that are capable of responding to the environment (ERS+ cells) would be depleted in an environmental stress condition?

Response: Thank you for this comment. We agree that the way we presented the experimental design and results in the original manuscript could indeed seem counterintuitive, and have revised the manuscript to clarify this point. When comparing full MS (1x) and half MS (0.5x), we initially described these media in terms of nutrient availability, which might suggest that half MS represents a stressful condition due to reduced nutrient content. However, both media are commonly used for standard seedling growth, and no obvious stress phenotype is observed under half MS. Full MS is a rich medium with a relatively high concentration of macronutrient and micronutrient salts, which can create an elevated osmotic strength environment. Half MS is very widely used and effective for Arabidopsis growth on agar, and has been suggested to better represent soil conditions. Therefore, we do not consider half MS as representing a nutrient limited condition, but rather simply relatively lower macronutrient and micronutrient levels than the full MS condition, which may also subject the plants to lower osmolarity than in full MS. Thus, nutrient availability is unlikely to be limiting or perceived as a strong stress by the plant. Therefore, we may interpret the enrichment of ERS+ cells in the full MS condition as an adaptation to the elevated nutrient/salt levels or osmolarity, both of which are known to induce ABA accumulation and signalling. The elevated nutrient/salt levels or the higher osmolarity of full MS might be slightly more demanding for water uptake and therefore perceived as relatively more challenging by the plant, requiring a higher proportion of ERS+ cells that exhibit expression of an additional set of genes.

In support of this, when we drastically reduce the water potential using the “hard agar” drought model - a much stronger stress than full MS - we observe a clear increase in the proportion of ERS+ cells (see responses to **Referee 3 - point 1** and **Referee suggestion 3**, above). This suggests that the differences observed between full and half MS may reflect variation in water potential rather than nutrient limitation per se.

We have revised the manuscript to use “nutrient availability” and “stress” more cautiously and to better convey that the plant modulates the proportion of cells acquiring this state in response to growth parameters, including, in more extreme cases, drought stress.

Referee 3 - point 4: In the published datasets, leveraging the cell-type identities transferring strategy, the authors could identify the similar two branches. I am wondering if they have performed the different expressed gene tests and GO analysis, and could the similar gene modules corresponding to environmental responses be identified as well? Also, if the two branches also existed in the previous datasets, what is the possible reason that they have not been identified?

Response: Thank you for the suggestion. We have now used a projection of our co-expression modules, as enabled by the hdWGCNA package, to show that similar gene modules are specifically enriched in the ERS+ population detected in other datasets (Farmer *et al*: **Resp. Fig. 31 / Ext. Data Fig. 12**; Wendrich *et al*: **Resp. Fig. 42 / Ext. Data Fig. 13**; Shahan *et al*: **Resp. Fig. 43 / Ext. Data Fig. 14**).

We focused our comparison analysis on three different datasets, and the reasons why this branching event has not been further explored vary between studies:

- In Wendrich *et al*.[1], the authors applied a hard UMI threshold to distinguish cells from damaged cells or background, discarding all data below this cutoff. While this was a common practice in early single-cell RNA-seq studies, it removed a substantial proportion of ERS− cells, making it difficult to detect the presence of the two populations. When we re-processed this dataset using our pipeline, which more carefully discriminates real cells from damaged cells or background (see response to Referee 2 - point 7), we recovered many more cells and were able to identify both ERS− and ERS+ populations across lineages (**Resp. Fig. 42 / Ext. Data Fig. 13**). Although our detailed comparison focused on this study, other datasets also applied strict filtering criteria that may have similarly led to the loss of biologically relevant information.
- In Farmer *et al*.[2], the use of isolated nuclei (rather than whole cells) from chopped root tips likely reduced the representation of nuclei from early developmental stages. This may have limited their ability to capture the branching event in the data. Importantly, as detailed in response to point 3, above, our new analyses of their snRNA-seq data in the revised manuscript clearly identifies both ERS+ and ERS− states and expected features (**Resp. Fig. 31 / Ext. Data Fig. 12**).
- In Shahan *et al*.[3], which currently represents the largest Arabidopsis root single-cell atlas, the branching of lineages is visible in the UMAP (**Resp. Fig. 43 / Ext. Data Fig. 14**) and briefly mentioned in the main text and a supplementary figure, but it was not explored further.

The identification of the ERS and its associated branching event in our study was enabled by several key aspects of our analytical approach: (1) a more careful strategy for filtering cells, which avoided excluding potentially informative populations (see further details in response to Referee 2 - point 7); (2) the use of Partition-based Graph Abstraction (PAGA) trajectory analysis, which is particularly well suited for robustly capturing branching events; and (3) the identification of gene co-expression modules across the entire dataset, which was crucial for describing the ERS as a shared transcriptional state across a subpopulation of cells in multiple lineages, rather than only focusing on differential expression along pseudotime within each lineage independently, as in previous studies.

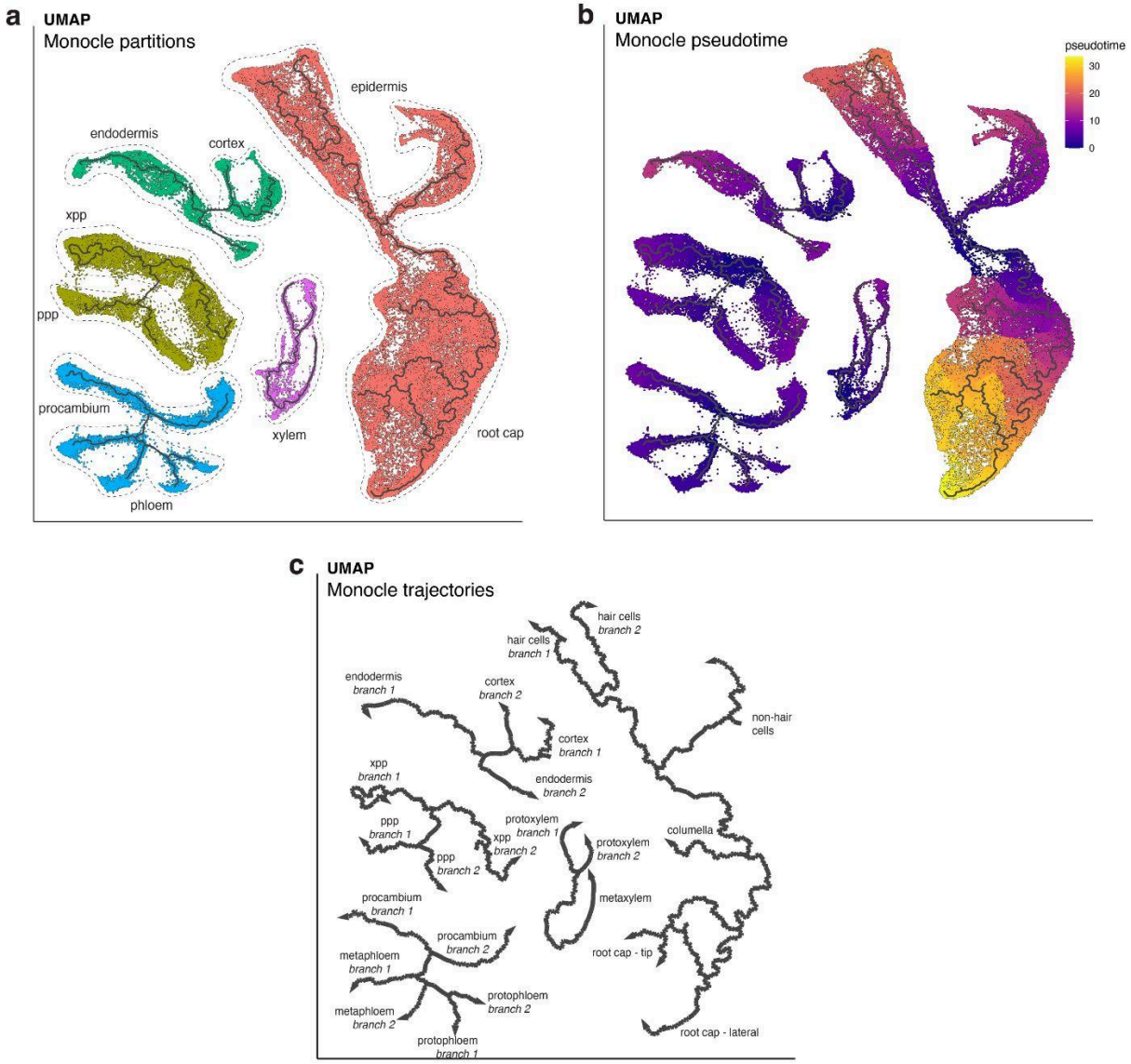
Referee 3 - point 5: Could the authors repeat the trajectory/pseudotime analysis (Fig. 1f) using some additional methods? For example, Monocle or RNA velocity based methods? Showing that these two branches can be seen when the data is analyzed with a different method (e.g. Monocle), and showing that the two branches can be found in the published datasets when those data are analyzed independently (i.e. not using cell label transferring alone) would show that these findings/branches are robust.

Response: We initially used the PAGA method from the Scanpy package because it is particularly well suited for detecting complex lineage trajectories, including convergence and divergence events, which we expected to observe in the root cap trajectory. In response to the referee's suggestion, we have now included an alternative trajectory analysis using Monocle (**Resp. Fig. 45 / Ext. Data Fig. 5**). Unlike our original approach in which we manually subset the dataset by lineage to define trajectories, Monocle automatically subdivides the data into partitions, computing independent trajectories within each. We ran Monocle on the full dataset and let it assign partitions unbiasedly (**Resp. Fig. 45a / Ext. Data Fig. 5a**).

Monocle grouped some related lineages together (e.g., endodermis with cortex, xylem pole pericycle with phloem pole pericycle, and procambium with phloem) and computed trajectories accordingly. Importantly, in all lineages where we previously observed the ERS+/ERS− branching event using PAGA (from hair cells in the epidermis to all inner layers), Monocle also independently detected the same branching event (**Resp. Fig. 45a-c / Ext. Data Fig. 5a-c**).

Interestingly, in cases where multiple lineages were grouped into the same partition, Monocle consistently connected them near the position we had previously defined as t=0. This convergence further supports the robustness of our t=0 assignment as representing early developmental stages shared among lineages, prior to their divergence into mature identities (**Resp. Fig. 45b / Ext. Data Fig. 5b**).

As mentioned in previous responses, we have also conducted a more thorough analysis of three published datasets. In all cases, we could identify the two branches, and the ERS+ population is characterized by the same gene modules as in our dataset.



Resp. Fig. 45 / Ext. Data Fig. 5: Monocle trajectory analysis validates the dichotomy in cell lineages. **a,** UMAP plot showing the partitions defined by Monocle, for which independent trajectory analyses were computed. Note that endodermis and cortex; xylem and phloem pole pericycle; and procambium and phloem are grouped within the same partition. **b,** UMAP plot showing the pseudotime ordering obtained with Monocle. **c,** UMAP plot showing the resulting trajectories from the Monocle analysis, recovering the same two branches identified in every cell type with the PAGA trajectory analysis.

Referee 3 - point 6: The starting point assigning strategy (Fig.1e & f) seems purely based on the computational strategies, is it possible to provide some experimental validations? For example, in the trajectory of XPP, the starting point could also possibly be the bottom rather than the middle. Again, the HCR results shown in Ext Fig. 4c only suggests the middle region is less differentiated relative to the top – it's necessary to provide more evidence about the bottom region.

Response: We thank the referee for raising this important point. In the revised manuscript, we now include additional experimental validations to support our pseudotime ordering and the assignment of *t = 0* in the trajectories. Specifically, we performed HCR on three different lineages (epidermis, endodermis, and xylem pole pericycle) using one gene expressed close to *t = 0* and another expressed at later pseudotime stages, based on our scRNA-seq data. In all three cases, we observed that the early-stage gene is expressed in cells near the root tip, while the late-stage gene is expressed further up the root, where cells are more mature (**Resp. Fig. 46 / Ext. Data Fig. 3**). This spatial pattern is consistent with the expected developmental progression and supports the pseudotime orientation.

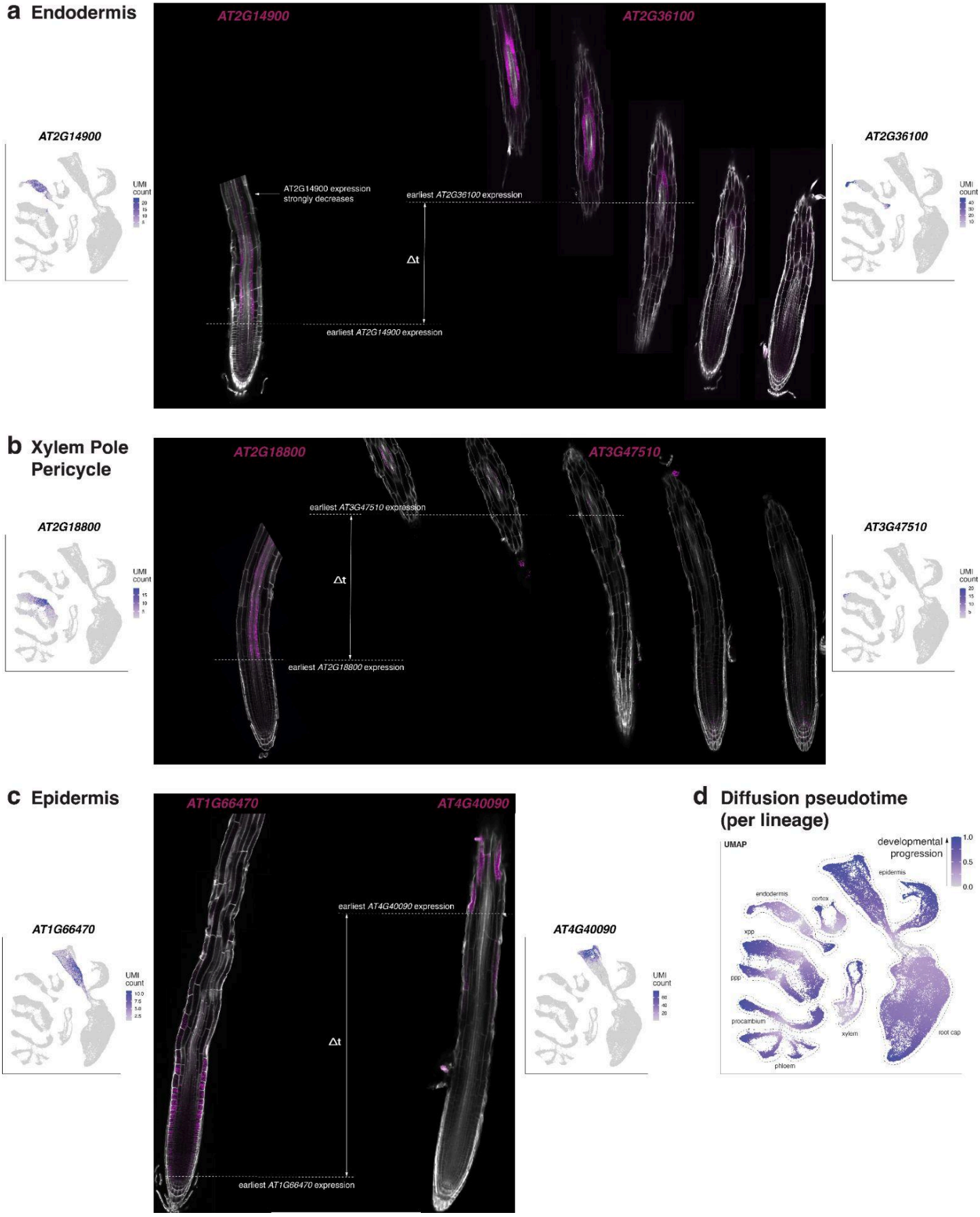
It is worth noting, however, that in the epidermis lineage, the branching between ERS+ and ERS−

populations occurs downstream of the assigned *t = 0* in the trajectory, whereas for other lineages the branching occurs essentially from the detected *t = 0* in the scRNA-seq data. This may be due to the fact that it is easier to release and capture these rare transient cell states in the outer layers of the root than in the less accessible internal layers during protoplasting. Consequently, some markers that are positioned close to *t = 0* in the scRNA-seq data may appear slightly further up in the root *in vivo*.

To further validate our *t = 0* assignment, we examined the expression of gene sets defined by Shahan *et al*.[3] as markers of the meristem, elongation, and maturation zones. We found that genes specific to the meristem zone are enriched at the beginning of our pseudotime trajectories, while elongation and maturation markers become enriched progressively later (**Resp. Fig. 47 / Ext. Data Fig. 4**), in agreement with developmental timing (see also our response to **Referee 1, point 5**).

Finally, in our Monocle-based trajectory analysis, we found that when multiple lineages are grouped into a single partition, they consistently connect near our assigned *t = 0* (**Resp. Fig. 45a-c / Ext. Data Fig. 5a-c**). This convergence across independent methods further supports that *t = 0* corresponds to an early, undifferentiated stage from which lineages diverge (see response to **Referee 3, point 5**).

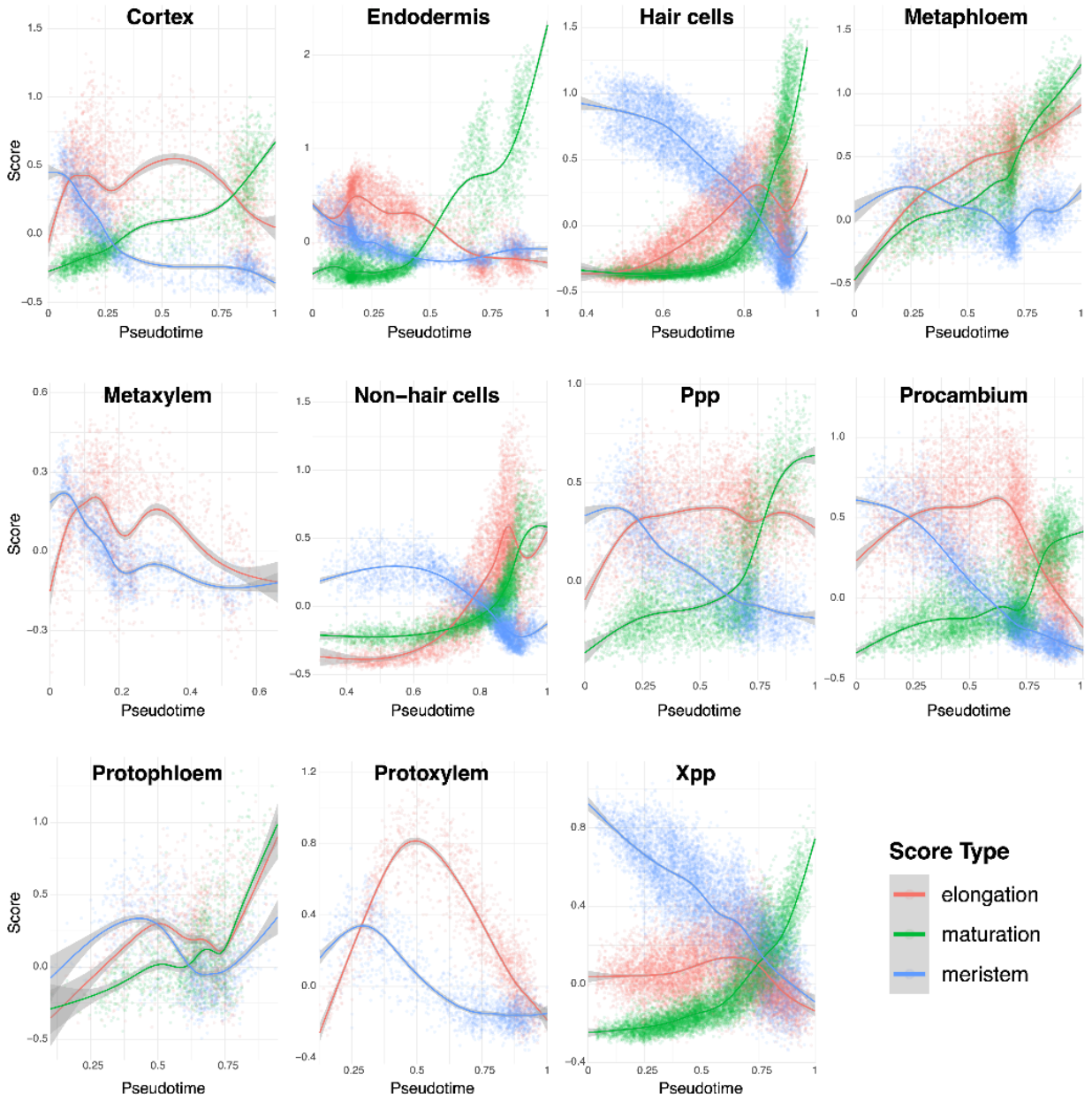
Taken together, these results provide strong support for the robustness and biological validity of our *t = 0* assignment in the pseudotime analyses.



Resp. Fig. 46 / Ext. Data Fig. 3: Validation of pseudotime assignments

a, UMAP plots and longitudinal optical sections of whole-mount HCR showing the expression of two endodermal markers, *AT2G14900* and *AT2G36100*, *in silico* and *in vivo*. *AT2G14900*, which is predicted to be expressed early according to the trajectory analysis (see pseudotime assignment in d), is detected closer to the meristem, whereas *AT2G36100*, a later marker, is detected further away, validating the pseudotime assignment. Multiple optical sections are shown when a complete longitudinal section of the root could not be

obtained at once due to minor root curvature during mounting. Dotted lines indicate the earliest developmental stage at which expression of each marker was detected. **b,** Similarly, in the xylem pole pericycle, *AT2G18800* (early marker) is detected nearer to the meristem and *AT3G47510* (late marker) is detected further away, consistent with the trajectory analysis. **c,** The trajectories for epidermal hair cells are also validated: *AT1G66470* (early marker) and *AT4G40090* (late marker) show concordant expression patterns *in silico* and *in vivo*. d, UMAP plot summarizing the pseudotime assignment per lineage in our reference atlas.



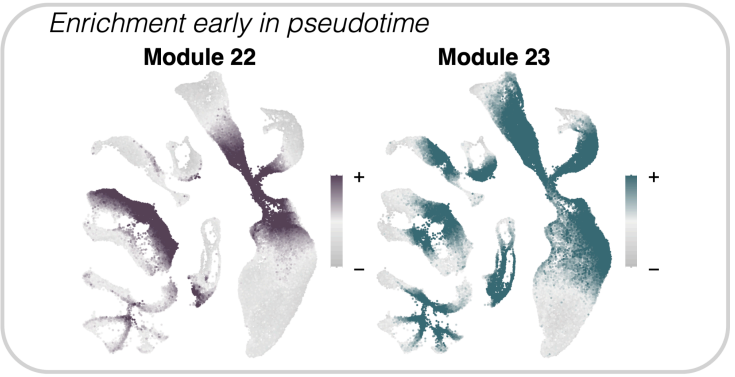
Resp. Fig. 47 / Ext. Data Fig. 4: Validation of trajectories using cell type- and developmental stage-specific markers from Shahan *et al.*
Scatter plots and regression lines showing the distribution of expression scores from our Col-0 scRNA-seq atlas for the top 50 genes specific to the meristematic, elongation, and maturation zones defined by Shahan *et al.*, plotted as a function of our pseudotime assignment for each cell type. Successive peaks of meristematic, elongation, and maturation scores are observed along the pseudotime axis, validating our trajectory analyses.

Referee 3 - point 7: As the remarkable difference of UMI counts between two branches (Ext Fig.5b & c), I am wondering how the authors convince the differential expressed genes between them are real rather than due to some background differences? Similarly, the high/low UMIs (Ext Fig.5b & c) between two branches might affect the strategy of finding the starting point. All the starting points seem to exist at the boundary between cells with different UMI counts.

The downsampling analysis alone doesn't seem convincing enough because genes that are never detected in branch 2 because it has too few UMIs might still be detected and called as "differentially expressed" in the branch 1 even after it has been downsampled?

Response: Thank you for raising this important point. It could indeed be hypothesised that the genes specific to the ERS+ population correspond to lowly expressed genes that are simply not detected in ERS− cells due to their lower UMI content, and that the downsampling approach might still propagate this artefact. To address this, we have verified that ERS-specific genes are not globally lowly expressed compared to genes expressed in both ERS+ and ERS− cells (**Resp. Fig. 44 / Ext. Data Fig. 10**).

Regarding the concern that the starting point is being identified at the boundary between two populations with different UMI contents: while it is true that some genes are detected only in one of the two populations, we also find genes expressed at similar levels in both branches, particularly at early stages, consistent with the expression pattern expected for meristematic genes. In our co-expression module analysis, we identified two of the large modules (Modules 22 and 23, **Resp. Fig. 48 / Fig. 2b**) that are enriched early in pseudotime across all lineages. This further supports the idea that, despite UMI and gene content differences, the early stages of all trajectories share a similar transcriptional signature.



Resp. Fig. 48 / Fig. 2b: UMAP of root tip scRNA-seq atlas showing module score for Modules 22 and 23, enriched early in pseudotime across all lineages.

As described above, we believe that early cells from inner root layers are less efficiently sampled due to their reduced accessibility during protoplasting, whereas in the outer layers like the epidermis, we do detect early-stage (meristematic) cells well. In this lineage specifically, we observe a *t = 0* region that clearly precedes the ERS+/− branching event, and this early region shares many genes with the region where *t = 0* is assigned in other lineages, near the ERS+/− boundary.

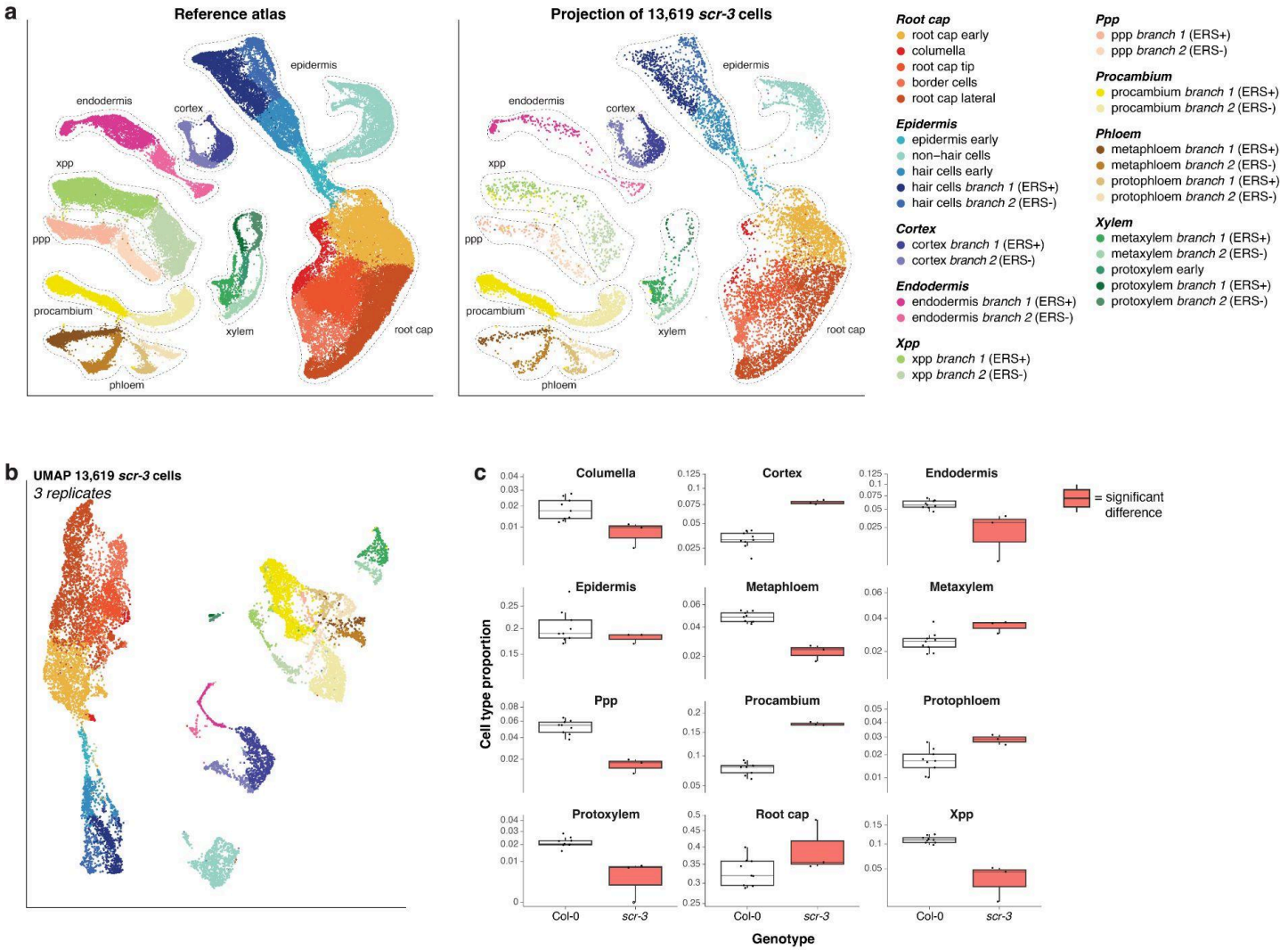
As detailed in our previous response (Referee 3 - point 6), we show that our t=0 assignment correlates with that of Shahan *et al*., where t=0 cells are connected on the UMAP and the two branches are clearly visible on the developmental trajectories. In addition, we provide experimental validation supporting our *t = 0* assignment.

Importantly, in our new drought model experiment, we observe a substantial increase in the ERS+ population through the scRNA-seq analyses, which is further validated *in vivo* by a large increase in the number of cells where ERS-specific transcripts are detected. This strongly supports that the ERS+ and ERS− populations reflect a true biological phenomenon rather than a technical artefact (see response to **Referee 3 - suggestion 3 (B-C)** and **Resp. Fig. 35d-g / Fig. 4d-g**).

We are therefore confident that the observed differences in gene expression between branches are not due to background variation, as they are systematically detected across datasets, as well as in snATAC-seq data, involve the same sets of genes and pathways, and can be modulated either by affecting the relevant pathways or by exposing roots to different conditions. Such functional validations would not be consistent with a background signal.

Referee 3 - point 8: Could the authors provide more replicates for scr-3 ecotypes? Otherwise, the cell composition analysis results are not fully convincing (Fig.3b).

Response: We have now three different replicates for *scr-3* that support the changes in cell composition described in the manuscript (**Resp. Fig. 49 / Ext. Data Fig. 20**).

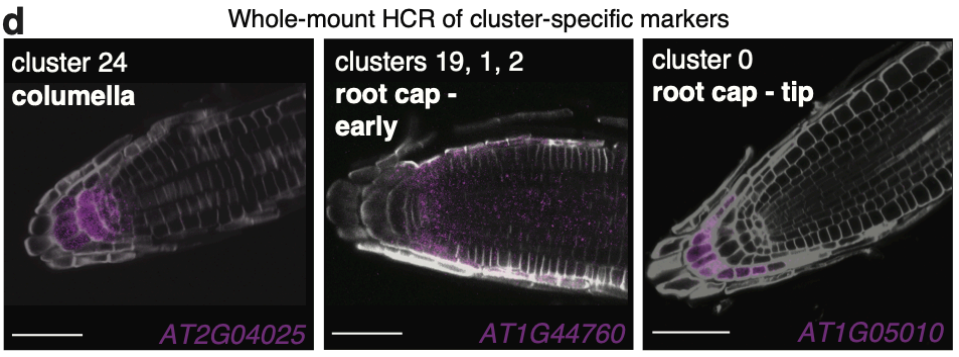


Resp. Fig. 49 / Ext. Data Fig. 20: The ERS is maintained upon developmental perturbations of cell identities in *scr-3*

a, UMAP plots showing our reference atlas and the projection of cells from *scr-3* replicates into the atlas UMAP embedding, with cells coloured by their assigned identity based on our atlas. **b,** *scr-3* cells atlas shown in their atlas UMAP embedding **c,** Box plots showing the distribution of cell types in our reference Col-0 atlas and in the *scr-3* mutant. Each dot represents an individual replicate. Tests showing a significant difference in cell type proportion compared to Col-0 are highlighted in pink (sccomp, FDR<0.05).

Referee 3 - point 9: Clusters 16 & 28 are not well annotated, which raises the possibility that they are not really distinct cell types, but rather under specific cell states or even low quality cells, for example, as shown in Ext Fig.3a, the cluster 28 is more likely driven by different cell-cycle phases.

Response: We have redefined the clusters and identities in our new integrated atlas. The population at the start of the root cap lineages that corresponds to actively dividing cells have been merged with the other "root cap - early" cluster (**Resp. Fig. 50 / Fig. 1d**). Please also see our response to **Referee 1 - point 5** for further discussion of these clusters and our improved annotation of them in the revised manuscript.



Resp. Fig. 50 / Fig. 1d *(part)*: HCR of cluster-specific markers.

Specific comments:

Referee 3 - point 10: In Fig.1b, there is no further discussion on the difference between clusters within each cell type (for example, both cluster 8 and 12 are non-hair cells), why not just merge those clusters and then label them by cell type names?
Response: In Fig. 1b, we show the cluster resolution required to identify all cell types by HCR. Subsequently, clusters corresponding to the same cell type were merged according to their developmental trajectories, as illustrated in Fig. 2a.

Referee 3 - point 11: In Fig.1b, a small cell population in green color (I guess the cells belong to cluster 14) is connected to cluster 13 on the left side, any comments on it?
Response: We do not know why some cells from the metaxylem cluster were positioned closer to the procambium than others, although some similarity between these adjacent tissues is expected. This small population that appeared "detached" from the main metaxylem cluster does not appear in our revised version, so we have not explored it further.

Referee 3 - point 12: In Fig.1c, some of the plots seem represented in Ext Fig.1c. In addition, 15 genes are shown in this panel, rather than 19 cell-cluster-specific marker genes talked about in the paper, which is confusing.
Response: We apologize for the confusion. The panel shown in Fig. 1c indeed contains the 15 genes that were used to identify the different cell types by HCR. We have corrected the text in the manuscript to read: *"We adapted a whole-mount HCR method for Arabidopsis root tips and combined it with a clearing technique (see Methods) to precisely map the expression of the 15 most specific cluster markers of our dataset."*

Referee 3 - point 13: In Fig.1d & f, cells are colored by sub-clusters, however, some of those colors are not easy to distinguish, especially some of them are reused in Fig.1b.
Response: We apologise for the challenges in discrimination of come colours. In the revised manuscript we have attempted to adjust the hues to enable better discrimination between different colours, while retaining a palette appropriate for various colour vision impairments. Hopefully the additional labelling on these figure panels also assists in more straightforward cluster identification.

Referee 3 - point 14: In Ext Fig.3b-c, I tend to see how the UMAP with cells colored by cell cycle phase (like the panel a) looks like after cell-cycle regression, rather than labeled by cluster id.
Response: We chose to colour cells by identity after cell cycle regression in order to demonstrate clearly that this regression did not affect the separation and distinction between the two branches within each lineage. This confirms that the observed branching is not the result of cell cycle variation, as can be seen in **Resp. Fig. 41c / Ext. Data Fig. 6c**.

Referee 3 - point 15: I don't fully understand what the authors wanted to indicate by Ext Fig.5a. Again, instead of showing the cell type transferring result, it would be better to directly show the co-embedding of UMAP between the current study with the published datasets.
Response: In the revised manuscript, we no longer present the figure that was originally in Ext. Data Fig. 5a. Instead, as suggested, we now directly show the co-embedding of the data from the other studies into our UMAP (Farmer *et al*: **Resp. Fig. 31 / Ext. Data Fig. 12**; Wendrich *et al*: **Resp. Fig. 42 / Ext. Data Fig. 13**), except, as mentioned above, for Shahan *et al*., which is a very large dataset where the branching is already clearly visible in their published UMAP (**Resp. Fig. 43 / Ext. Data Fig. 14**).

Referee 3 - point 16: In Fig.3b, could the authors provide a box plot or bar plot instead, which I think would be more straightforward to see the cell composition changes?
Response: Thank you for this suggestion, which does improve the interpretability. In the revised manuscript we now present this analysis as a box plot (**Resp. Fig. 49c / Ext. Data Fig. 20c**).

Referee 3 - point 17: There appears to be a random letter "c" in the middle of the plot in extended data figure 10C.
Response: Thank you for pointing this out. We have reprocessed all figures and addressed this.

Referee 3 - point 18: Relatively minor, but personally didn't find the schematic in figure 5 to be super necessary in order to understand the experiment.
Response: We have removed this element from the Figure.

Referee 3 - point 19: Might be nice to include extended data figure 10a with Fig.5d only maybe split the two different nutrient conditions into two UMAPs so the reader can easily/qualitatively see how the proportion of cell types and the proportion of ERS+ to ERS- cells changes between the two conditions/UMAPs? There is a stark change in the number of hair cells that can be seen in the images in 5a – would be cool (though maybe not critical to the main findings of the paper) to recapitulate that with UMAP.
Or, could have two UMAPs for the two conditions and highlight cells by the expression of a branch 1 gene that has a strong change in expression in response to the nutrient condition?
Response: Thank you for the suggestion. In this revised version of the manuscript, the MS experiment was included in the reference atlas, so replicates for paired experiments comparing 1× MS and 0.5× MS were incorporated into the UMAP embedding. In **Resp. Fig. 35b / Fig. 4b**, we show the replicates from the 1× MS and 0.5× MS conditions separately. For some cell types, it is very clear from the UMAP that there is enrichment in the ERS+ trajectory under full MS, and consequently enrichment in the ERS– trajectory under half MS, as confirmed by the statistical analysis in **Resp. Fig. 36 / Ext. Data Fig. 22**.

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Point by point response to comments on Nature manuscript 2022-06-09447A

We extend our sincere thanks for the constructive feedback and time and effort in considering our manuscript. We believe we have addressed all comments and requested changes, which has helped us improve our study considerably. Below we provide a point-by-point response to all comments and suggestions.

Referee #1

Summary: The authors have strengthened the manuscript by performing orthogonal analyses to support the presence of two transcriptional branches, incorporating additional biological replicates, and testing the role of ABA in regulating the proportion of ERS^{+} cells. However, several aspects of the data interpretation and presentation would benefit from further clarification and additional controls to ensure that the conclusions are fully supported. In particular, clearer definitions of the ERS^{+}/ERS^{-} states, validation of HCR-based transcript detection, and improved visualization of comparative datasets would enhance the overall robustness and clarity of the study.

Response: We thank the referee for this constructive summary and for recognising the additional analyses incorporated in the revised manuscript. In response to these comments, we have revised the manuscript to clarify the conceptual framework underlying the ERS+/ERS− states and revising the text throughout to better explain that environmental conditions modulate the frequency with which cells acquire the ERS state, rather than defining intrinsically responsive versus unresponsive populations.

We have also expanded the orthogonal experimental validation of the ERS-associated heterogeneity by incorporating additional HCR controls and quantitative analyses, including co-detection of ubiquitous internal control transcripts, 3D segmentation-based volumetric fluorescence quantification, and experimental validation of mutually exclusive ERS+/ERS− epidermal markers *in vivo*. Together, these analyses provide direct quantitative support for the existence of distinct ERS+ and ERS− populations.

In parallel, we have strengthened the computational and comparative analyses by adding quantitative cross-method trajectory comparisons, RNA velocity-based validation, robustness statistics for cross-dataset label transfer, revised UMI downsampling analyses, and additional controls addressing potential technical confounders including transcript capture, filtering thresholds, and developmental stage effects.

Collectively, we believe these additions substantially improve both the clarity and robustness of the manuscript and provide strong orthogonal support that the ERS-associated bifurcation described in this study reflects a genuine biological feature of root cell differentiation.

Major comments:

Referee 1 - point 1: The authors claim in the abstract that "*The discovery of this alternate fate trajectory, induced by a highly active and environmentally responsive cell state, reveals an additional layer of root cell identity that may underpin the adaptability of roots to challenging environments.*" However, there are several aspects of this claim that were not sufficiently made. First, while cells in an ERS+ and ERS- state were identified for specific tissue types, the authors have not shown that these cells are especially responsive to the environment, only that the proportion of cells in this state increases with stress. It is not clear how cells transition into and out of the ERS+/- states and whether cells with an ERS+ state are any more responsive to stress than ERS- cells. One possible approach would be to use ABA FRET sensors (e.g., ABACUS or ABAleon) to visualize basal ABA levels in individual root hair cells, then apply an abiotic stress treatment to determine whether cells with higher basal ABA concentrations indeed exhibit faster or stronger ABA responses.

Response: Thank you for raising this point and for the opportunity to clarify our statements in the manuscript. We agree that understanding the dynamics by which cells acquire or exit the ERS, and how this relates to the ABA signalling pathway, would be very interesting and highly informative. These have been highlighted as important follow-up questions in the Discussion section of the manuscript.

The experiment suggested in this point, however, appears to stem from an interpretation of the ERS concept that differs from what we intended to convey in the manuscript. In our study, ERS+ and ERS− cells are not defined as cells that are intrinsically more or less capable of responding to environmental stimuli. Rather, ERS+ cells are those that have acquired a highly transcriptionally active state characterised by the expression of ERS-associated gene modules, whereas ERS− cells have not acquired this state. What our data show is that environmental conditions influence the proportion of cells that acquire this state. For example, by altering the medium composition - either by increasing MS concentration or, more strongly, under a model

drought condition - the plant adjusts the number of cells in the root that acquire the ERS (termed 'ERS+ cells'). If ERS+ cells represented a population intrinsically specialised for environmental responsiveness, stress would be expected to primarily increase the activity of these cells rather than alter their relative abundance. Instead, a clear increase in the proportion of cells acquiring the ERS is observed.

To further examine this point, we have now tested whether ERS+ cells show a stronger transcriptional response to drought stress than other cells. We compared both UMI content and the number of genes expressed between control and drought conditions (**Response Figure 1**). While drought increases overall transcript abundance (UMI count) and genes detected in the root, this effect is observed across all cell populations and is not specific to ERS+ cells. In fact, the magnitude of the increase is comparable or greater in ERS− and non-ERS cells (note: non-ERS cells are present in root cap and early developmental stages before the branching, where distinct ERS+ and ERS− states are not observed). The median fold-change in UMI content is 1.32 for ERS+ cells, 1.50 for ERS− cells, and 1.71 for non-ERS cells, while the median fold-change in the number of genes expressed is 1.24 for ERS+ cells, 1.32 for ERS− cells, and 1.62 for non-ERS cells (**Response Figure 1**). These results further support the interpretation that, rather than ERS+ cells specifically responding to drought, all cells exhibit a transcriptional response, while drought shifts a larger proportion of cells into the highly transcriptionally active ERS state already present in a subset of the cells under control conditions.

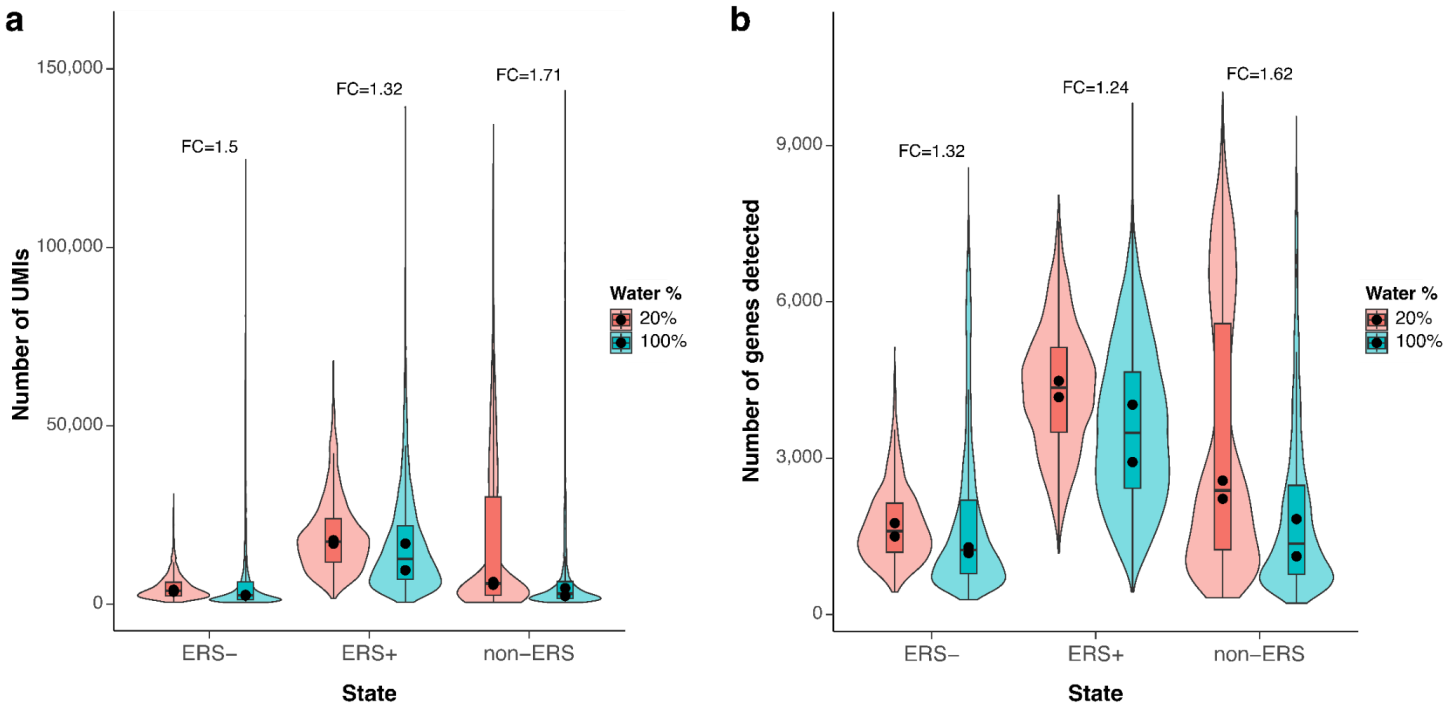
We recognise that some of the wording in the manuscript may have contributed to this ambiguity. In particular, the term *"ERS+ cells"* could be interpreted as referring to *"environmentally responsive cells"* (which would imply that *"ERS− cells"* are not environmentally responsive cells), and the sentence from the Abstract that is mentioned in this comment, which refers to an *"alternate fate trajectory"*, could be interpreted as implying that certain cells are developmentally predisposed to acquire this state. This was not our intention. To avoid this misunderstanding and better convey our conceptual framework, the relevant sections of the manuscript have been revised as follows:

- Page 2, line 12: We have replaced *"we found that most lineage trajectories exhibit a stereotyped bifid topology, with two cell developmental paths rather than one"* with "*we found that most lineage trajectories exhibit a stereotyped bifid topology, with two cell identity paths rather than one*" in the abstract.
- Page 2, line 20: We have replaced "*The discovery of this alternate fate trajectory*" with "*The discovery of this alternate trajectory*" in the abstract.
- Page 11, line 24: We have clarified the definition of the ERS, and ERS-positive and ERS-negative cells, by adding the following sentence when these terms are first introduced: *"These terms reflect the acquisition or absence of this transcriptional state at the single-cell level, rather than any intrinsic difference in the capacity of individual cells to respond to environmental signals*."
- Page 16, line 19: We have replaced *"3) the presence or absence of this additional environmentally responsive state, which varies with environmental conditions"* with *"3) the presence or absence of this additional transcriptional state, whose prevalence is modulated by environmental conditions"* in the Discussion section.

The experiment proposed in this comment - that is, using ABA FRET sensors such as ABACUS or ABAleon to test whether cells with higher basal ABA levels exhibit stronger ABA responses - would address the hypothesis that ERS+ cells are intrinsically more responsive to stress signalling. Because this is not the model proposed in our manuscript, such an experiment would not directly test the central claims of the study.

More broadly, experimentally resolving how cells transition into and out of the ERS state would require the generation and validation of live reporters for ERS-associated genes and the analysis of their spatiotemporal dynamics *in vivo*. While we think that this would be a fascinating experiment to further explore the nature and dynamics of this new cell state phenomenon, developing and characterising such constructs and reporter lines would be a significant undertaking that would not be possible to complete within the limited time available in this revision period (a few months). We therefore agree with Referee 2 that testing these dynamics experimentally is beyond the scope of the present manuscript.

To address this point, the manuscript has been revised to clarify the conceptual framework of the ERS and to emphasise that environmental stress increases the frequency with which cells acquire this transcriptional state. The potential dynamics of ERS acquisition and its relationship to stress signalling pathways, including ABA, are also discussed as important directions for future investigation.

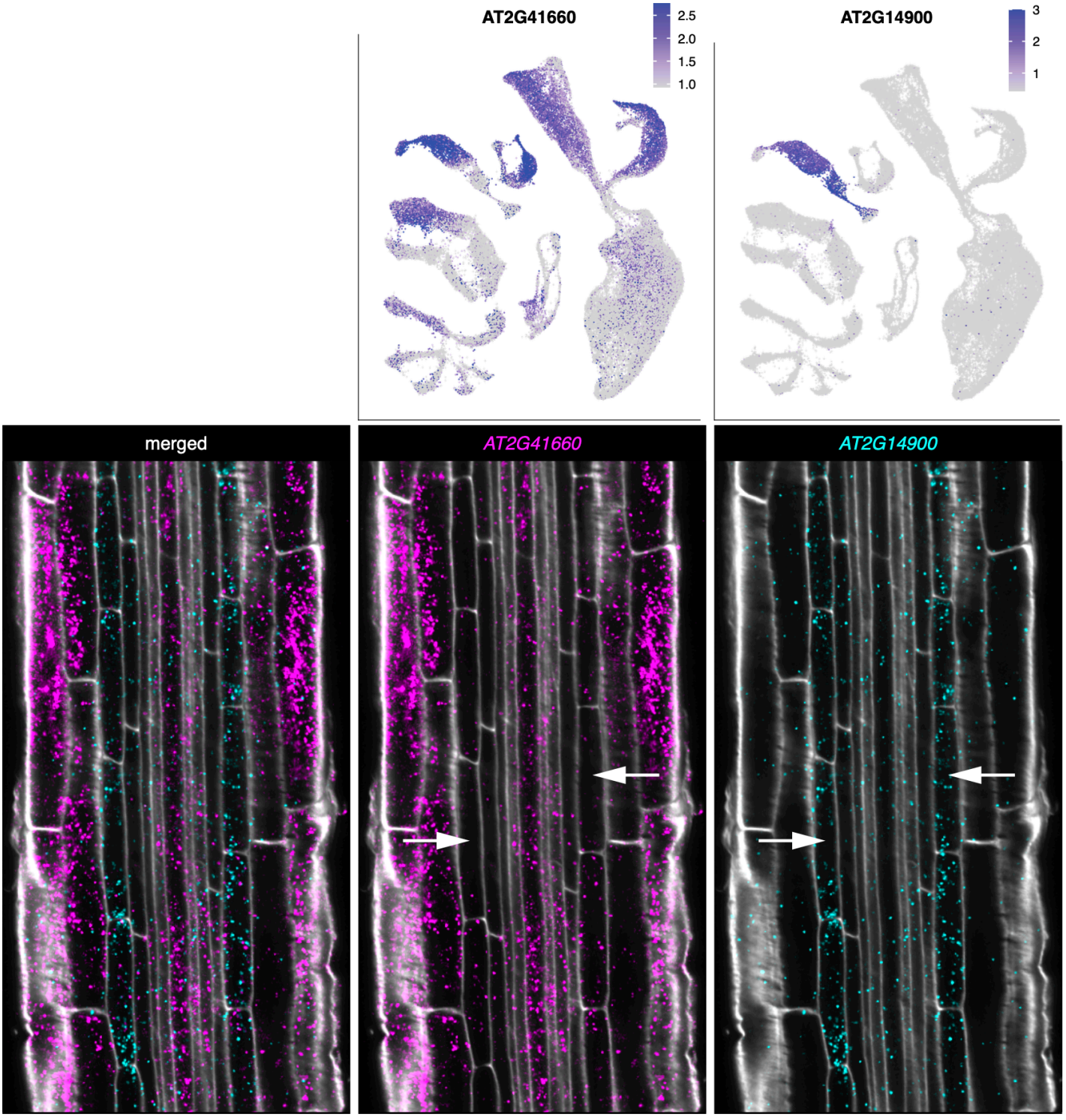


Response Figure 1: Comparison of **a,** UMI content and **b,** the number of genes expressed between control (100% water) and drought (20% water) conditions, for different cell states (ERS−, ERS+, and non-ERS cells). The fold change (FC) between 100% and 20% water conditions is indicated for each cell state.

Referee 1 - point 2: HCR-based transcript detection is a critical component of validating the manuscript's conclusions regarding the ERS^{+} cell state. It is therefore essential to demonstrate that the observed differences in signal intensity truly reflect biological variation rather than experimental artifacts. I recommend that, in addition to detecting ERS^{+} state markers, the authors include simultaneous detection of ubiquitously expressed genes that are expected to be present in both branches of the cell type as internal controls for probe performance and hybridization efficiency. It would also strengthen the analysis to present images across multiple Z-planes, confirming that the apparent absence of signal in certain cells is not due to differences in the chosen focal plane.

Response: This is an important point, and we agree that it is essential to demonstrate that the differential HCR signal observed between ERS+ and ERS− cells reflects genuine biological variation rather than technical artefact.

To address *probe performance and hybridisation efficiency*, we have now performed a new control experiment in the endodermis in which the ERS marker *AT2G41660* was co-detected with *AT2G14900*, a marker expressed at a similar level in both branches of the endodermal layer (**Resp. Fig. 2 / Extended Data Figure 20**). While *AT2G41660* shows the expected heterogeneous ERS-associated expression pattern, *AT2G14900* is robustly detected in both the ERS+ and ERS− cells of the endodermis. This demonstrates that cells lacking ERS marker signal are nonetheless efficiently permeabilised, hybridised, and their transcripts detected by HCR, and therefore that the absence of ERS marker signal in ERS− cells is not attributable to technical failure (**Resp. Fig. 2 / Ext. Data Fig. 20**).

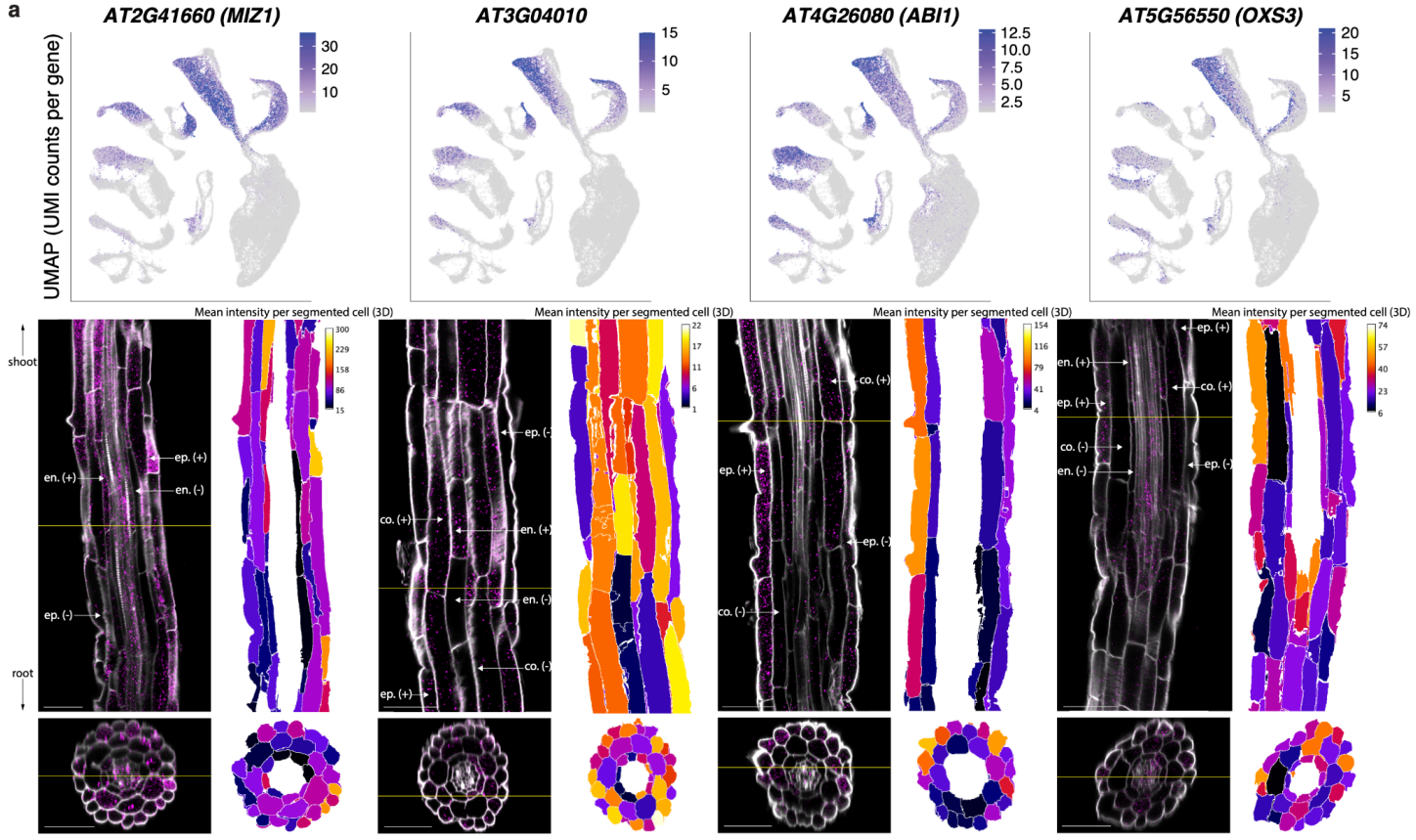


Resp. Fig. 2 / Extended Data Figure 20: Absence of ERS marker signal in negative cells is not due to failed HCR detection. UMAP plots showing the expression pattern of the ERS-associated marker *AT2G41660* and the ubiquitously expressed endodermal marker *AT2G14900* in the reference atlas. *AT2G41660* is enriched specifically in ERS+ endodermal cells, whereas *AT2G14900* is expressed throughout the endodermis independently of ERS state. Representative whole-mount HCR images showing simultaneous detection of *AT2G41660* (magenta) and *AT2G14900* (cyan) in Arabidopsis root endodermal cells. While *AT2G41660* signal is restricted to a subset of endodermal cells, consistent with ERS-associated expression, *AT2G14900* is robustly detected in both *AT2G41660*-positive and *AT2G41660*-negative cells. Arrows indicate examples of endodermal cells lacking detectable *AT2G41660* signal but retaining clear *AT2G14900* expression. These results demonstrate that the absence of ERS marker signal reflects genuine biological variation rather than differences in probe performance or hybridisation efficiency.

Regarding *image presentation*, we note that in the initial submission of the manuscript we had displayed multiple optical transverse cross-sections as suggested here. However, during the previous review round we were encouraged to favour longitudinal views, as root cells are thin and highly elongated, and

fluorescence puncta are more readily missed in individual transverse sections than in longitudinal optical sections spanning a larger proportion of the cell volume. Longitudinal sections are also the view in which positive and negative cells can most readily be appreciated within the same tissue layer. While adding multiple transverse cross-sections could increase confidence, this alone would still not fully exclude focal plane-related bias and would require a large number of sections across many cells to be convincing.

Therefore, in the revised manuscript, we have now adopted a more quantitative strategy by performing 3D segmentation of individual cells in the outer root layers (epidermis, cortex, and endodermis), where cell boundaries can be robustly delineated, and performing volumetric fluorescence quantification of the HCR signal on a per-cell basis. In the revised figure, segmented cells are colour-coded by mean HCR fluorescence intensity, allowing direct visualisation of heterogeneous ERS marker signal at single-cell resolution (**Resp. Fig. 3 / Fig. 3a**). This analysis reveals substantial differences in ERS marker abundance between cells within the same layer, with up to 20-fold difference between positive and negative cells.



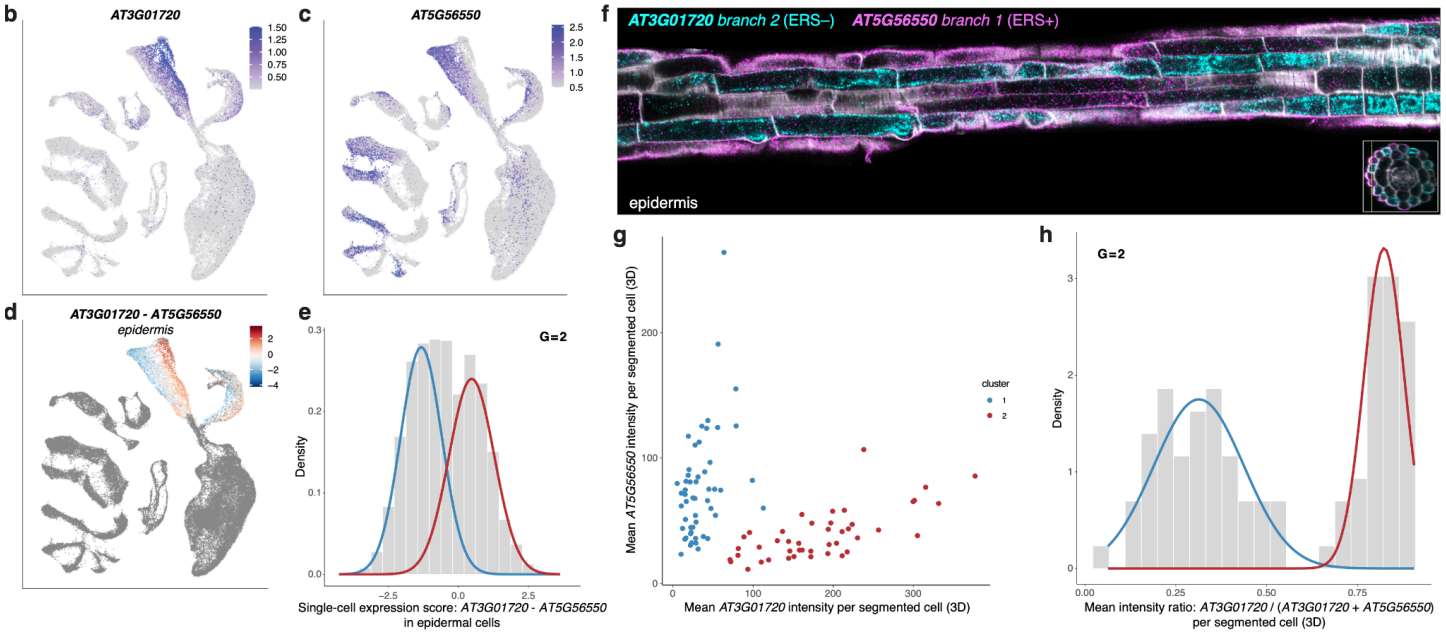
Resp. Fig. 3 / Fig. 3a: Whole-mount HCR validates the bifid topologies and associated branch-specific genes. a, UMAP plots and optical sections with associated segmentation showing transcript detection of four 'branch 1' markers, *AT2G41660* (*MIZ1*), *AT3G04010*, *AT4G26080* (*ABI1*), and *AT5G56550* (*OXS3*), both *in silico* from scRNA-seq data and *in vivo* by whole-mount *in situ* Hybridization Chain Reaction (HCR). Yellow lines indicate the relative positions of longitudinal and transverse cross sections. Example cells that are positive (+) or negative (–) for each transcript in the epidermis (ep.), cortex (co.), or endodermis (en.) are indicated with arrows. The associated segmented images show the same optical section after segmentation of the root layers (epidermis, cortex, endodermis), in which positive and negative cells were identified. Cells are colored by mean intensity signal (measured in 3D). Scale bar = 50 µm.

One of the challenges in experimentally validating the existence of the two branches is that the ERS− cells do not exhibit a clear branch-specific transcriptional programme analogous to the ERS-associated modules. Rather, ERS+ and ERS− cells share the canonical markers of their underlying cell type, with ERS+ cells distinguished primarily by the additional expression of the ERS-associated transcriptional programme. However, upon closer inspection of epidermal markers, we have identified a small number of genes with expression patterns indicative of ERS− enrichment. In particular, *AT3G01720* shows an expression pattern that is largely complementary to the ERS marker *AT5G56550* in epidermal cells, with both genes expressed at levels suitable for *in situ* detection (**Resp. Fig. 4b-d / Fig. 3b-d**).

We therefore tested this pair experimentally by HCR and found that the two transcripts display largely mutually exclusive expression patterns *in vivo* (**Resp. Fig. 4f / Fig. 3f**). Volumetric quantification of segmented epidermal cells across three independent roots reveals two distinct populations of epidermal cells, one

enriched for *AT3G01720* and the other for *AT5G56550* (**Resp. Fig. 4g / Fig. 3g**), closely matching the ERS− and ERS+ epidermal populations inferred from scRNA-seq. Consistent with this, Gaussian mixture modelling of both scRNA-seq expression scores and HCR-derived fluorescence ratios supports the presence of two discrete epidermal populations (**Resp. Fig. 4e,h / Fig. 3e,h**).

Together, these orthogonal controls and quantitative imaging analyses demonstrate that the heterogeneous HCR signal observed for ERS markers reflects *bona fide* biological heterogeneity between neighbouring cells rather than focal plane effects or technical variability in probe detection, thereby providing strong independent experimental support for the existence of distinct ERS+ and ERS− cell states *in vivo*.



Resp. Fig. 4b-h / Fig. 3b-h: Whole-mount HCR validates the bifid topologies and associated branch-specific genes. b–e, A 'branch 2' (ERS–)-specific gene (*AT3G01720*), mutually exclusive to the 'branch 1' (ERS+) marker *AT5G56550*, is identified in the epidermis. UMAPs showing the expression pattern of *AT3G01720* (b), *AT5G56550* (c), and the expression score *AT3G01720* − *AT5G56550* in the epidermis (d). Distribution of the expression score across epidermal cells and Gaussian mixture modelling support the existence of two distinct populations (G = 2; Bayesian information criterion (BIC) favoured two over one component), represented by the blue and red lines (e). **f–h**, The two epidermal branches and associated markers are also detected by whole-mount HCR. Optical section showing simultaneous transcript detection of *AT3G01720* ('branch 2' marker) and *AT5G56550* ('branch 1' marker) in the epidermis. The yellow line in the inset transverse optical section of the same root indicates the plane of the longitudinal optical section, which passes through 4-5 epidermal cell files. Distribution of mean signal intensity for *AT3G01720* and *AT5G56550* (g), and of the mean intensity ratio *AT3G01720* / (*AT3G01720* + *AT5G56550*) (h) per epidermal cell measured after 3D segmentation. Gaussian mixture modelling supports the existence of two distinct populations (G = 2; Bayesian information criterion (BIC) favoured two over one component), represented by clusters in (g) and red lines in (h). Quantifications were performed on 97 epidermal cells from three independent roots.

Referee 1 - point 3: The identification of two trajectories with both PAGA and Monocle is encouraging. To strengthen the claim of a true bifurcation, I recommend quantifying agreement between methods (e.g., overlap of branch assignments and correlation of local pseudotime). And since both PAGA and Monocle are graph-based analyses, it will be better to use a graph independent approach, like RNA velocity models, to confirm the trajectories identified in PAGA and Monocle. Additionally, please include pathway enrichment for the Monocle trajectories. Do similar GO terms appear in the Monocle-based analysis as in the PAGA-inferred branches?

Response: The trajectory framework used in the manuscript combines PAGA to infer trajectory topology with diffusion pseudotime (DPT) to order cells along those trajectories. PAGA was selected because it is particularly well suited for resolving complex developmental topologies, including the bifurcations and convergence events observed in several lineages such as the epidermis and root cap. While PAGA itself is graph-based, the subsequent DPT computation used to order cells is not graph-based and independently supports the presence of two trajectories. Specifically, in all affected lineages, the inferred origin of the trajectory (t = 0) is positioned centrally within the lineage, while two distinct terminal populations are assigned t

= 1 (**see Fig. 1f**), consistent with the presence of two developmental endpoints and therefore two separate trajectories.

To further address this point, we also tested an RNA velocity-based approach using scVelo. This method was not initially prioritised because intronic signal in Arabidopsis scRNA-seq datasets is very limited, resulting in low signal-to-noise ratios and reduced robustness of velocity inference. In our atlas, the median proportion of unspliced reads is only ~3%, and fewer than 7,000 of the 33,977 detected genes passed filtering thresholds for sufficient spliced and unspliced counts to support kinetic modelling. Nevertheless, latent time inferred using the scVelo dynamical model showed strong agreement with our DPT ordering, with Spearman correlation coefficients >0.7 in six of the eight lineages that exhibit ERS-associated bifurcation (**Resp. Fig. 5e,f / Ext. Data Fig. 5e,f**). For the xylem pole pericycle and procambium, the lower correlation reflects a shift in the inferred t = 0 assignment rather than disagreement in trajectory structure. In all cases, latent time increases toward the termini of both branches, consistent with the bifurcating developmental trajectories identified in our analysis.

Regarding comparison between the PAGA- and Monocle-inferred trajectories, we agree that the initial manuscript relied primarily on visual concordance of the trajectory topologies and pseudotime progression. To provide a more quantitative assessment, we compared pseudotime values inferred by Scanpy/DPT and Monocle across all lineages. This analysis revealed strong concordance between methods, with pseudotime correlations exceeding 0.83 in all lineages except the endodermis (**Resp. Fig. 5a-d / Ext. Data Fig. 5a-d**). In the endodermis, one branch shows lower correlation because Monocle places the trajectory root slightly deeper within that branch, thereby shifting the relative pseudotime scale without altering the overall bifurcating topology.

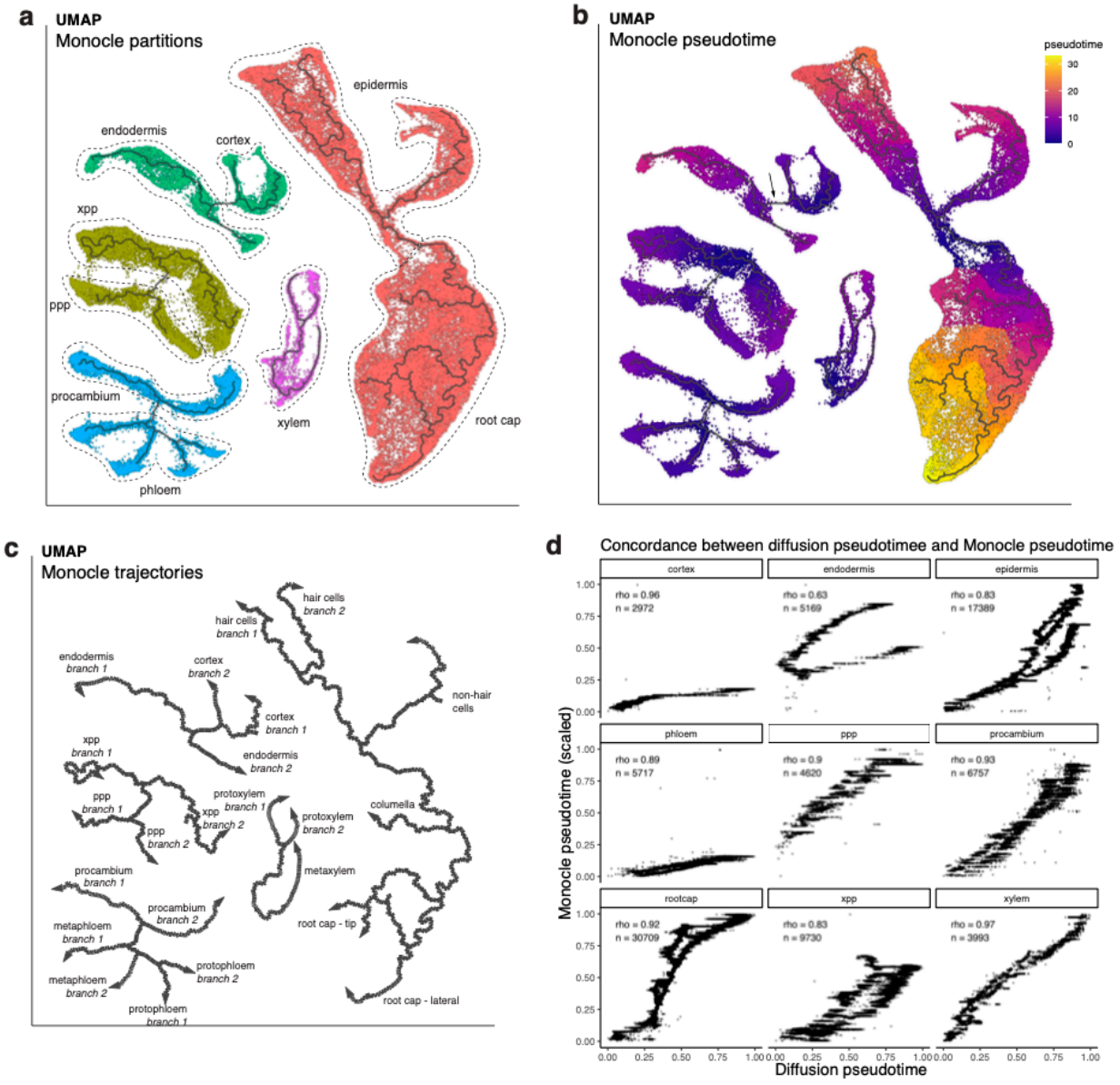
Direct comparison of branch assignments between PAGA and Monocle is complicated by the fact that Monocle frequently introduces additional minor nodes and local branches in complex datasets such as ours, beyond the principal global bifurcation of interest. As branch identities in Monocle must therefore be reconstructed manually from graph topology rather than being directly output as discrete branch labels, robust per-cell branch assignment comparisons (e.g. ARI, Adjusted Rand Index) are not straightforward or necessarily meaningful in this context. This complexity was one of the reasons PAGA is favoured as the primary topology inference method in the study.

Regarding pathway enrichment, the GO term analyses presented in the manuscript are not derived from differential expression testing between branches defined by a specific trajectory inference method. Rather, they are based on co-expression modules identified across the entire dataset, independently of trajectory or branch assignments. This approach was intentionally chosen because it is more robust to minor differences in pseudotime ordering or branch boundary placement than branch-specific differential expression analyses, which can be sensitive to methodological noise and assignment thresholds. Notably, the ERS-associated gene modules describe a cell state that is superimposed across all lineages rather than being confined to a single trajectory branch or cell type, and this global, lineage-independent nature of the signal is better captured by a dataset-wide co-expression framework than by per-branch differential expression within individual lineages.

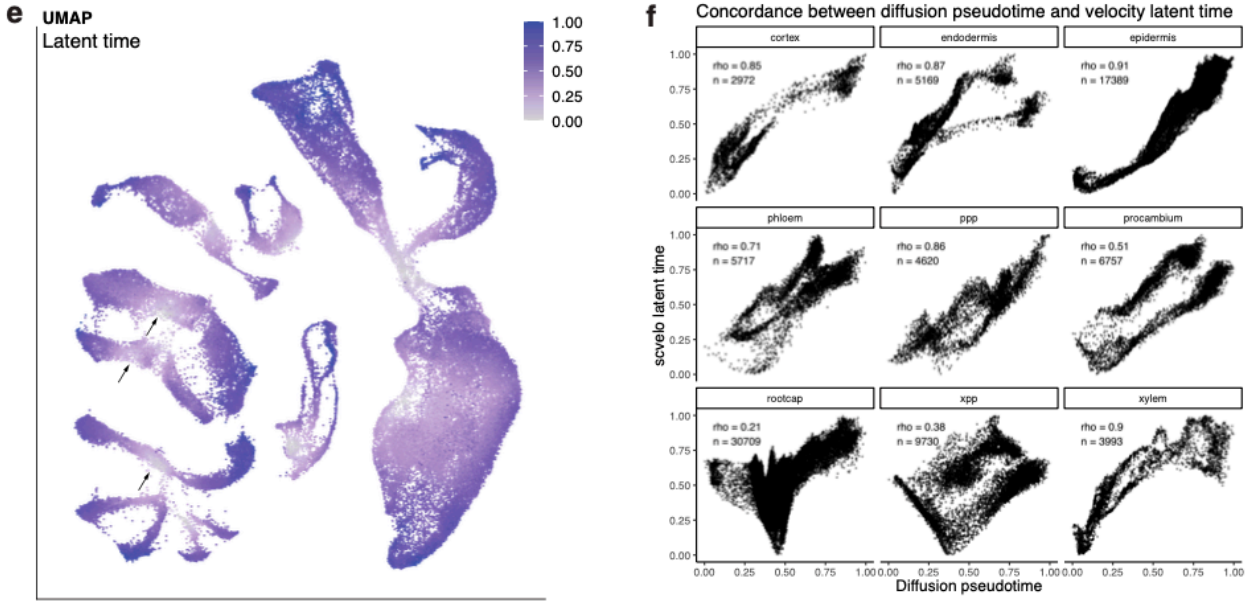
Importantly, since the UMAP embedding underlying both the PAGA and Monocle analyses is identical, projecting the co-expression module scores onto the Monocle trajectory yields the same spatial pattern of ERS-associated gene activity by definition. The GO enrichments are therefore not merely consistent between methods but are the same enrichments, derived from the same cells and visualised on the same embedding. Given this, and given the strong concordance observed between PAGA, Monocle, and RNA velocity-based trajectory inference, the functional interpretation of the ERS-associated branch does not rely on a specific trajectory inference framework but instead emerges from a trajectory-independent co-expression analysis that is robust to minor differences in cell ordering across methods.

Together, these analyses demonstrate that the bifurcating developmental trajectories reported in this study are robustly recovered across multiple independent trajectory inference frameworks, including graph-based, non-graph-based, and RNA velocity-derived approaches, strongly supporting the conclusion that the observed ERS-associated branching reflects a genuine and biologically meaningful feature of root cell differentiation rather than a methodological artefact.

Monocle



scvelo



Resp. Fig. 5 / Ext. Data Fig. 5: Monocle and scvelo trajectory analyses validate the dichotomy in cell lineages. a, UMAP plot showing the partitions defined by Monocle, for which independent trajectory analyses were computed. Note that endodermis and cortex; xylem and phloem pole pericycle; and procambium and phloem are grouped within the same partition. **b,** UMAP plot showing the pseudotime ordering obtained with Monocle. Arrow indicates shift in inferred t=0 placement relative to the reference atlas. **c,** UMAP plot showing the resulting trajectories from the Monocle analysis, recovering the same two branches identified in every cell type with the PAGA trajectory analysis. **d,** Scatter plot showing Monocle pseudotime (scaled from 0 to 1) and diffusion pseudotime assignment per cell, by cell type, demonstrating good correlation between the two methods. Spearman correlation and number of cells are indicated in the top left. **e,** UMAP plot showing the latent time assignment obtained with velocity analysis by scVelo. Arrows indicate shift in inferred t=0 placement relative to the reference atlas. **f,** Scatter plot showing latent time and diffusion pseudotime assignment per cell, by cell type. Spearman correlation and number of cells are indicated in the top left. Lower correlation between the two methods is mainly due to a slight shift in t = 0 in the velocity analysis compared to the other methods.

Referee 1 - point 4: Extended Data Fig.2. It is unclear what is validated in this figure. If published reporter lines support the reported expression patterns, the corresponding references should be included. Note also that AT1G11190 is BFN1, not BNF1.

Response: Thank you for pointing out this lack of clarity and for noting the typographical error in the gene name. We have corrected *BNF1* to *BFN1*.

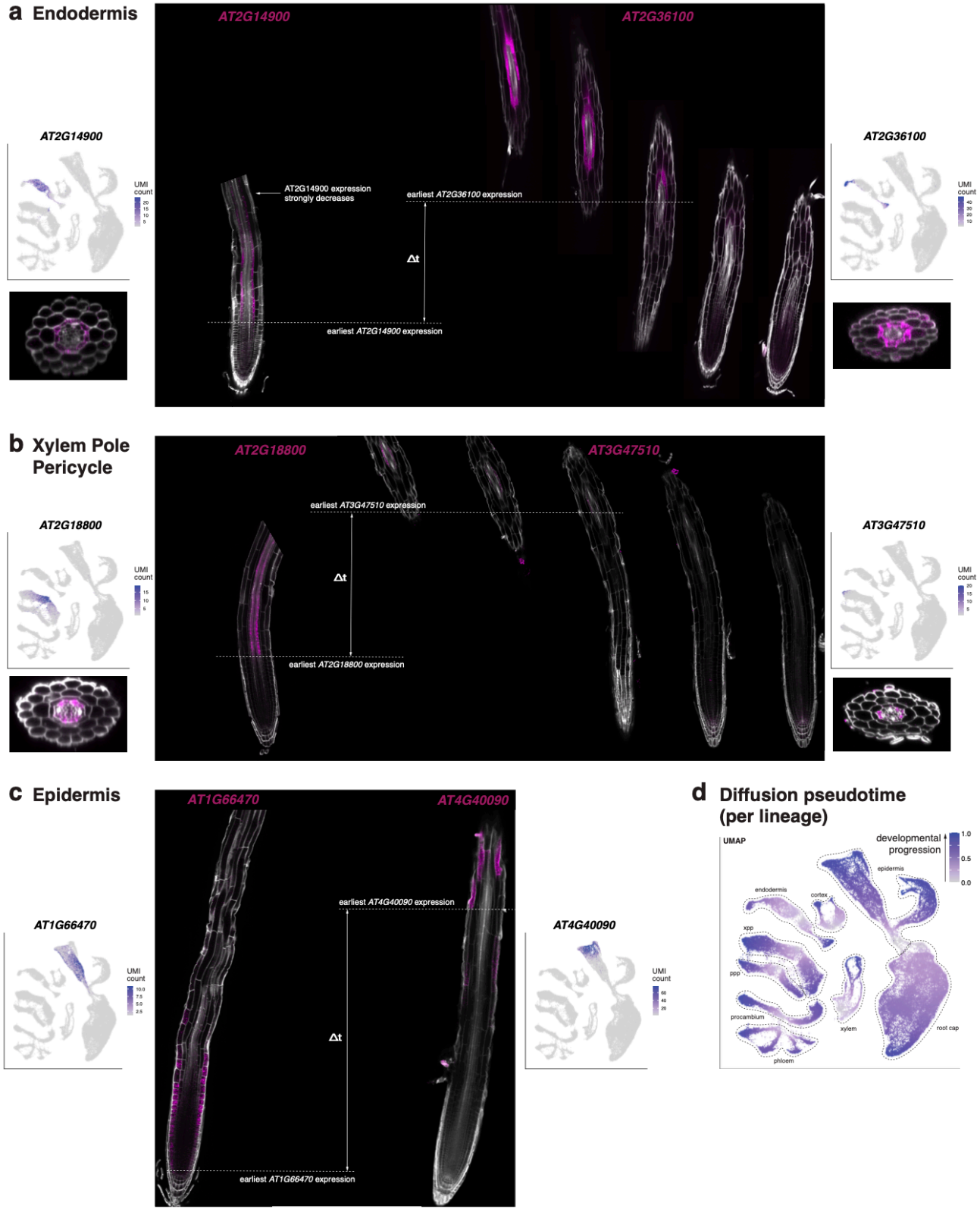
Extended Data Figure 2 is intended to validate the biological interpretation of the inferred root cap trajectory topology by showing that the distinct branches identified in our trajectory analysis are associated with the expected expression patterns of marker genes known to be expressed at specific stages or subdomains of root cap development. Specifically, the expression patterns of *CDF4*, *FEZ*, *RCPG*, and *BFN1* in the scRNA-seq data are consistent with their previously reported *in vivo* expression domains and support the interpretation that the inferred branches correspond to known developmental events within the root cap lineage.

The corresponding references for these published expression patterns were already cited in the main text (refs. 22–24), but we recognise that this may not have been sufficiently explicit in relation to Extended Data Figure 2. To improve clarity, we have revised the manuscript text and figure legend to state more explicitly that these previously characterised marker genes are used to validate the biological identity of the inferred trajectories based on their known expression patterns during root cap development. The corresponding references have also been added to the Extended Data Figure 2 legend.

Together, these marker expression patterns provide independent biological support that the complex root cap topology inferred by our trajectory analysis recapitulates *bona fide* developmental branching events rather than representing computational artefacts.

Referee 1 - point 5: In Extended Data Fig.3, the images shown for genes AT2G36100, AT3G47510 can be made clear by showing optical cross sections at different distances from the root tip. Currently, it is difficult to determine, for example, whether AT2G36100 is expressed in the endodermis.

Response: Thank you for this suggestion. To improve the clarity of the expression patterns shown in Extended Data Figure 3, we have now added additional optical cross-sections to make the spatial localisation of the signal clearer and facilitate interpretation of the corresponding cell-type-specific expression patterns (**Resp. Fig. 6 / Ext. Data Fig. 3**).



Resp. Fig. 6 / Ext. Data Fig. 3: Validation of pseudotime assignments. a, UMAP plots, and transverse and longitudinal optical sections of whole-mount HCR showing the expression of two endodermal markers, *AT2G14900* and *AT2G36100*, *in silico* and *in vivo*. *AT2G14900*, which is predicted to be expressed early according to the trajectory analysis (see pseudotime assignment in d), is detected closer to the meristem, whereas *AT2G36100*, a later marker, is detected further away, validating the pseudotime assignment. Multiple optical sections are shown when a complete longitudinal section of the root could not be obtained at once due to minor root curvature during mounting. Dotted lines indicate the earliest developmental stage at which expression of each marker was detected. **b,** Similarly, in the xylem pole pericycle, *AT2G18800* (early marker) is detected nearer to the meristem and *AT3G47510* (late marker) is detected further away, consistent with the trajectory analysis. **c,** The trajectories for epidermal hair cells are also validated: *AT1G66470* (early marker) and *AT4G40090* (late marker) show concordant expression patterns *in silico* and *in vivo*. **d,** UMAP plot summarizing the pseudotime assignment per lineage in our reference atlas.

Referee 1 - point 6: In the author response, the statement "ERS$^-$ does not mean that these cells are unresponsive to the environment; rather, it means they have not acquired the specific transcriptional state that we define as 'environmentally responsive' (ERS$^+$)" remains conceptually ambiguous. In the hard-agar drought stress experiment, the increase in ERS$^+$ cells is accompanied by a decrease in ERS$^-$ cells, suggesting that both states are, in fact, responsive to environmental cues. To avoid confusion, I recommend refraining from using the term "environmentally responsive" to describe the ERS$^+$ state, as it implies that ERS$^-$ cells are environmentally inert.

Response: We thank the referee for this comment and agree that the terminology we used may lead to ambiguity if interpreted as implying that ERS− cells are environmentally inert. As clarified in **Response to Referee 1 - point 1**, above, this is not the conceptual framework intended in the manuscript. The term "environmentally responsive" refers to the transcriptional state itself, which is characterised by the coordinated activation of genes associated with environmental and stress responses. In addition, the probability of a cell acquiring this transcriptional state is modulated by environmental conditions, with drought resulting in an increased proportion of ERS+ cells across multiple lineages.

We do not intend the term "environmentally responsive" to imply that ERS+ cells are uniquely capable of responding to environmental cues. Indeed, as shown in Response Fig. 1, drought affects both ERS+ and ERS− cells, including increased transcript abundance and numbers of detected genes in both populations. However, the ERS− cells that remain under these conditions still do not exhibit the characteristic ERS-associated transcriptional signature identified by our co-expression analysis.

Because cells within a given lineage are classified as either ERS+ or ERS−, any increase in the proportion of ERS+ cells under stress necessarily results in a corresponding decrease in the proportion of ERS− cells. This does not indicate that ERS− cells are environmentally unresponsive, but simply reflects the reciprocal nature of the classification.

We acknowledge that the terminology may be open to misinterpretation and have therefore revised the manuscript to clarify this conceptual framework more explicitly throughout the text (detailed in **Response to Referee 1 - point 1**, above), including when first defining the ERS and when referring to ERS+ and ERS− populations. While alternative terminology was considered, we found that other options introduced similar ambiguities while reducing continuity with the terminology used throughout the manuscript and review process. As this concern was not raised by the other referees, we have retained the term ERS while clarifying its intended meaning.

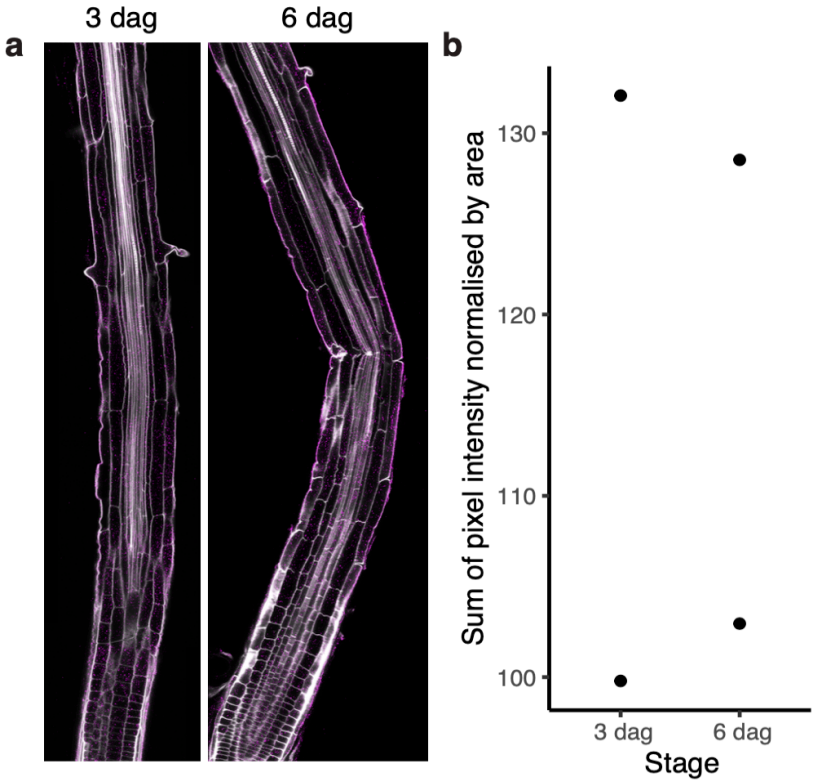
If the Editor and referees feel that renaming this state would nevertheless improve the clarity of the manuscript, we would of course be happy to revise the terminology accordingly.

Referee 1 - point 7: In the drought stress and full MS/half MS experiments, could the authors clarify whether seeds were germinated directly on the respective media or if seedlings were transferred to these conditions after initial germination? The method of establishing the treatment is important, as transfer-related stress or developmental stage differences could influence the proportion of ERS$^+$ cells and the interpretation of the results.

Response: Thank you for pointing out this potential source of bias. Seeds were sown directly on their respective media and germinated under those conditions; seedlings were not transferred between media prior to analysis.

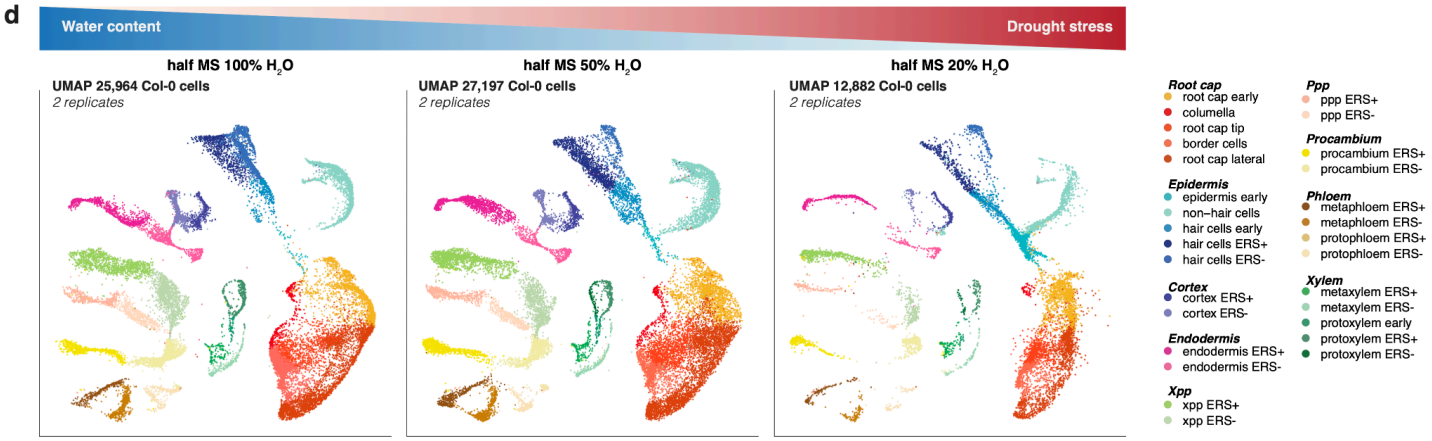
Because drought-stress medium delays germination and reduces root growth, drought plates were transferred to the growth room 48h earlier so that samples from all conditions could be harvested simultaneously at comparable developmental stages relative to germination. In addition, all samples were collected by harvesting the same region from the root tip, ensuring that comparable developmental zones were analysed even when total root length differed between conditions.

To ensure that reduced root growth itself does not account for the observed changes in ERS-associated gene expression, we examined ERS marker expression in younger control roots at 3 days after germination (dag), when the roots are substantially smaller and more comparable in size to the drought-treated roots. This showed that *AT3G04010* fluorescence intensity at the root tip remained comparable between 3 and 6 dag, indicating that root length does not explain the increased fluorescence intensity observed under drought conditions. Quantification supporting this control has now been added to the revised manuscript (**Resp. Fig. 7 / Ext. Data Fig. 28**).



Resp. Fig. 7 / Ext. Data Fig. 28: Increased ERS marker expression under drought conditions is not explained by reduced root size. a, Representative whole-mount HCR images showing expression of the ERS marker *AT3G04010* in control roots at 3 and 6 days after germination (dag). Despite the substantially shorter length of 3 dag roots, fluorescence intensity at the root tip remains comparable between stages. **b**, Quantification of *AT3G04010* HCR signal intensity normalised by root area in 3 and 6 dag control roots. Similar fluorescence levels were observed at both developmental stages, indicating that reduced root size does not explain the increased ERS-associated expression observed under drought conditions.

Referee 1 - point 8: In Figure 4d, it would be helpful to project the datasets from the different drought stress conditions onto a single UMAP embedding. Presenting the data in a shared UMAP space would allow for a more direct comparison of the cellular distributions and ERS⁺ cell frequencies across conditions.
Response: Thank you for this suggestion. We have now projected the drought-stress datasets onto the reference atlas UMAP embedding and included these analyses in the revised manuscript. This shared embedding allows direct visual comparison across conditions and clearly shows an enrichment of ERS+ cells, with a corresponding decrease in ERS− cells, across multiple cell types under drought stress relative to control conditions (**Resp. Fig. 8 / Fig. 5d**). Please note the change in figure numbering in the revised manuscript.

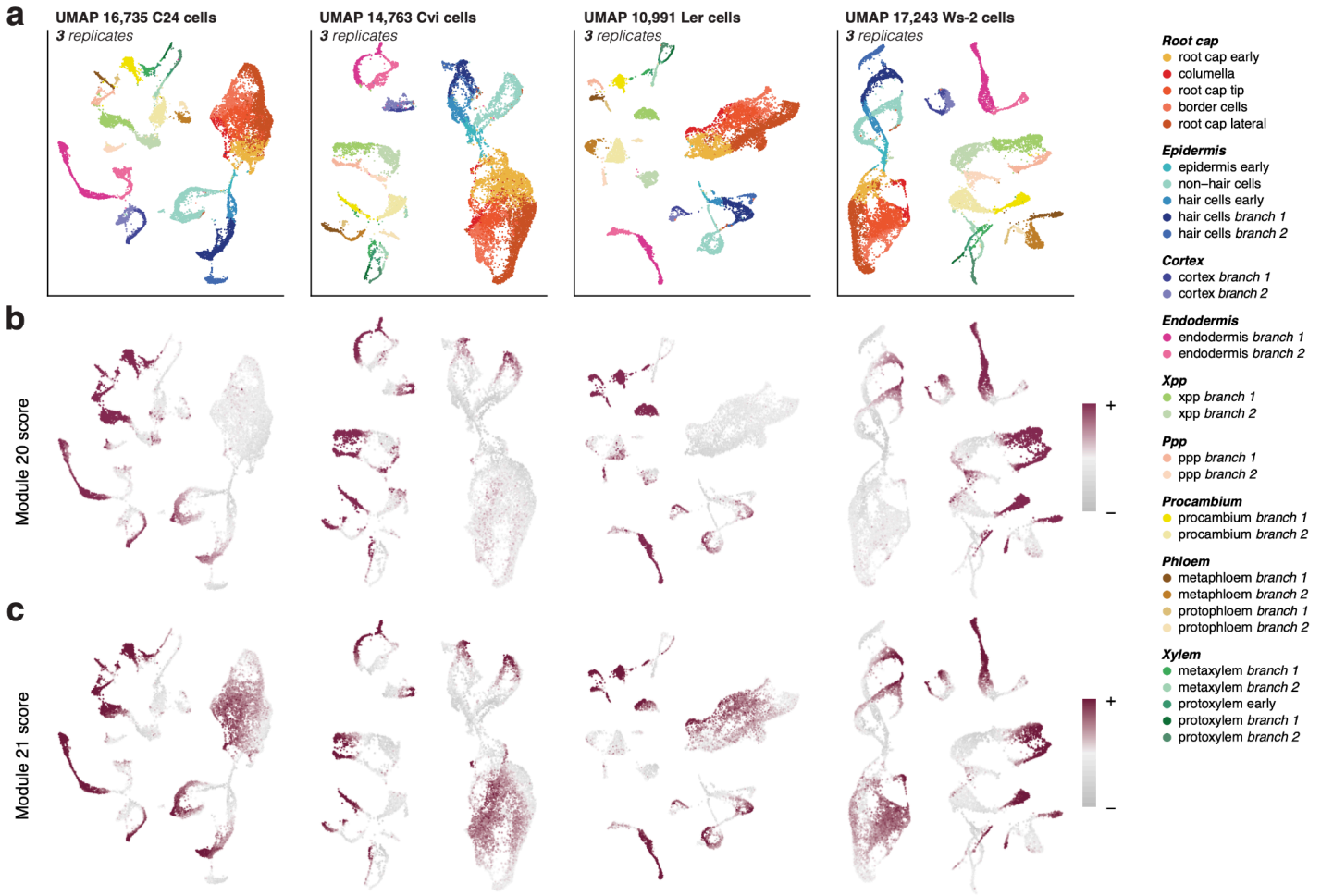


Resp. Fig. 8 / Fig. 5d: Acquisition of the ERS is modulated under drought stress. d, UMAP plots showing assigned cell identities from scRNA-seq profiling of roots grown under control conditions (half MS, 100% H_2O) and in the "hard agar" drought model, in which water content in half MS medium was reduced to 50% (half MS, 50% H_2O) or 20% (half MS, 20% H_2O). Cells were projected onto our reference cell atlas UMAP embedding.

Referee 1 - point 9: In Extended Data Fig. 15b–d, do the authors observe that branch 1–specific genes across the different ecotypes are enriched for ABA- or circadian-related pathways? Clarifying whether this enrichment is consistent among ecotypes would help determine if the observed transcriptional divergence reflects conserved ABA/circadian regulation or ecotype-specific differences in environmental responsiveness.
Response: Thank you for this suggestion. To identify the branch 1-specific genes associated with ABA- and circadian-related GO terms, we did not perform differential expression analysis between branch 1 and branch

2 cells. Instead, as described in **Response to Referee 1 - point 3**, we identified co-expression modules across the full dataset independently of branch assignments, an approach that is more robust to minor variation in branch boundaries and cell ordering than branch-specific differential expression analyses.

Accordingly, to determine whether the same transcriptional programme is associated with branch 1 cells across ecotypes, we assessed the expression of Modules 20 and 21 (the branch 1-specific co-expression modules identified in the Col-0 reference atlas and associated with ABA- and circadian-related GO terms) in the other ecotypes. We found that, similarly to Col-0, these modules remain specifically enriched in branch 1 cells across all ecotypes examined (**Resp. Fig. 9a-c / Ext. Data Fig. 16a-c**). This indicates that the transcriptional programme associated with the branch 1/ERS+ state is broadly conserved across ecotypes and that the observed transcriptional divergence is not unique to Col-0.



Resp. Fig. 9a-c / Ext. Data Fig. 16a-c: Branches are robustly detected in all five tested Arabidopsis ecotypes. a, UMAP plots showing the atlases from C24, Cvi-0, Ler, and Ws-2 cells, coloured by identity assigned using our reference atlas. **b-c,** UMAP plots showing the expression patterns of the 'branch 1'-specific gene modules (Modules 20 and 21) identified in our Col-0 reference atlas in the other four ecotypes, demonstrating the same enrichment in 'branch 1' cells.

Referee 1 - point 10: Data on root hair number are not presented in the manuscript. The corresponding section in the Methods should therefore be removed unless these results are included.

Response: Thank you for this comment. Root hair density is not presented as a major result of the manuscript; however, these data were included to demonstrate that, although full MS and half MS media are both common and generally considered mild growth conditions in Arabidopsis studies, the reduction in medium concentration nevertheless induces a measurable physiological response. Specifically, the increase in root hair density provides an independent phenotypic indication that the half MS condition is perceived by the plant and elicits a physiological adjustment, consistent with the moderate shift in ERS proportion observed under this condition.

Accordingly, these data are referred to in the manuscript in the context of supporting the interpretation that altered medium composition induces a biologically relevant response, rather than as a standalone result. The relevant sentence in the manuscript reads: *"This range is commonly used in Arabidopsis studies and does*

not drastically affect root growth, but the decrease in medium concentration induces a physiological response reflected by an increase in root hair density (Extended Data Fig. 21).”

We retained the associated Methods section because these data were originally part of the main figures in earlier versions of the manuscript and remained included in the revised supplementary data. However, if the Editor and Referees feel that these data are not sufficiently central to warrant inclusion, we would be happy to remove both the figure and corresponding Methods section.

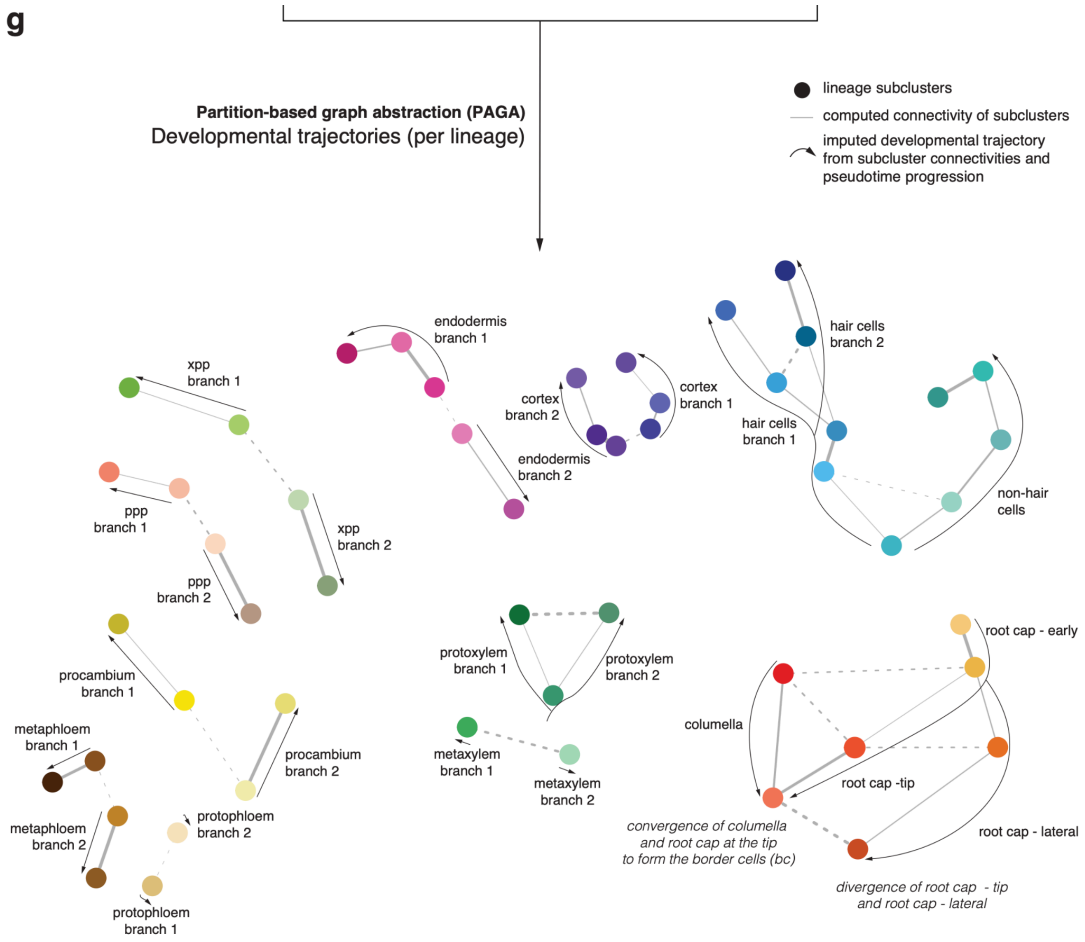
Referee 1 - point 11: Since the edge_threshold parameter in the PAGA analysis was “adapted to each lineage,” it would be important to specify the exact thresholds used or provide a clear rationale for how these values were determined, as this choice can strongly influence the inferred trajectory topology.

Response: Thank you for raising this point. In the PAGA analysis, each lineage is first subdivided into smaller clusters prior to topology inference. Because transcriptional similarity can reflect both developmental progression and shared developmental stage, weaker connections are sometimes detected between subclusters belonging to different branches but occupying similar pseudotime positions. These likely reflect shared stage-specific transcriptional programs rather than the principal developmental progression within a branch.

The purpose of the edge_threshold parameter is therefore to filter out these weaker secondary connections while retaining the stronger edges that define the principal developmental trajectories. Thresholds were adjusted independently for each lineage because the relative strength of within- versus between-branch connections varies depending on lineage complexity, cluster number, and transcriptional heterogeneity.

In some cases, these weaker inter-branch connections could not be completely removed without disrupting the principal trajectory structure and were therefore retained; these are shown as dashed lines in **Resp. Fig. 10 / Fig. 1g**. Importantly, these dashed edges represent only a small subset of the detected relationships. With the exception of the protoxylem and the more complex root cap trajectories, which contain multiple closely related sub-cell types in close spatial and developmental proximity near the root tip, these residual inter-branch connections are largely restricted to the earliest stages of the bifurcation, when branch 1 and branch 2 cells are only beginning to separate transcriptionally. The overall branching topology is therefore not driven by thresholding itself, but reflects the dominant connectivity structure present in the data. Consistent with this, similar bifurcating developmental structures are also apparent in independent Arabidopsis root trajectory analyses from Shahan *et al.*, despite the use of different datasets and trajectory inference approaches, supporting that these branching trajectories represent reproducible biological structure rather than an artefact of parameter selection. (**Resp. Fig. 10 / Fig. 1g**).

All edge_threshold values used for each lineage are provided in the associated analysis script available in the dedicated github repository.



Resp. Fig. 10 / Fig. 1g: Developmental trajectories obtained by combining the connectivity between the subclusters shown in Fig. 1e computed in a partition based graph abstraction (PAGA) and the pseudotime inference calculated in Fig. 1f reveal two distinct developmental branches affecting all cell lineages (named 'branch 1' and 'branch 2'). Dashed lines indicate weak inter-branch connections, at similar developmental stages.

Referee 1 - point 12: The cross-dataset validation should come with supporting statistics. The authors used extensive projection-based comparisons to determine if ERS+ and ERS- populations were also present in previously published scRNA-seq/snRNA-seq datasets. However, these projections were known to vary dramatically when using different parameters. The authors need to show corresponding statistics for the stability of these projections.

Response: Thank you for raising this important point. To assess the robustness of the projection-based ERS assignments in external datasets, we have now systematically evaluated label transfer stability across a range of parameter settings for the two principal parameters influencing Seurat label transfer, namely the number of principal components used and the k.anchor parameter.

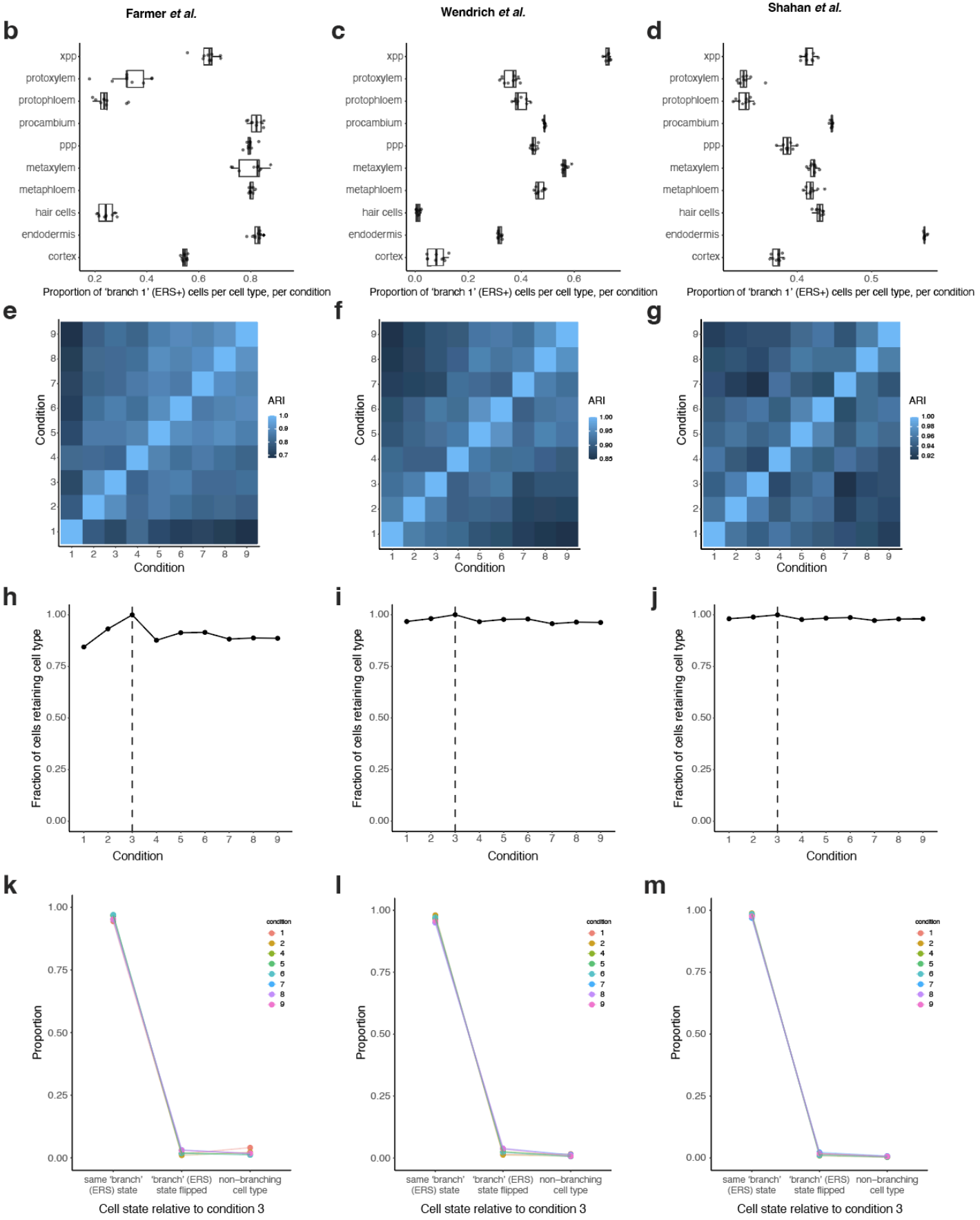
Specifically, projections were repeated using 30, 40, or 50 principal components and k.anchor values of 5, 10, or 20, generating nine parameter combinations in total (**Resp. Fig. 11a / Ext. Data Fig. 15a**). Across all three external datasets examined, the proportion of ERS+ cells assigned to each cell type remained highly consistent across parameter sets (**Resp. Fig. 11b-d / Ext. Data Fig. 15b-d**). Pairwise Adjusted Rand Index (ARI) comparisons between parameter sets further demonstrated strong agreement in transferred cell type and ERS assignments (**Resp. Fig. 11e-g / Ext. Data Fig. 15e-g**), with all ARI values remaining above 0.7. Similarly, more than 75% of cells retained their baseline cell-type assignment across all parameter sets, with most conditions for most datasets close to 100% (**Resp. Fig. 11h-j / Ext. Data Fig. 15h-j**).

The slightly lower ARI values and cell-type assignment stability observed for the Farmer *et al.* dataset likely reflect the fact that this dataset is based on snRNA-seq rather than whole-cell scRNA-seq, and therefore differs more substantially from our scRNA-seq reference atlas in transcript capture characteristics and overall data structure. Nevertheless, the stability remains high despite this expected modality-related variability.

Importantly, ERS state assignment proved markedly more robust than fine cell-type assignment: across all three datasets, including the Farmer *et al.* snRNA-seq dataset, ERS+/ERS− assignments were retained for nearly 100% of projected cells across parameter sets, with only negligible proportions of cells switching ERS state (**Resp. Fig. 11k-m / Ext. Data Fig. 15k-m**). This demonstrates that the identification of the ERS dichotomy in external datasets is highly stable and does not reflect instability of the projection procedure.

a **Parameter sweep**

parameters	condition	1	2	3	4	5	6	7	8	9
	dims	30	40	50	30	40	50	30	40	50
	k.anchor	5	5	5	10	10	10	20	20	20



Resp. Fig. 11 / Ext. Data Fig. 15: 'Branch' assignments are robust to label transfer parameter selection across external datasets. a, Parameter combinations used for label transfer stability analysis, showing the nine combinations of dimensionality and k.anchor values tested. **b–d**, Proportion of 'branch 1' (ERS+) cells within each cell type across all parameter combinations in the Farmer *et al.*, Wendrich *et al.*, and Shahan *et al.* datasets, respectively. The relative proportion of 'branch 1' (ERS+) cells within each lineage remains highly consistent across parameter settings. **e–g**, Pairwise Adjusted Rand Index (ARI) comparisons between all parameter combinations for transferred labels in the Farmer *et al.*, Wendrich *et al.*, and Shahan *et al.* datasets, respectively, showing strong global agreement across projections. **h–j**, Fraction of cells retaining their baseline cell type assignment across parameter combinations in the Farmer *et al.*, Wendrich *et al.*, and Shahan *et al.* datasets, respectively. Dashed lines indicate the baseline parameter set used throughout the study (50 principal components, k.anchor = 5; condition 3). **k–m**, Proportion of cells retaining the same 'branch' (ERS) state, switching 'branch' (ERS) state, or losing the 'branch' (ERS) assignment relative to the baseline parameter set in the Farmer *et al.*, Wendrich *et al.*, and Shahan *et al.* datasets, respectively. Across all datasets and parameter combinations, the vast majority of cells retained both their cell type identity and 'branch' (ERS) state assignment, indicating that the detected ERS bifurcation is robust to label transfer parameter selection.

Referee #2

Summary: I appreciate the authors' careful and thoughtful revisions in response to my initial review. The manuscript has improved substantially in providing evidence for the validation of the ERS:
- Validation of cell type and ERS+ marker genes by HCR
- Provide additional analysis that demonstrate that ERS+ cells are not analytical artifacts
- Characterization of the ERS in previous single cell/nuclei data sets
- The establishment of an ERS is modular and adaptable under varying nutrient (full MS or ½ MS) or stress (e.g. drought) conditions

My previous concerns and suggestions have been adequately addressed. The authors have provided new data and clear responses to all points raised in my initial review, incorporating the necessary revisions throughout the manuscript. The identification, characterization, and validation of the ERS as an additional cellular characteristic—beyond cell type lineage and developmental state—remain highly novel. The finding that drought stress can modulate the establishment of this state across multiple cell types, and that ABA biosynthesis and receptor mutants exhibit reduced numbers of ERS$^+$ cells, represents a particularly interesting and significant contribution.

The source genomic data are complete, and the methods are described in adequate detail to ensure transparency and reproducibility. The provided code further enhances reproducibility. The statistical analyses are appropriate, and there are no apparent data that would require additional statistical testing. The reporting of error bars and probability values is clear and consistently presented in figure legends and relevant text sections.

The following point is an optional suggestion that could further refine a point of discussion, though it does not affect the overall novelty or conclusions of the study:

-Page 16 Line 5-8: I agree with the authors` conclusion that the underlying regulatory networks of the ERS could vary between cell types, which causes the described degree of acquisition of the ERS-state between cell types. However, as the ERS is regulated by ABA, differences in ABA sensitivity or signaling among cells within and between cell types could also offer a plausible explanation. I understand that testing this hypothesis is beyond the scope of the present manuscript, but this argument could be added to the current statement to provide a more comprehensive view of the possible regulatory mechanisms influencing ERS establishment.

Response: We thank the referee for the very positive assessment of the revised manuscript and for recognising the substantial additional work incorporated in the revision. We are pleased that the referee considers the previous concerns to have been adequately addressed and that the novelty and significance of the ERS framework are appreciated.

We also thank the referee for this thoughtful suggestion regarding potential differences in ABA sensitivity or signalling between and within cell types as an additional factor influencing ERS establishment. We agree that this represents an important and plausible complementary mechanism to differences in the underlying regulatory networks controlling ERS acquisition. While experimentally dissecting this possibility is beyond the scope of the present study, we agree that it provides a valuable additional perspective on the potential determinants of ERS establishment.

We have therefore revised the Discussion to incorporate this possibility and now explicitly note that variation in ABA sensitivity or signalling competence between cells and cell types may also contribute to the

differential propensity of cells to acquire the ERS state, as follows: "*Differences in ABA sensitivity or signaling capacity among cells within and between cell types could equally contribute to this variability, and represent an important avenue for future investigation.*"

Minor comments:

Referee 2 - point 1: Page 15 Line 9: "sRNA-seq" should be scRNA-seq
Response: Thank you, this has now been fixed.

Referee 2 - point 2: Page 32 Line 16: "Putative TF targets genes" should be "Putative TF target genes"
Response: Thank you, this has now been fixed.

Referee 2 - point 3: Figure 3 panel D: the y-axis should be "Cell state proportion"
Response: Thank you, this has now been fixed.

Referee #3

Summary: The authors have done extensive and commendable additional work in this revision, including generating new datasets, performing new experiments, and developing new analyses under specific conditions. These efforts substantially strengthen the study. However, our main concern remains whether branch 2 represents a true biological state or a technical artifact. There are some additional analyses suggested below that could be helpful and are not "major lifts". Depending on the authors' take on our concerns, we could imagine these being addressed through minor revisions & textual changes that reflect the concerns or remaining uncertainty.
Response: We thank the referee for the thoughtful summary and for recognising the substantial additional work performed in this revision. We appreciate that the central remaining question is whether branch 2/ERS− represents a *bona fide* biological state rather than a technical artefact, and we have focused this revision on addressing that concern as directly and comprehensively as possible.

In response, we have substantially strengthened the manuscript through multiple new orthogonal analyses specifically aimed at testing the biological validity of branch 2/ERS− cells. These include improved quantitative analyses addressing low-UMI/damaged-cell hypotheses, revised UMI downsampling and regression-based modelling of expected transcript loss under reduced-capture scenarios, robustness analyses across external datasets and projection parameters, and additional biological controls that exclude alternative explanations such as cell cycle and developmental-stage effects.

Most importantly, we have now added direct experimental evidence for the existence of ERS− cells *in vivo* by identifying an ERS−-enriched epidermal marker and demonstrating, through multiplex HCR and quantitative 3D fluorescence segmentation, the presence of two mutually exclusive epidermal populations corresponding to ERS+ and ERS− states. Together with the existing HCR, ABA/drought perturbation, and cross-dataset validation analyses, we believe these additions (detailed below) provide strong and convergent evidence that branch 2/ERS− represents a genuine biological cell state rather than a technical artefact.

Major comments:

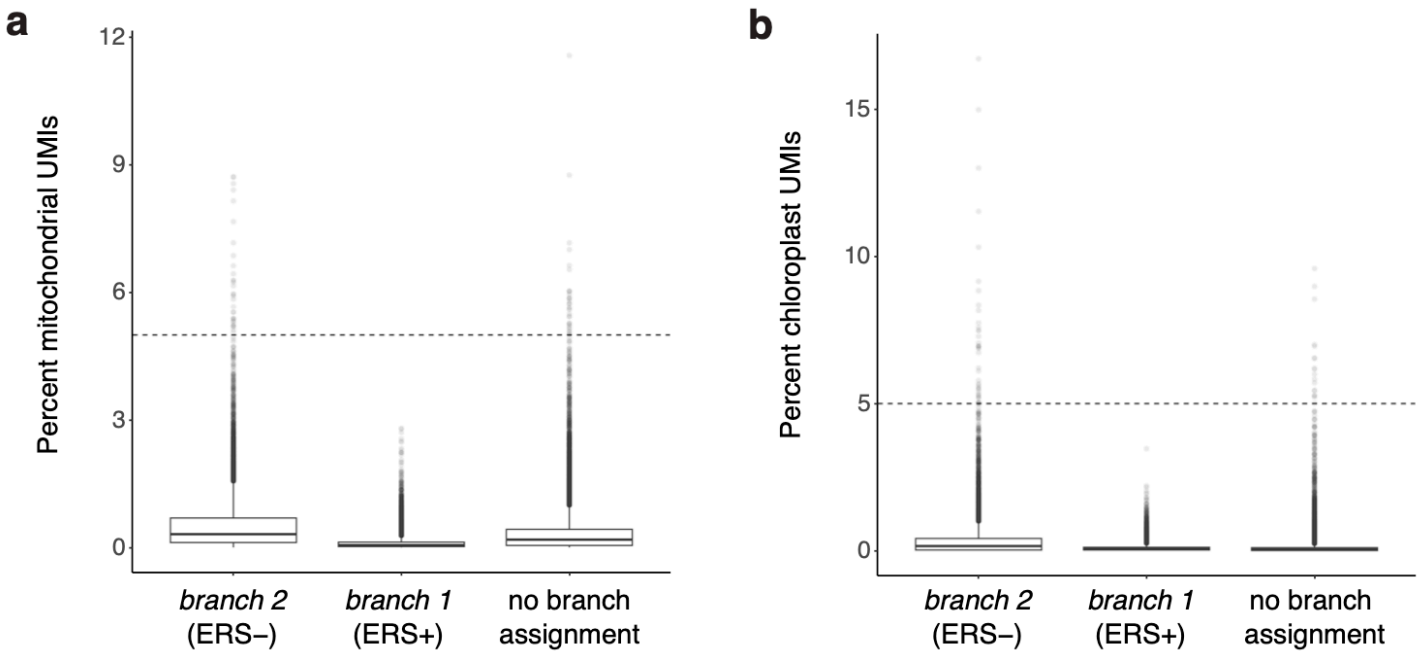
Referee 3 - point 1: Low UMI / High Mitochondrial %
Branch 2 cells have low UMI counts and high mitochondrial percentages, a common issue in scRNA-seq where low-quality cells form spurious clusters. In Extended Fig. 9, Branch 2 appears to show higher mitochondrial % and chloroplastic % than Branch 1, despite the text stating they are comparable. The overplotted jitter makes it difficult to see the distribution clearly; a statistical comparison (and perhaps clearer violin or density plots) would help.
Response: Thank you for the opportunity to clarify the intent of this figure. Because branch 2 cells have lower overall UMI content, mitochondrial and chloroplastic reads represent a larger fraction of their transcriptomes, resulting in higher mitochondrial/chloroplastic percentages relative to branch 1 cells. Our intention was not to suggest that these percentages are identical between branches, but rather to show that branch 2 cells do not exhibit mitochondrial/chloroplastic proportions consistent with the levels typically used to define low-quality or damaged cells in plant scRNA-seq studies.

Specifically, only a very small minority (0.16% for mitochondrial reads and 0.19% for chloroplastic

reads) of branch 2 cells exceed the 5% mitochondrial/chloroplastic percentage thresholds commonly applied as hard filtering cut-offs in published plant single-cell studies (**Resp. Fig. 12 / Ext. Data Fig. 9**). Thus, the vast majority of branch 2 cells would not have been excluded based on these criteria. This analysis was included in response to concerns raised during the previous revision regarding whether branch 2 cells might represent damaged cells based on organellar read content.

Importantly, other abundant cell populations in our dataset that are not affected by the ERS dichotomy (named 'non-ERS' cells), most notably root cap cells, also display relatively low UMI content and correspondingly elevated mitochondrial/chloroplastic percentages comparable to or exceeding those of branch 2 cells (**Resp. Fig. 12 / Ext. Data Fig. 9**), yet are clearly *bona fide* biological populations rather than low-quality cells. This further supports the conclusion that the observed organellar read fractions in branch 2 cells reflect lower overall transcript capture rather than cell damage.

We agree that the overplotted jitter representation was not optimal for conveying these distributions. We have therefore revised the figure to display the data using boxplots (**Resp. Fig. 12 / Ext. Data Fig. 9**), which more clearly illustrate the distribution of mitochondrial and chloroplastic percentages across populations.



Resp. Fig. 12 / Ext. Data Fig. 9: Extended Data Fig. 9: 'Branch 2' cells do not show mitochondrial or chloroplast markers of low-quality cells. Distribution of the percentage of mitochondrial and chloroplast genes per category: cells from the 'branch 1' (ERS+) trajectories, cells from the 'branch 2' (ERS–) trajectories, and cells not assigned to these branches (root cap and early stages before branching). Only a few dozen cells in our reference atlas of >87,000 cells exceed 5%, a commonly used threshold for low-quality cells. Moreover, 'branch 2' cells, which have lower UMI content, display mitochondrial and chloroplast gene proportions comparable to root cap and early cells, suggesting they are unlikely to represent damaged cells.

Referee 3 - point 2: Lack of distinct gene expression features

Most of the analyses (e.g., scRNA-seq across ecotypes, snATAC-seq) primarily strengthen evidence for Branch 1. Since the central question is whether Branch 2 is real, a more direct approach would be to identify specific genes that are up- or downregulated in Branch 2 with biological interpretability. To our reading, there are still no clear gene expression features defining Branch 2. In analogous datasets, distinct clusters are characterized by specific genes that can usually be either connected to the literature or some function in a clear, believable way (e.g. known/clear markers are best, but GO modules, though not our favorite, and the like often suffice). This facilitates gaining confidence (for authors, reviewers and readers) that a given cluster is real rather than an artifact. The authors provide some explanations in their response why distinct features were not found. We found these points to be reasonable but not entirely convincing. We recognize that this concern is not really addressable if the given reasons are valid, but it is another reason for caveating Branch 2.

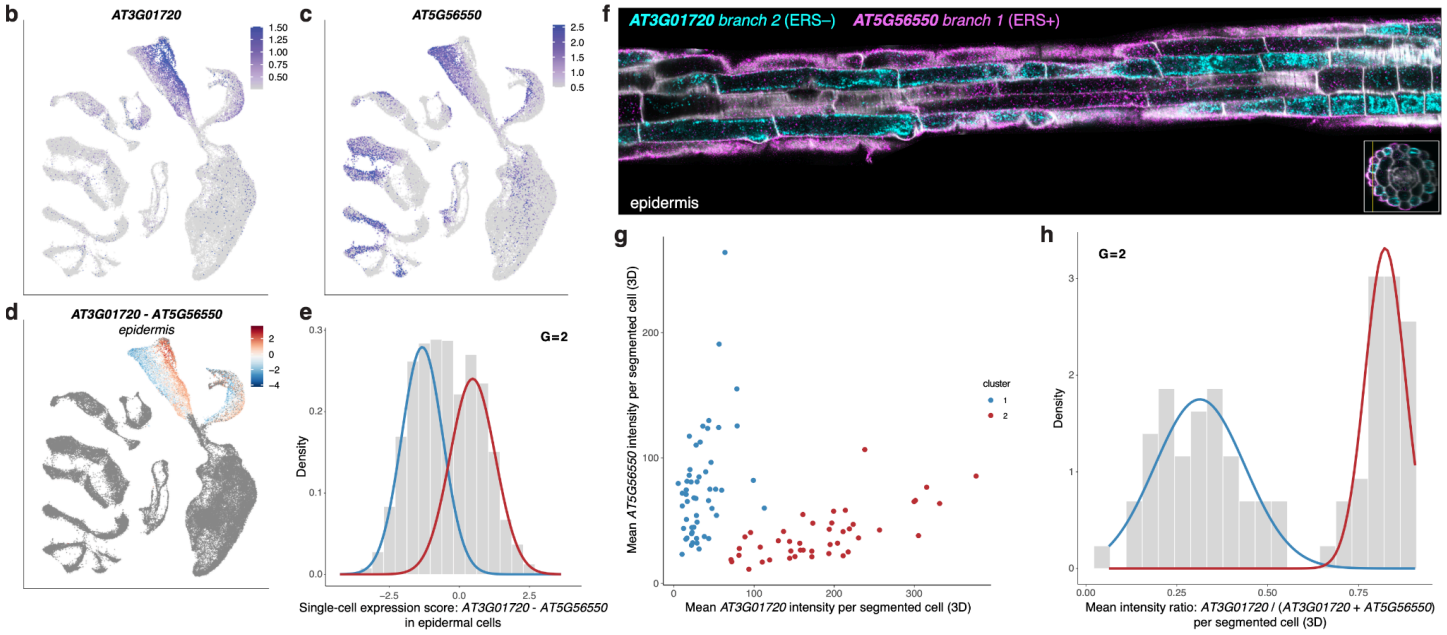
Response: We agree that one of the central challenges of the framework described in this manuscript is that, unlike many discrete cell states identified in single-cell studies, ERS− cells are not characterised by a strong branch-specific transcriptional programme. Rather, ERS+ and ERS− populations share the canonical developmental and cell-type markers of their underlying lineage, with ERS+ cells distinguished primarily by the superimposition of an additional large co-expressed transcriptional programme. As a result, validation of the

existence of two distinct populations has largely relied on demonstrating that 'branch 2'/ERS− cells do not represent technical artefacts.

While this overall conceptual framework remains unchanged and no broad ERS−-specific transcriptional programme has been identified, closer inspection of epidermis-specific genes revealed a small number of candidates enriched in ERS− cells. In particular, *AT3G01720* shows enrichment in ERS− epidermal cells and is expressed at levels comparable to the ERS marker *AT5G56550*, with the two genes displaying largely mutually exclusive expression patterns in the epidermis in the scRNA-seq data (**Resp. Fig. 13b-d / Fig. 3b-d**). Gaussian mixture modelling of their single-cell expression profiles further supported the presence of two distinct epidermal populations based on these markers (**Resp. Fig. 13e / Fig. 3e**), making them strong candidates for orthogonal experimental validation.

We therefore performed simultaneous whole-mount HCR detection of *AT3G01720* and *AT5G56550*, which revealed a clear mutually exclusive expression pattern *in vivo* (***Resp. Fig. 13f / Fig. 3f***). Segmentation and volumetric fluorescence quantification of epidermal cells across three independent roots confirmed the presence of two distinct populations, one enriched for *AT3G01720* and the other for *AT5G56550* (**Resp. Fig. 13g / Fig. 3g**), with Gaussian mixture modelling of the HCR-derived per-cell fluorescence ratios again supporting bimodality (**Resp. Fig. 13h / Fig. 3h**).

Thus, although ERS− cells are not defined by a broad alternative transcriptional programme analogous to the ERS-associated signature of ERS+ cells, these new orthogonal imaging data provide direct experimental evidence for the existence of a distinct ERS−/branch 2 epidermal population *in vivo*, strengthening the conclusion that branch 2 reflects a *bona fide* biological cell state rather than a technical artefact.



Resp. Fig. 13b-h / Fig. 3b-h: Whole-mount HCR validates the bifid topologies and associated branch-specific genes. b–e, A 'branch 2' (ERS–)-specific gene (*AT3G01720*), mutually exclusive to the 'branch 1' (ERS+) marker *AT5G56550*, is identified in the epidermis. UMAPs showing the expression pattern of *AT3G01720* (b), *AT5G56550* (c), and the expression score *AT3G01720* − *AT5G56550* in the epidermis (d). Distribution of the expression score across epidermal cells and Gaussian mixture modelling support the existence of two distinct populations (G = 2; Bayesian information criterion (BIC) favoured two over one component), represented by the blue and red lines (e). **f–h**, The two epidermal branches and associated markers are also detected by whole-mount HCR. Optical section showing simultaneous transcript detection of *AT3G01720* ('branch 2' marker) and *AT5G56550* ('branch 1' marker) in the epidermis. The yellow line in the inset transverse optical section of the same root indicates the plane of the longitudinal optical section, which passes through 4-5 epidermal cell files. Distribution of mean signal intensity for *AT3G01720* and *AT5G56550* (g), and of the mean intensity ratio *AT3G01720* / (*AT3G01720* + *AT5G56550*) (h) per epidermal cell measured after 3D segmentation. Gaussian mixture modelling supports the existence of two distinct populations (G = 2; Bayesian information criterion (BIC) favoured two over one component), represented by clusters in (g) and red lines in (h). Quantifications were performed on 97 epidermal cells from three independent roots.

Referee 3 - point 3: Extended Figures 9 & 10 Interpretation

We do not see how Extended Fig. 10 supports the authors' statement, which is very confusing. From Extended Figs. 9 and 10, it seems the authors have not fully ruled out the possibility that the second branch represents

damaged cells with lower UMI capture (characterized by high mito% and low UMI ***which is a a common issue*** in scRNA-seq analysis. On a related point, Extended Fig. 10 is intended to show that genes enriched in Branch 1 span a similar expression range to those expressed in both branches, suggesting that Branch 1-specific genes aren't simply missed in low-UMI cells. Conceptually this makes sense, but the data are overplotted, and many Branch 1-specific genes appear lowly expressed. Two violin plots (one for "branch 1-specific" and one for "branch 1 + Branch 2-specific" genes) showing their average expression in Branch 1 might make this comparison clearer. Currently, this figure feels more qualitative than quantitative.

Response: Thank you for this suggestion. We agree that the original presentation of Extended Data Fig. 10 was not sufficiently clear and remained overly qualitative. We have therefore revised the figure and analysis to improve interpretability and to present the comparison more quantitatively.

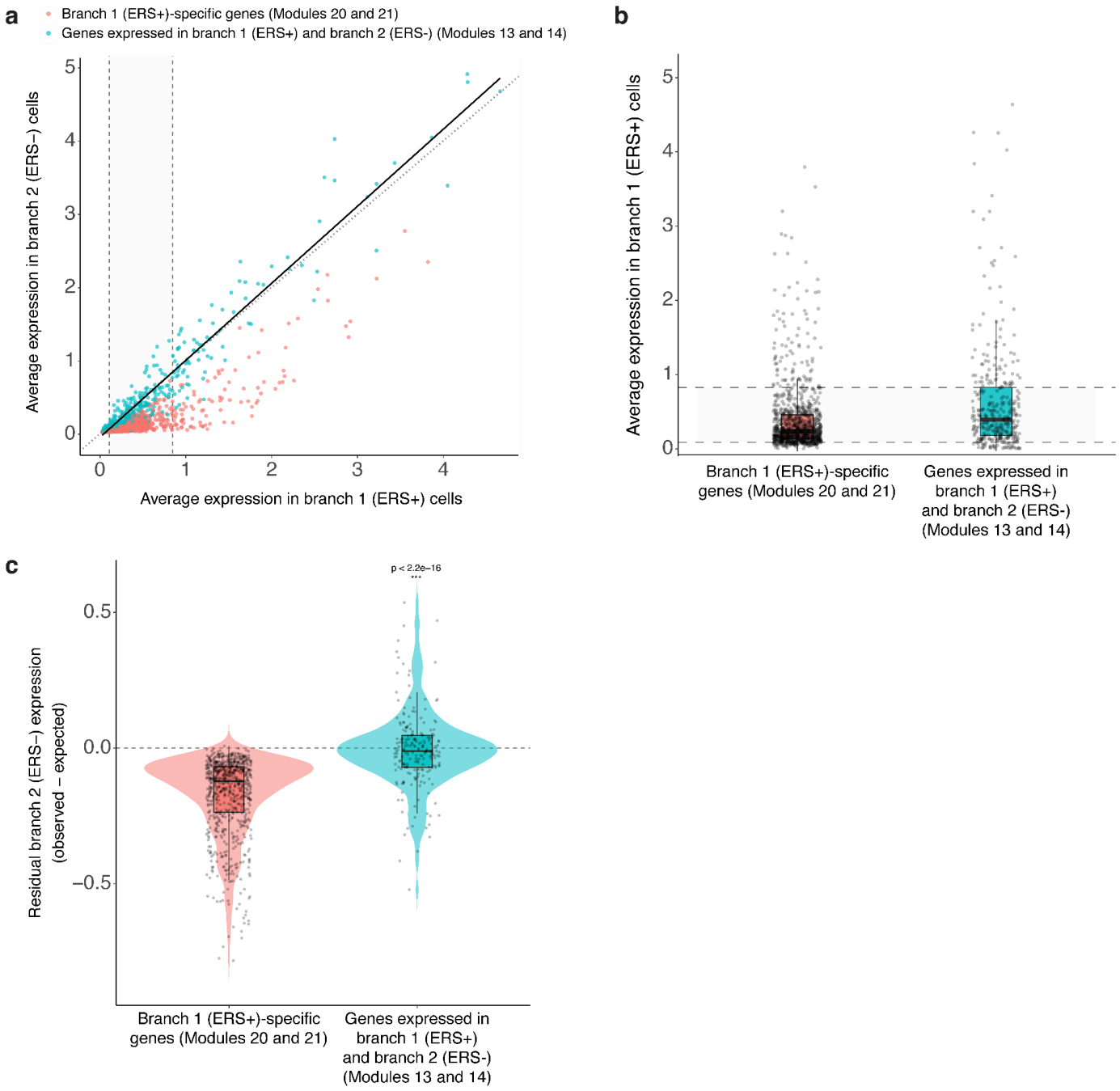
The purpose of this analysis was to test whether the apparent absence of ERS-associated genes in ERS− cells could be explained simply by lower transcript capture in these cells, i.e. whether ERS− cells might represent damaged or partially captured counterparts of ERS+ cells in which lower-expressed ERS genes fall below the detection threshold.

As noted by the referee, genes from modules expressed in both branches are on average more highly expressed than branch 1-specific genes. However, the lower tails of the two expression distributions overlap substantially (**Resp. Fig. 14a,b / Ext. Data Fig. 10a,b**), indicating that both groups contain genes expressed at similarly low levels in ERS+ cells. This argues that many ERS-associated genes should remain detectable in ERS− cells if their absence were solely due to reduced transcript capture.

To test this more quantitatively, we modelled the expected ERS− expression based on the behaviour of genes expressed in both branches. Using xylem pole pericycle, the largest lineage affected by the ERS dichotomy, as a test case, we fitted a linear model describing the relationship between ERS+ and ERS− expression for genes from modules expressed in both branches. This model therefore represents the ERS− expression expected under a simple reduced-capture scenario. We then calculated residual ERS− expression values (observed minus expected expression from the fitted model) for all genes and compared the residual distributions of branch 1-specific genes and genes expressed in both branches within the shared expression range of the two categories. Branch 1-specific genes showed significantly more negative residual ERS− expression values than genes expressed in both branches (**Resp. Fig. 14c / Ext. Data Fig. 10c**), indicating that these genes are depleted in ERS− cells beyond what would be expected from reduced transcript capture alone. Together, these analyses demonstrate that the absence of ERS-associated genes in ERS− cells is inconsistent with a simple low-UMI or damaged-cell explanation.

Regarding Extended Data Fig. 9, this figure was included in response to requests during the previous review round to assess whether ERS− cells would have been excluded by conventional mitochondrial/chloroplastic filtering thresholds (**see Response to Referee 3 - point 1**). While we agree that neither Extended Data Fig. 9 nor 10 alone constitutes definitive proof of biological relevance, these analyses form part of a broader series of quality-control analyses aimed at systematically excluding technical artefact explanations.

Importantly, these computational analyses are complemented by multiple independent biological validations, including: (i) *in vivo* HCR imaging demonstrating neighbouring ERS marker-positive and -negative cells within the same tissue layers; (ii) decrease in ERS+ cell proportion in ABA-pathway mutants, consistent with the predicted regulatory associations of ERS-associated genes; (iii) strong environmentally induced increase in ERS acquisition under drought stress observed by both scRNA-seq and HCR; and (iv) most importantly, the new identification and experimental validation of an ERS−-enriched epidermal marker, enabling direct HCR-based detection of two mutually exclusive epidermal populations *in vivo* (**see Response to Referee 3 – point 2**). Together, these analyses strongly support the conclusion that the ERS− cells represent a *bona fide* biological cell population rather than damaged cells.

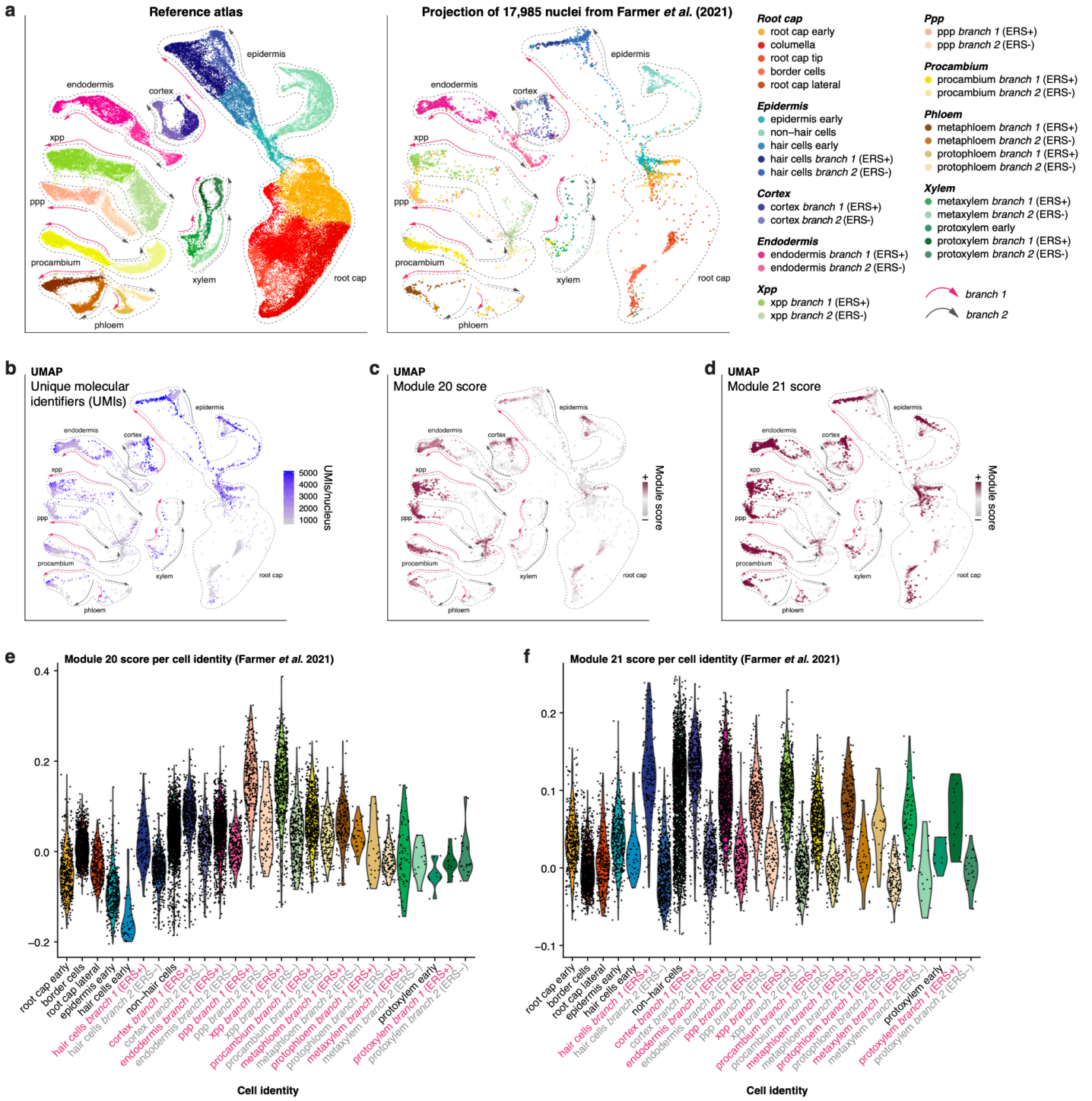


Resp. Fig. 14 / Ext. Data Fig. 10: Differences in gene content between 'branch 1' and 'branch 2' are not driven by lowly expressed genes. a, Scatter plot showing, for the xylem pole pericycle, the average expression in 'branch 1' (ERS+) versus 'branch 2' (ERS−) cells for genes from two categories of co-expression modules: those that are 'branch 1'-specific (Modules 20 and 21) and those expressed in both branches (Modules 13 and 14). The dotted line indicates the identity line (x = y), and the solid black line indicates the linear regression fitted using genes expressed in both branches. Dashed vertical lines indicate the overlapping range of 'branch 1' expression between the two gene categories, defined from the common 10th to 90th percentile range and used for the controlled analysis in panel c. **b,** Distribution of average expression in 'branch 1' (ERS+) cells for the two gene categories. Dashed horizontal lines indicate the same overlapping expression range shown in panel a. Although genes expressed in both branches extend further into the upper tail of the distribution, both gene categories include genes within the same lower expression range. **c,** Residual 'branch 2' (ERS−) expression, calculated as the observed 'branch 2' expression minus the expression expected from the regression fitted in panel a. Residuals are shown for genes within the overlapping 'branch 1' expression range indicated in panels a and b. 'Branch 1'-specific genes show significantly lower residual 'branch 2' expression than genes expressed in both branches (Wilcoxon rank-sum test), indicating that their absence from 'branch 2' cannot be explained solely by lower expression or reduced transcript detection in cells with lower UMI content.

Referee 3 - point 4: Integration Analysis

The evidence from the integration analysis seems somewhat arbitrary. First, the first dataset (Extended Fig. 13) did not detect Branch 2. Second, the second dataset (Extended Fig. 14) was not analyzed with the same integration approach as the first. Third, it is unclear what cell filtering criteria were applied to each dataset; applying different UMI or mito% cutoffs could probably determine whether Branch 2 appears or disappears.

Response: Thank you for the opportunity to clarify this point. To avoid potential confusion with regard to referencing figure numbers in this response, we would note that in the previous manuscript version, the 'first dataset' was presented in Ext. Data Fig. 12 (the Farmer *et al.* dataset). We would first note that branch 2/ERS− cells were detected in this Farmer *et al.* dataset (**Resp. Fig. 15 / Ext. Data Fig. 12**), as evidenced by the distribution of ERS-associated module scores for each individual nucleus (**Resp. Fig. 15e-f / Ext. Data Fig. 12e-f)**, as well as numerous nuclei projected onto branch 2 for almost all lineages in the UMAP embeddings (**Resp. Fig. 15a-d / Ext. Data Fig. 12a-d**). However, because this dataset is based on snRNA-seq rather than whole-cell scRNA-seq, nuclei were isolated by tissue chopping, a process that likely under-samples the small populations of early developmental cells near the root tip. As a consequence, early branch cells and the full continuity of the branching topology are less clearly represented in the embedding, even though ERS+/ERS− populations remain detectable by module score and label transfer. We would further note that we detected branch 2/ERS− cells in all three previously published datasets: Farmer *et al.*, Wendrich *et al.*, and Shahan *et al.* (Ext. Data Figs. 12, 13, and 14).



Resp. Fig. 15 / Ext. Data Fig. 12: Branches and their associated 'branch 1'-specific genes are detected in the snRNA-seq dataset from Farmer *et al*. **a,** UMAPs showing our reference atlas and the projection of nuclei from Farmer *et al*. into the atlas UMAP embedding, with cells coloured by their assigned identity based on our atlas. **b,** UMAP showing the distribution of UMI content per nucleus in Farmer *et al*. **c–f,** UMAPs (c, d) and violin plots (e, f) showing the distribution of expression scores for our 'branch 1'-specific modules 20 and 21 in the snRNA-seq data from Farmer *et al*.

Regarding the integration strategy, all label transfer analyses were performed using the same integration/projection framework across the three external datasets, as described in the Methods. The only technical difference is that for the Shahan *et al.* dataset (Extended Data Fig. 14), we were unable to project the transferred labels directly onto our reference atlas UMAP embedding due to compatibility issues between the downloadable Seurat object version provided by that study and the Seurat version used in our analysis. We therefore retained the original Shahan *et al.* embedding for visualisation. Importantly, this does not affect the label transfer itself. Indeed, we believe that retaining their original embedding is advantageous, as their trajectory structure differs from ours in that the lineages converge more clearly to a common t = 0 before each separates into two branches, providing an independent visual representation of the same bifurcating topology.

With respect to filtering criteria, we agree that stringent filtering can strongly influence whether ERS−/branch 2 cells are retained, and indeed this was precisely the point illustrated by our reanalysis of the Wendrich *et al.* dataset (Extended Data Fig. 13). In that study, the authors applied a hard UMI threshold during preprocessing, which removed the ERS− population from the processed dataset. By reprocessing the raw data using our own filtering strategy, we were able to recover these cells and identify the ERS dichotomy. In contrast, the Farmer *et al.* and Shahan *et al.* datasets already retained ERS− cells in their publicly available processed objects and therefore did not require reprocessing. For the Farmer *et al*. dataset, we used the publicly available Cell Ranger output matrices without additional filtering. For the Shahan *et al*. dataset, the authors had already applied quality-control filtering using the COPILOT pipeline, including removal of low-quality cells based on mitochondrial transcript enrichment and similarity to low-quality expression profiles, exclusion of UMI-count outliers and predicted doublet.

Thus, while preprocessing choices in some cases affected whether ERS− cells were retained in the published processed data, the downstream integration and label transfer analyses were performed consistently across all three external datasets.

Referee 3 - point 5: UMI Downsampling

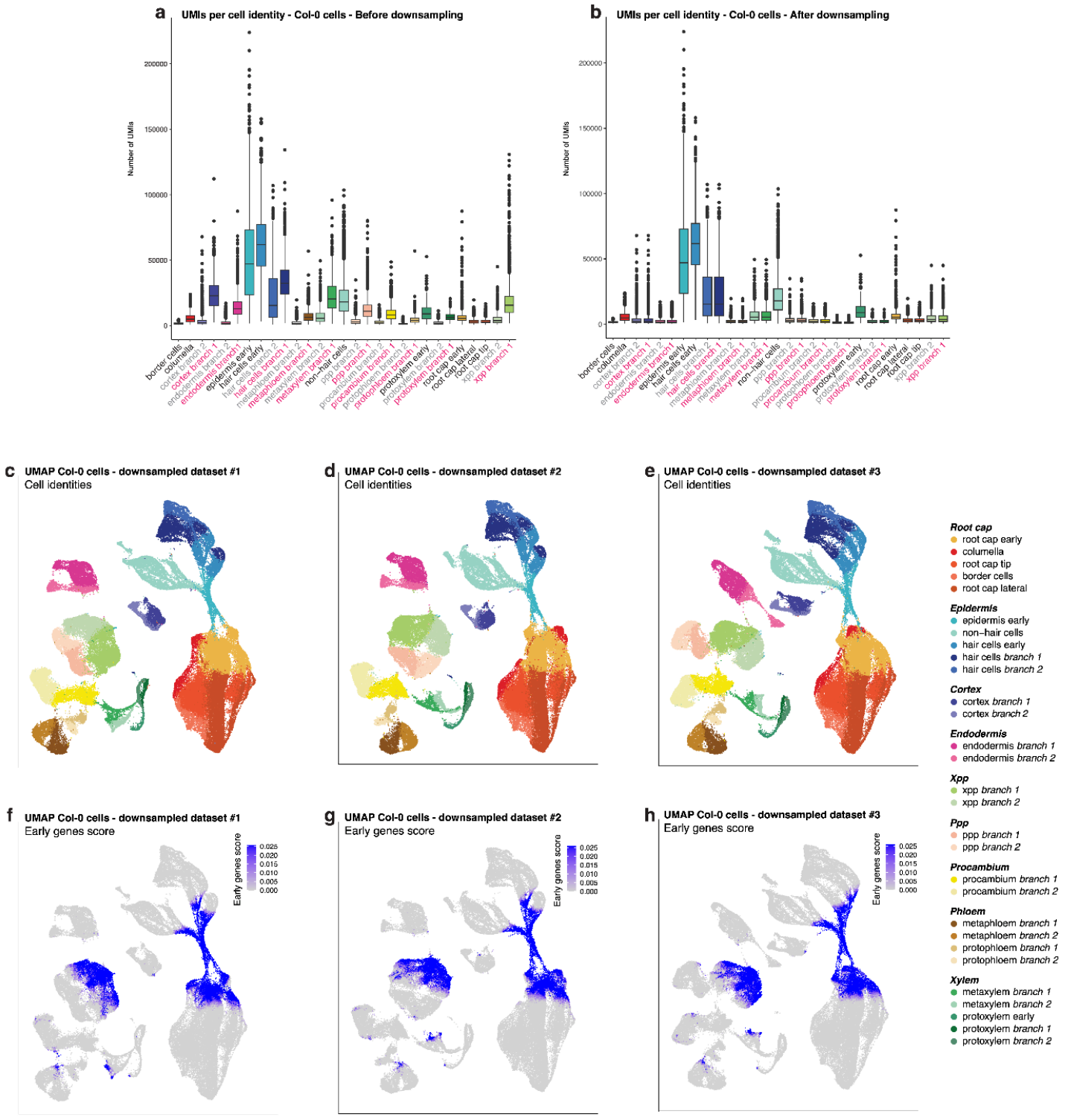
To test whether branch separation is driven by UMI differences, the authors downsampled cells with >1,000 UMIs to 1,000 while leaving lower-UMI cells unchanged. It's not clear why 1,000 was chosen (perhaps it corresponds to the average of Branch 2?) but ideally, downsampling would match the full UMI distribution of Branch 2 rather than applying a fixed cutoff. Even after downsampling, Branch 2 remains slightly lower and retains a pronounced low-UMI tail not seen in Branch 1.

Response: Thank you for this helpful suggestion. We agree that matching the full UMI distribution of branch 2/ERS− cells provides a more appropriate test of whether the observed bifurcation could be driven by differences in transcript capture alone.

Accordingly, we have now revised our downsampling strategy such that branch 1/ERS+ cells are no longer downsampled to a fixed UMI threshold. Instead, branch 1/ERS+ cells are now randomly downsampled to match the full UMI distribution of their branch 2/ERS− counterparts within each lineage. This procedure was repeated independently three times.

Importantly, the conclusions remain unchanged. In all three downsampling replicates, the two branch populations remain clearly separable, and cells with the highest meristematic scores consistently localise between the two branches, maintaining the inferred t = 0 position and supporting the bifurcating trajectory topology (**Resp. Fig. 16 / Extended Data Fig. 11**).

These results further demonstrate that the ERS-associated branching structure cannot be explained solely by differences in UMI content between branch 1 and branch 2 cells.



Resp. Fig. 16 / Ext. Data Fig. 11: Branching events are not an artefact caused by coverage. a-b, Box plots showing the UMI content distribution per cell identity in our reference atlas, before and after UMI downsampling. UMI content was downsampled in 'branch 1' (ERS+) cells to span the same range as their 'branch 2' (ERS–) counterparts. **c–h**, UMAP plots showing the cell identities and 'meristematic score' used for pseudotime assignment, after three independent UMI downsampling computations. Branches are detected in all three downsampling replicates, with the cells with the highest 'meristematic score' per lineage consistently positioned between branches, as in the reference atlas.

Referee 3 - point 6: Cell Cycle Effects (Extended Fig. 6b)
The text states that branch separation is not explained by cell cycle stage, which may be true, but there appears to be a consistent difference in the proportion of G1 cells between branches. Although the two branches remain after regressing out cell cycle effects, regression may not fully remove all related variation?
Response: We agree that a modest difference in cell-cycle phase proportions between branches is visible in some lineages. However, if the observed bifurcation were primarily driven by cell-cycle state, a substantially stronger segregation by cell-cycle phase would be expected. Importantly, regression of cell-cycle scores does

not abolish the branching structure, indicating that cell-cycle-associated transcriptional variation is not sufficient to explain the observed dichotomy.

More broadly, we consider a cell-cycle-based explanation unlikely for biological reasons. First, if ERS+ and ERS− populations primarily reflected different cell-cycle stages, clear differences in cell size and morphology would be expected in the HCR images between ERS marker-positive and -negative cells. No such systematic differences are observed.

Second, previous studies using mitotic/cell division markers have shown that cell division in the Arabidopsis primary root is largely confined to the meristematic zone near the root tip and becomes rare once cells enter elongation and differentiation zones, where the ERS+/ERS− dichotomy is most prominently observed[1,2]. Thus, the spatial position of the ERS-associated branching is inconsistent with the expected localisation of proliferative cell-cycle effects.

Together, these observations strongly suggest that while minor cell-cycle differences may contribute modestly to transcriptional variation between branches, they do not account for the ERS-associated bifurcation observed in our data.

Referee 3 - point 7: HCR Validation (Fig. 2e)

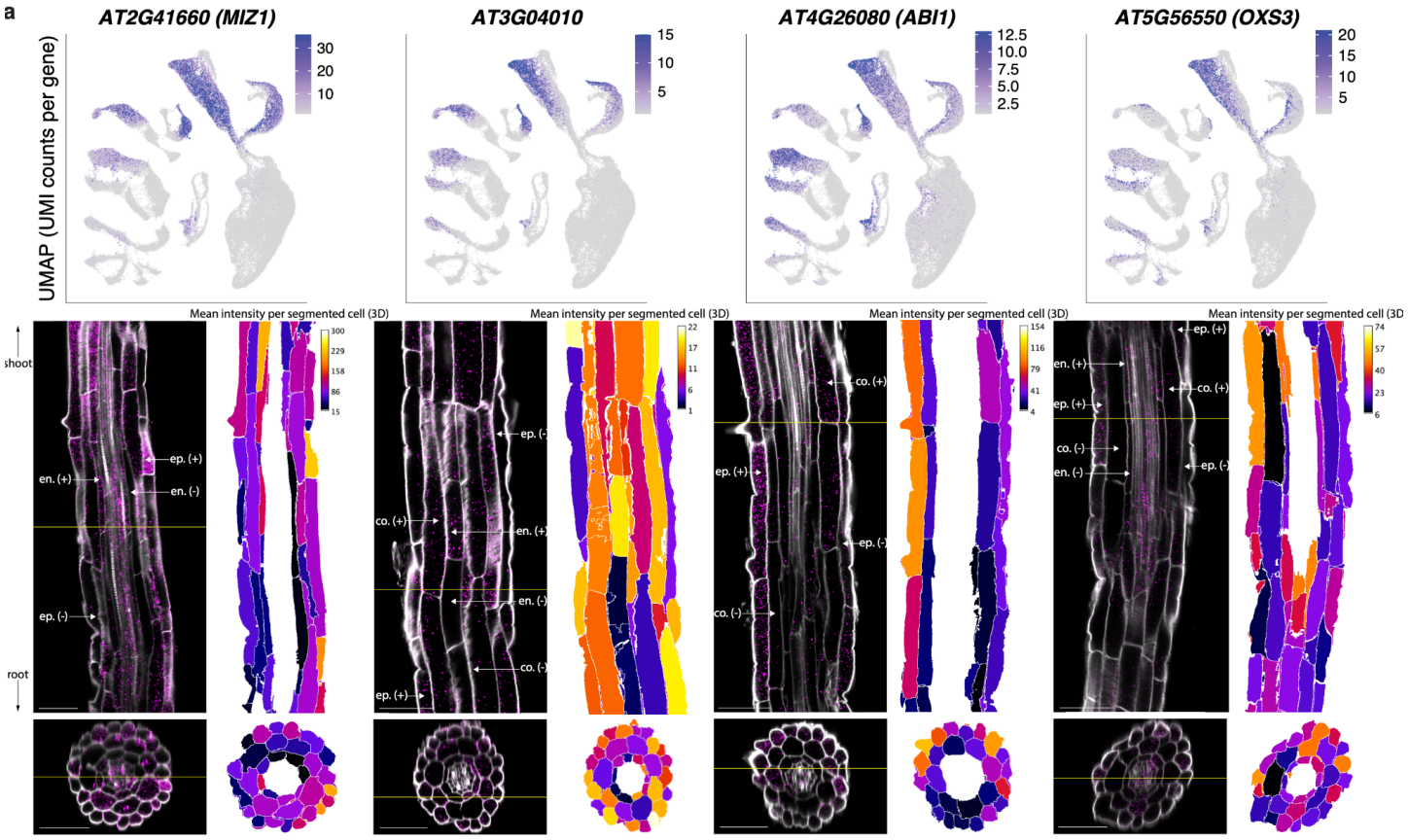
The HCR experiments validating Branch 1–specific genes are among the most promising evidence. We suggest adding quantitative analyses and results, rather than only highlighting selected examples through visualization, as this would provide stronger support.

Response: Thank you for the positive comment regarding the HCR validation. We agree that quantitative analysis provides stronger support than representative optical sections alone.

We initially did not include segmentation-based quantification because the thin, elongated cells located in the inner tissues of the root are very difficult to segment reliably in three dimensions. However, for the markers shown in Fig. 2 (now Fig. 3 in the revised manuscript), the relevant positive and negative cells are located in the outer root layers (epidermis, cortex, and endodermis), where cell boundaries are larger and more readily segmented.

We have therefore now performed 3D segmentation of these outer tissue layers in the roots presented in Fig. 3 of the revised manuscript, and quantified volumetric fluorescence intensity on a per-cell basis. In the revised figure, segmented cells are colour-coded by mean fluorescence intensity, providing quantitative support for the positive and negative cells highlighted in the representative images (**Resp. Fig. 17 / Fig. 3a**). For all four ERS-associated markers, the cells visually identified as positive and negative show clear and substantial differences in fluorescence intensity, with cells within the same layer differing by up to 20 fold-change in signal intensity (**Resp. Fig. 17 / Fig. 3a**). Furthermore, we have also performed 3D segmentation and per-cell quantification of volumetric fluorescence intensity for the new HCR experiments comparing the ERS+ (*AT5G56550*) and ERS- (*AT3G01720*) markers in epidermal cells (see **Resp. Fig. 13f-h / Fig. 3f-h**).

These quantitative analyses confirm that the heterogeneous HCR patterns highlighted in the representative images reflect robust and reproducible differences in transcript abundance between neighbouring cells, providing strong orthogonal support for the existence of ERS-associated transcriptional heterogeneity *in vivo*.



Resp. Fig. 17 / Fig. 3a: Whole-mount HCR validates the bifid topologies and associated branch-specific genes. a, UMAP plots and optical sections with associated segmentation showing transcript detection of four 'branch 1' markers, *AT2G41660* (*MIZ1*), *AT3G04010*, *AT4G26080* (*ABI1*), and *AT5G56550* (*OXS3*), both *in silico* from scRNA-seq data and *in vivo* by whole-mount *in situ* Hybridization Chain Reaction (HCR). Yellow lines indicate the relative positions of longitudinal and transverse cross sections. Example cells that are positive (+) or negative (–) for each transcript in the epidermis (ep.), cortex (co.), or endodermis (en.) are indicated with arrows. The associated segmented images show the same optical section after segmentation of the root layers (epidermis, cortex, endodermis), in which positive and negative cells were identified. Cells are colored by mean intensity signal (measured in 3D). Scale bar = 50 µm.

Response references:

1. Verbelen, J.-P., De Cnodder, T., Le, J., Vissenberg, K. & Baluska, F. The root apex of Arabidopsis thaliana consists of four distinct zones of growth activities: Meristematic zone, transition zone, fast elongation zone and growth terminating zone. *Plant Signal. Behav.* **1**, 296–304 (2006).
2. Lavrekha, V. V., Pasternak, T., Ivanov, V. B., Palme, K. & Mironova, V. V. 3D analysis of mitosis distribution highlights the longitudinal zonation and diarch symmetry in proliferation activity of the Arabidopsis thaliana root meristem. *Plant J.* **92**, 834–845 (2017).

Point by point response to comments on Nature manuscript 2022-06-09447A

We extend our sincere thanks for the constructive feedback and time and effort in considering our manuscript. We believe we have addressed all comments and requested changes, which has helped us improve our study considerably. Below we provide a point-by-point response to all comments and suggestions.

Referee #1

Summary: The authors have strengthened the manuscript by performing orthogonal analyses to support the presence of two transcriptional branches, incorporating additional biological replicates, and testing the role of ABA in regulating the proportion of ERS^{+} cells. However, several aspects of the data interpretation and presentation would benefit from further clarification and additional controls to ensure that the conclusions are fully supported. In particular, clearer definitions of the ERS^{+}/ERS^{-} states, validation of HCR-based transcript detection, and improved visualization of comparative datasets would enhance the overall robustness and clarity of the study.

Response: We thank the referee for this constructive summary and for recognising the additional analyses incorporated in the revised manuscript. In response to these comments, we have revised the manuscript to clarify the conceptual framework underlying the ERS+/ERS− states and revising the text throughout to better explain that environmental conditions modulate the frequency with which cells acquire the ERS state, rather than defining intrinsically responsive versus unresponsive populations.

We have also expanded the orthogonal experimental validation of the ERS-associated heterogeneity by incorporating additional HCR controls and quantitative analyses, including co-detection of ubiquitous internal control transcripts, 3D segmentation-based volumetric fluorescence quantification, and experimental validation of mutually exclusive ERS+/ERS− epidermal markers *in vivo*. Together, these analyses provide direct quantitative support for the existence of distinct ERS+ and ERS− populations.

In parallel, we have strengthened the computational and comparative analyses by adding quantitative cross-method trajectory comparisons, RNA velocity-based validation, robustness statistics for cross-dataset label transfer, revised UMI downsampling analyses, and additional controls addressing potential technical confounders including transcript capture, filtering thresholds, and developmental stage effects.

Collectively, we believe these additions substantially improve both the clarity and robustness of the manuscript and provide strong orthogonal support that the ERS-associated bifurcation described in this study reflects a genuine biological feature of root cell differentiation.

Major comments:

Referee 1 - point 1: The authors claim in the abstract that "*The discovery of this alternate fate trajectory, induced by a highly active and environmentally responsive cell state, reveals an additional layer of root cell identity that may underpin the adaptability of roots to challenging environments.*" However, there are several aspects of this claim that were not sufficiently made. First, while cells in an ERS+ and ERS- state were identified for specific tissue types, the authors have not shown that these cells are especially responsive to the environment, only that the proportion of cells in this state increases with stress. It is not clear how cells transition into and out of the ERS+/- states and whether cells with an ERS+ state are any more responsive to stress than ERS- cells. One possible approach would be to use ABA FRET sensors (e.g., ABACUS or ABAleon) to visualize basal ABA levels in individual root hair cells, then apply an abiotic stress treatment to determine whether cells with higher basal ABA concentrations indeed exhibit faster or stronger ABA responses.

Response: Thank you for raising this point and for the opportunity to clarify our statements in the manuscript. We agree that understanding the dynamics by which cells acquire or exit the ERS, and how this relates to the ABA signalling pathway, would be very interesting and highly informative. These have been highlighted as important follow-up questions in the Discussion section of the manuscript.

The experiment suggested in this point, however, appears to stem from an interpretation of the ERS concept that differs from what we intended to convey in the manuscript. In our study, ERS+ and ERS− cells are not defined as cells that are intrinsically more or less capable of responding to environmental stimuli. Rather, ERS+ cells are those that have acquired a highly transcriptionally active state characterised by the expression of ERS-associated gene modules, whereas ERS− cells have not acquired this state. What our data show is that environmental conditions influence the proportion of cells that acquire this state. For example, by altering the medium composition - either by increasing MS concentration or, more strongly, under a model

drought condition - the plant adjusts the number of cells in the root that acquire the ERS (termed 'ERS+ cells'). If ERS+ cells represented a population intrinsically specialised for environmental responsiveness, stress would be expected to primarily increase the activity of these cells rather than alter their relative abundance. Instead, a clear increase in the proportion of cells acquiring the ERS is observed.

To further examine this point, we have now tested whether ERS+ cells show a stronger transcriptional response to drought stress than other cells. We compared both UMI content and the number of genes expressed between control and drought conditions (**Response Figure 1**). While drought increases overall transcript abundance (UMI count) and genes detected in the root, this effect is observed across all cell populations and is not specific to ERS+ cells. In fact, the magnitude of the increase is comparable or greater in ERS− and non-ERS cells (note: non-ERS cells are present in root cap and early developmental stages before the branching, where distinct ERS+ and ERS− states are not observed). The median fold-change in UMI content is 1.32 for ERS+ cells, 1.50 for ERS− cells, and 1.71 for non-ERS cells, while the median fold-change in the number of genes expressed is 1.24 for ERS+ cells, 1.32 for ERS− cells, and 1.62 for non-ERS cells (**Response Figure 1**). These results further support the interpretation that, rather than ERS+ cells specifically responding to drought, all cells exhibit a transcriptional response, while drought shifts a larger proportion of cells into the highly transcriptionally active ERS state already present in a subset of the cells under control conditions.

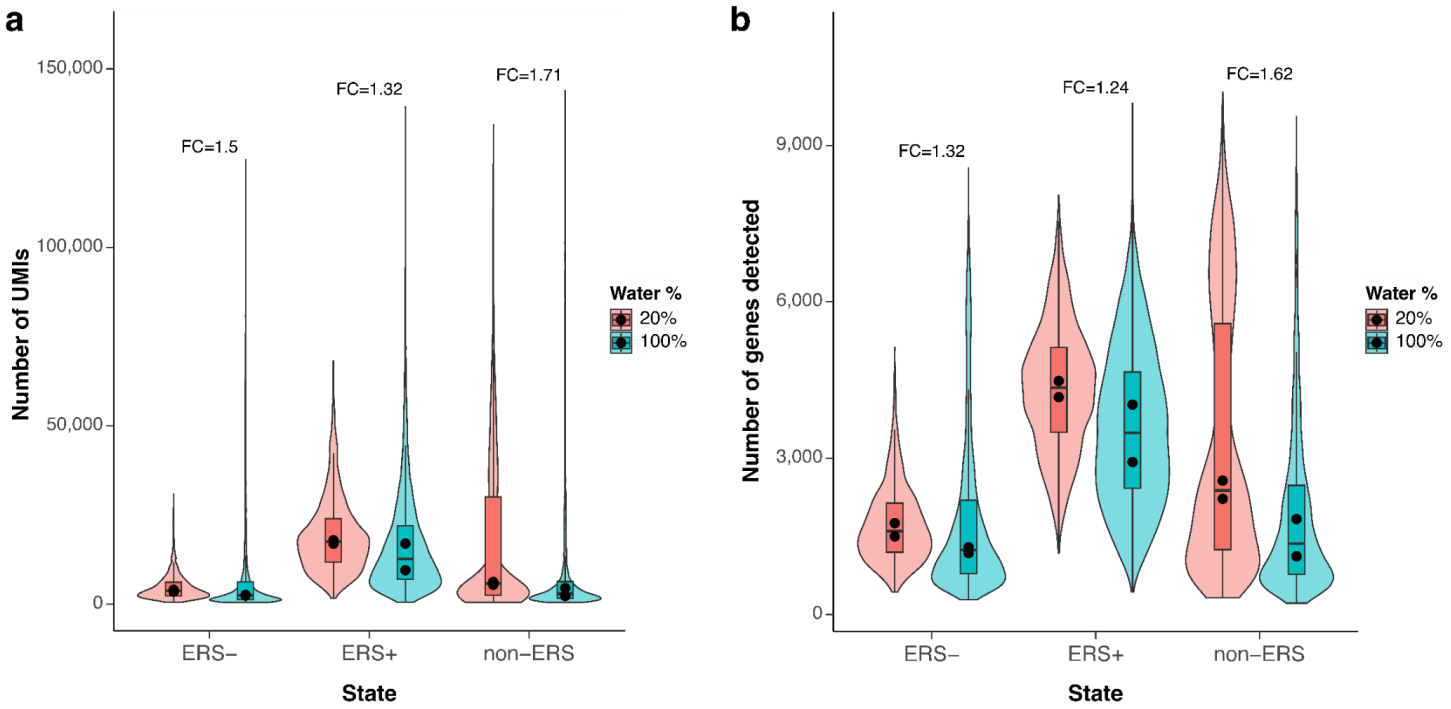
We recognise that some of the wording in the manuscript may have contributed to this ambiguity. In particular, the term *"ERS+ cells"* could be interpreted as referring to *"environmentally responsive cells"* (which would imply that *"ERS− cells"* are not environmentally responsive cells), and the sentence from the Abstract that is mentioned in this comment, which refers to an *"alternate fate trajectory"*, could be interpreted as implying that certain cells are developmentally predisposed to acquire this state. This was not our intention. To avoid this misunderstanding and better convey our conceptual framework, the relevant sections of the manuscript have been revised as follows:

- Page 2, line 12: We have replaced *"we found that most lineage trajectories exhibit a stereotyped bifid topology, with two cell developmental paths rather than one"* with "*we found that most lineage trajectories exhibit a stereotyped bifid topology, with two cell identity paths rather than one*" in the abstract.
- Page 2, line 20: We have replaced "*The discovery of this alternate fate trajectory*" with "*The discovery of this alternate trajectory*" in the abstract.
- Page 11, line 24: We have clarified the definition of the ERS, and ERS-positive and ERS-negative cells, by adding the following sentence when these terms are first introduced: *"These terms reflect the acquisition or absence of this transcriptional state at the single-cell level, rather than any intrinsic difference in the capacity of individual cells to respond to environmental signals*."
- Page 16, line 19: We have replaced *"3) the presence or absence of this additional environmentally responsive state, which varies with environmental conditions"* with *"3) the presence or absence of this additional transcriptional state, whose prevalence is modulated by environmental conditions"* in the Discussion section.

The experiment proposed in this comment - that is, using ABA FRET sensors such as ABACUS or ABAleon to test whether cells with higher basal ABA levels exhibit stronger ABA responses - would address the hypothesis that ERS+ cells are intrinsically more responsive to stress signalling. Because this is not the model proposed in our manuscript, such an experiment would not directly test the central claims of the study.

More broadly, experimentally resolving how cells transition into and out of the ERS state would require the generation and validation of live reporters for ERS-associated genes and the analysis of their spatiotemporal dynamics *in vivo*. While we think that this would be a fascinating experiment to further explore the nature and dynamics of this new cell state phenomenon, developing and characterising such constructs and reporter lines would be a significant undertaking that would not be possible to complete within the limited time available in this revision period (a few months). We therefore agree with Referee 2 that testing these dynamics experimentally is beyond the scope of the present manuscript.

To address this point, the manuscript has been revised to clarify the conceptual framework of the ERS and to emphasise that environmental stress increases the frequency with which cells acquire this transcriptional state. The potential dynamics of ERS acquisition and its relationship to stress signalling pathways, including ABA, are also discussed as important directions for future investigation.



Response Figure 1: Comparison of **a,** UMI content and **b,** the number of genes expressed between control (100% water) and drought (20% water) conditions, for different cell states (ERS−, ERS+, and non-ERS cells). The fold change (FC) between 100% and 20% water conditions is indicated for each cell state.

Referee 1 - point 2: HCR-based transcript detection is a critical component of validating the manuscript's conclusions regarding the ERS⁺ cell state. It is therefore essential to demonstrate that the observed differences in signal intensity truly reflect biological variation rather than experimental artifacts. I recommend that, in addition to detecting ERS⁺ state markers, the authors include simultaneous detection of ubiquitously expressed genes that are expected to be present in both branches of the cell type as internal controls for probe performance and hybridization efficiency. It would also strengthen the analysis to present images across multiple Z-planes, confirming that the apparent absence of signal in certain cells is not due to differences in the chosen focal plane.

Response: This is an important point, and we agree that it is essential to demonstrate that the differential HCR signal observed between ERS+ and ERS− cells reflects genuine biological variation rather than technical artefact.

To address *probe performance and hybridisation efficiency*, we have now performed a new control experiment in the endodermis in which the ERS marker *AT2G41660* was co-detected with *AT2G14900*, a marker expressed at a similar level in both branches of the endodermal layer (**Extended Data Figure 10c**). While *AT2G41660* shows the expected heterogeneous ERS-associated expression pattern, *AT2G14900* is robustly detected in both the ERS+ and ERS− cells of the endodermis. This demonstrates that cells lacking ERS marker signal are nonetheless efficiently permeabilised, hybridised, and their transcripts detected by HCR, and therefore that the absence of ERS marker signal in ERS− cells is not attributable to technical failure (**Ext. Data Fig. 10c**).

Regarding *image presentation*, we note that in the initial submission of the manuscript we had displayed multiple optical transverse cross-sections as suggested here. However, during the previous review round we were encouraged to favour longitudinal views, as root cells are thin and highly elongated, and fluorescence puncta are more readily missed in individual transverse sections than in longitudinal optical sections spanning a larger proportion of the cell volume. Longitudinal sections are also the view in which positive and negative cells can most readily be appreciated within the same tissue layer. While adding multiple transverse cross-sections could increase confidence, this alone would still not fully exclude focal plane-related bias and would require a large number of sections across many cells to be convincing.

Therefore, in the revised manuscript, we have now adopted a more quantitative strategy by performing 3D segmentation of individual cells in the outer root layers (epidermis, cortex, and endodermis), where cell boundaries can be robustly delineated, and performing volumetric fluorescence quantification of the HCR signal on a per-cell basis. In the revised figure, segmented cells are colour-coded by mean HCR fluorescence intensity, allowing direct visualisation of heterogeneous ERS marker signal at single-cell resolution (**Fig. 3a**). This analysis reveals substantial differences in ERS marker abundance between cells within the same layer, with up to 20-fold difference between positive and negative cells.

One of the challenges in experimentally validating the existence of the two branches is that the ERS− cells do not exhibit a clear branch-specific transcriptional programme analogous to the ERS-associated modules. Rather, ERS+ and ERS− cells share the canonical markers of their underlying cell type, with ERS+ cells distinguished primarily by the additional expression of the ERS-associated transcriptional programme. However, upon closer inspection of epidermal markers, we have identified a small number of genes with expression patterns indicative of ERS− enrichment. In particular, *AT3G01720* shows an expression pattern that is largely complementary to the ERS marker *AT5G56550* in epidermal cells, with both genes expressed at levels suitable for *in situ* detection (**Fig. 3b-d**).

We therefore tested this pair experimentally by HCR and found that the two transcripts display largely mutually exclusive expression patterns *in vivo* (**Fig. 3f**). Volumetric quantification of segmented epidermal cells across three independent roots reveals two distinct populations of epidermal cells, one enriched for *AT3G01720* and the other for *AT5G56550* (**Fig. 3g**), closely matching the ERS− and ERS+ epidermal populations inferred from scRNA-seq. Consistent with this, Gaussian mixture modelling of both scRNA-seq expression scores and HCR-derived fluorescence ratios supports the presence of two discrete epidermal populations (**Fig. 3e,h**).

Together, these orthogonal controls and quantitative imaging analyses demonstrate that the heterogeneous HCR signal observed for ERS markers reflects *bona fide* biological heterogeneity between neighbouring cells rather than focal plane effects or technical variability in probe detection, thereby providing strong independent experimental support for the existence of distinct ERS+ and ERS− cell states *in vivo*.

Referee 1 - point 3: The identification of two trajectories with both PAGA and Monocle is encouraging. To strengthen the claim of a true bifurcation, I recommend quantifying agreement between methods (e.g., overlap of branch assignments and correlation of local pseudotime). And since both PAGA and Monocle are graph-based analyses, it will be better to use a graph independent approach, like RNA velocity models, to confirm the trajectories identified in PAGA and Monocle. Additionally, please include pathway enrichment for the Monocle trajectories. Do similar GO terms appear in the Monocle-based analysis as in the PAGA-inferred branches?

Response: The trajectory framework used in the manuscript combines PAGA to infer trajectory topology with diffusion pseudotime (DPT) to order cells along those trajectories. PAGA was selected because it is particularly well suited for resolving complex developmental topologies, including the bifurcations and convergence events observed in several lineages such as the epidermis and root cap. While PAGA itself is graph-based, the subsequent DPT computation used to order cells is not graph-based and independently supports the presence of two trajectories. Specifically, in all affected lineages, the inferred origin of the trajectory (t = 0) is positioned centrally within the lineage, while two distinct terminal populations are assigned t = 1 (**see Fig. 1f**), consistent with the presence of two developmental endpoints and therefore two separate trajectories.

To further address this point, we also tested an RNA velocity-based approach using scVelo. This method was not initially prioritised because intronic signal in Arabidopsis scRNA-seq datasets is very limited, resulting in low signal-to-noise ratios and reduced robustness of velocity inference. In our atlas, the median proportion of unspliced reads is only ~3%, and fewer than 7,000 of the 33,977 detected genes passed filtering thresholds for sufficient spliced and unspliced counts to support kinetic modelling. Nevertheless, latent time inferred using the scVelo dynamical model showed strong agreement with our DPT ordering, with Spearman correlation coefficients >0.7 in six of the eight lineages that exhibit ERS-associated bifurcation (**Ext. Data Fig. 2f,g**). For the xylem pole pericycle and procambium, the lower correlation reflects a shift in the inferred t = 0 assignment rather than disagreement in trajectory structure. In all cases, latent time increases toward the termini of both branches, consistent with the bifurcating developmental trajectories identified in our analysis.

Regarding comparison between the PAGA- and Monocle-inferred trajectories, we agree that the initial manuscript relied primarily on visual concordance of the trajectory topologies and pseudotime progression. To provide a more quantitative assessment, we compared pseudotime values inferred by Scanpy/DPT and Monocle across all lineages. This analysis revealed strong concordance between methods, with pseudotime correlations exceeding 0.83 in all lineages except the endodermis (**Ext. Data Fig. 2b-e**). In the endodermis, one branch shows lower correlation because Monocle places the trajectory root slightly deeper within that branch, thereby shifting the relative pseudotime scale without altering the overall bifurcating topology.

Direct comparison of branch assignments between PAGA and Monocle is complicated by the fact that Monocle frequently introduces additional minor nodes and local branches in complex datasets such as ours, beyond the principal global bifurcation of interest. As branch identities in Monocle must therefore be reconstructed manually from graph topology rather than being directly output as discrete branch labels, robust per-cell branch assignment comparisons (e.g. ARI, Adjusted Rand Index) are not straightforward or necessarily meaningful in this context. This complexity was one of the reasons PAGA is favoured as the primary topology inference method in the study.

Regarding pathway enrichment, the GO term analyses presented in the manuscript are not derived from differential expression testing between branches defined by a specific trajectory inference method. Rather, they are based on co-expression modules identified across the entire dataset, independently of trajectory or branch assignments. This approach was intentionally chosen because it is more robust to minor differences in pseudotime ordering or branch boundary placement than branch-specific differential expression analyses, which can be sensitive to methodological noise and assignment thresholds. Notably, the ERS-associated gene modules describe a cell state that is superimposed across all lineages rather than being confined to a single trajectory branch or cell type, and this global, lineage-independent nature of the signal is better captured by a dataset-wide co-expression framework than by per-branch differential expression within individual lineages.

Importantly, since the UMAP embedding underlying both the PAGA and Monocle analyses is identical, projecting the co-expression module scores onto the Monocle trajectory yields the same spatial pattern of ERS-associated gene activity by definition. The GO enrichments are therefore not merely consistent between methods but are the same enrichments, derived from the same cells and visualised on the same embedding. Given this, and given the strong concordance observed between PAGA, Monocle, and RNA velocity-based trajectory inference, the functional interpretation of the ERS-associated branch does not rely on a specific trajectory inference framework but instead emerges from a trajectory-independent co-expression analysis that is robust to minor differences in cell ordering across methods.

Together, these analyses demonstrate that the bifurcating developmental trajectories reported in this study are robustly recovered across multiple independent trajectory inference frameworks, including graph-based, non-graph-based, and RNA velocity-derived approaches, strongly supporting the conclusion that the observed ERS-associated branching reflects a genuine and biologically meaningful feature of root cell differentiation rather than a methodological artefact.

Referee 1 - point 4: Extended Data Fig.2. It is unclear what is validated in this figure. If published reporter lines support the reported expression patterns, the corresponding references should be included. Note also that AT1G11190 is BFN1, not BNF1.

Response: Thank you for pointing out this lack of clarity and for noting the typographical error in the gene name. We have corrected *BNF1* to *BFN1*.

Extended Data Figure 2 is intended to validate the biological interpretation of the inferred root cap trajectory topology by showing that the distinct branches identified in our trajectory analysis are associated with the expected expression patterns of marker genes known to be expressed at specific stages or subdomains of root cap development. Specifically, the expression patterns of *CDF4*, *FEZ*, *RCPG*, and *BFN1* in the scRNA-seq data are consistent with their previously reported *in vivo* expression domains and support the interpretation that the inferred branches correspond to known developmental events within the root cap lineage.

The corresponding references for these published expression patterns were already cited in the main text (refs. 22–24), but we recognise that this may not have been sufficiently explicit in relation to Extended Data Figure 2. To improve clarity, we have revised the manuscript text and figure legend to state more explicitly that these previously characterised marker genes are used to validate the biological identity of the inferred trajectories based on their known expression patterns during root cap development. The corresponding references have also been added to the Extended Data Figure 2 legend.

Together, these marker expression patterns provide independent biological support that the complex root cap topology inferred by our trajectory analysis recapitulates *bona fide* developmental branching events rather than representing computational artefacts.

Referee 1 - point 5: In Extended Data Fig.3, the images shown for genes AT2G36100, AT3G47510 can be made clear by showing optical cross sections at different distances from the root tip. Currently, it is difficult to determine, for example, whether AT2G36100 is expressed in the endodermis.

Response: Thank you for this suggestion. To improve the clarity of the expression patterns shown in Extended Data Figure 3, we have now added additional optical cross-sections to make the spatial localisation of the signal clearer and facilitate interpretation of the corresponding cell-type-specific expression patterns (**Ext. Data Fig. 1b-c**).

Referee 1 - point 6: In the author response, the statement "ERS$^-$ does not mean that these cells are unresponsive to the environment; rather, it means they have not acquired the specific transcriptional state that we define as 'environmentally responsive' (ERS$^+$)" remains conceptually ambiguous. In the hard-agar drought stress experiment, the increase in ERS$^+$ cells is accompanied by a decrease in ERS$^-$ cells, suggesting that both states are, in fact, responsive to environmental cues. To avoid confusion, I recommend refraining from

using the term “environmentally responsive” to describe the ERS⁺ state, as it implies that ERS⁻ cells are environmentally inert.

Response: We thank the referee for this comment and agree that the terminology we used may lead to ambiguity if interpreted as implying that ERS− cells are environmentally inert. As clarified in **Response to Referee 1 - point 1**, above, this is not the conceptual framework intended in the manuscript. The term “environmentally responsive” refers to the transcriptional state itself, which is characterised by the coordinated activation of genes associated with environmental and stress responses. In addition, the probability of a cell acquiring this transcriptional state is modulated by environmental conditions, with drought resulting in an increased proportion of ERS+ cells across multiple lineages.

We do not intend the term “environmentally responsive” to imply that ERS+ cells are uniquely capable of responding to environmental cues. Indeed, as shown in Response Fig. 1, drought affects both ERS+ and ERS− cells, including increased transcript abundance and numbers of detected genes in both populations. However, the ERS− cells that remain under these conditions still do not exhibit the characteristic ERS-associated transcriptional signature identified by our co-expression analysis.

Because cells within a given lineage are classified as either ERS+ or ERS−, any increase in the proportion of ERS+ cells under stress necessarily results in a corresponding decrease in the proportion of ERS− cells. This does not indicate that ERS− cells are environmentally unresponsive, but simply reflects the reciprocal nature of the classification.

We acknowledge that the terminology may be open to misinterpretation and have therefore revised the manuscript to clarify this conceptual framework more explicitly throughout the text (detailed in **Response to Referee 1 - point 1**, above), including when first defining the ERS and when referring to ERS+ and ERS− populations. While alternative terminology was considered, we found that other options introduced similar ambiguities while reducing continuity with the terminology used throughout the manuscript and review process. As this concern was not raised by the other referees, we have retained the term ERS while clarifying its intended meaning.

If the Editor and referees feel that renaming this state would nevertheless improve the clarity of the manuscript, we would of course be happy to revise the terminology accordingly.

Referee 1 - point 7: In the drought stress and full MS/half MS experiments, could the authors clarify whether seeds were germinated directly on the respective media or if seedlings were transferred to these conditions after initial germination? The method of establishing the treatment is important, as transfer-related stress or developmental stage differences could influence the proportion of ERS⁺ cells and the interpretation of the results.

Response: Thank you for pointing out this potential source of bias. Seeds were sown directly on their respective media and germinated under those conditions; seedlings were not transferred between media prior to analysis.

Because drought-stress medium delays germination and reduces root growth, drought plates were transferred to the growth room 48h earlier so that samples from all conditions could be harvested simultaneously at comparable developmental stages relative to germination. In addition, all samples were collected by harvesting the same region from the root tip, ensuring that comparable developmental zones were analysed even when total root length differed between conditions.

To ensure that reduced root growth itself does not account for the observed changes in ERS-associated gene expression, we examined ERS marker expression in younger control roots at 3 days after germination (dag), when the roots are substantially smaller and more comparable in size to the drought-treated roots. This showed that *AT3G04010* fluorescence intensity at the root tip remained comparable between 3 and 6 dag, indicating that root length does not explain the increased fluorescence intensity observed under drought conditions. Quantification supporting this control has now been added to the revised manuscript (**Ext. Data Fig. 12f**).

Referee 1 - point 8: In Figure 4d, it would be helpful to project the datasets from the different drought stress conditions onto a single UMAP embedding. Presenting the data in a shared UMAP space would allow for a more direct comparison of the cellular distributions and ERS⁺ cell frequencies across conditions.

Response: Thank you for this suggestion. We have now projected the drought-stress datasets onto the reference atlas UMAP embedding and included these analyses in the revised manuscript. This shared embedding allows direct visual comparison across conditions and clearly shows an enrichment of ERS+ cells, with a corresponding decrease in ERS− cells, across multiple cell types under drought stress relative to control conditions (**Fig. 5d**). Please note the change in figure numbering in the revised manuscript.

Referee 1 - point 9: In Extended Data Fig. 15b–d, do the authors observe that branch 1–specific genes across the different ecotypes are enriched for ABA- or circadian-related pathways? Clarifying whether this

enrichment is consistent among ecotypes would help determine if the observed transcriptional divergence reflects conserved ABA/circadian regulation or ecotype-specific differences in environmental responsiveness.
Response: Thank you for this suggestion. To identify the branch 1-specific genes associated with ABA- and circadian-related GO terms, we did not perform differential expression analysis between branch 1 and branch 2 cells. Instead, as described in **Response to Referee 1 - point 3**, we identified co-expression modules across the full dataset independently of branch assignments, an approach that is more robust to minor variation in branch boundaries and cell ordering than branch-specific differential expression analyses.

Accordingly, to determine whether the same transcriptional programme is associated with branch 1 cells across ecotypes, we assessed the expression of Modules 20 and 21 (the branch 1-specific co-expression modules identified in the Col-0 reference atlas and associated with ABA- and circadian-related GO terms) in the other ecotypes. We found that, similarly to Col-0, these modules remain specifically enriched in branch 1 cells across all ecotypes examined (**Ext. Data Fig. 8a-c**). This indicates that the transcriptional programme associated with the branch 1/ERS+ state is broadly conserved across ecotypes and that the observed transcriptional divergence is not unique to Col-0.

Referee 1 - point 10: Data on root hair number are not presented in the manuscript. The corresponding section in the Methods should therefore be removed unless these results are included.
Response: Thank you for this comment. Root hair density is not presented as a major result of the manuscript; however, these data were included to demonstrate that, although full MS and half MS media are both common and generally considered mild growth conditions in Arabidopsis studies, the reduction in medium concentration nevertheless induces a measurable physiological response. Specifically, the increase in root hair density provides an independent phenotypic indication that the half MS condition is perceived by the plant and elicits a physiological adjustment, consistent with the moderate shift in ERS proportion observed under this condition.

Accordingly, these data are referred to in the manuscript in the context of supporting the interpretation that altered medium composition induces a biologically relevant response, rather than as a standalone result. The relevant sentence in the manuscript reads: *"This range is commonly used in Arabidopsis studies and does not drastically affect root growth, but the decrease in medium concentration induces a physiological response reflected by an increase in root hair density (Extended Data Fig. 21)."*

We retained the associated Methods section because these data were originally part of the main figures in earlier versions of the manuscript and remained included in the revised supplementary data. However, if the Editor and Referees feel that these data are not sufficiently central to warrant inclusion, we would be happy to remove both the figure and corresponding Methods section.

Referee 1 - point 11: Since the edge_threshold parameter in the PAGA analysis was "adapted to each lineage," it would be important to specify the exact thresholds used or provide a clear rationale for how these values were determined, as this choice can strongly influence the inferred trajectory topology.
Response: Thank you for raising this point. In the PAGA analysis, each lineage is first subdivided into smaller clusters prior to topology inference. Because transcriptional similarity can reflect both developmental progression and shared developmental stage, weaker connections are sometimes detected between subclusters belonging to different branches but occupying similar pseudotime positions. These likely reflect shared stage-specific transcriptional programs rather than the principal developmental progression within a branch.

The purpose of the edge_threshold parameter is therefore to filter out these weaker secondary connections while retaining the stronger edges that define the principal developmental trajectories. Thresholds were adjusted independently for each lineage because the relative strength of within- versus between-branch connections varies depending on lineage complexity, cluster number, and transcriptional heterogeneity.

In some cases, these weaker inter-branch connections could not be completely removed without disrupting the principal trajectory structure and were therefore retained; these are shown as dashed lines in **Fig. 1g**. Importantly, these dashed edges represent only a small subset of the detected relationships. With the exception of the protoxylem and the more complex root cap trajectories, which contain multiple closely related sub-cell types in close spatial and developmental proximity near the root tip, these residual inter-branch connections are largely restricted to the earliest stages of the bifurcation, when branch 1 and branch 2 cells are only beginning to separate transcriptionally. The overall branching topology is therefore not driven by thresholding itself, but reflects the dominant connectivity structure present in the data. Consistent with this, similar bifurcating developmental structures are also apparent in independent Arabidopsis root trajectory analyses from Shahan *et al.*, despite the use of different datasets and trajectory inference approaches, supporting that these branching trajectories represent reproducible biological structure rather than an artefact of parameter selection. (**Fig. 1g**).

All edge_threshold values used for each lineage are provided in the associated analysis script available in the dedicated github repository.

Referee 1 - point 12: The cross-dataset validation should come with supporting statistics. The authors used extensive projection-based comparisons to determine if ERS+ and ERS- populations were also present in previously published scRNA-seq/snRNA-seq datasets. However, these projections were known to vary dramatically when using different parameters. The authors need to show corresponding statistics for the stability of these projections.

Response: Thank you for raising this important point. To assess the robustness of the projection-based ERS assignments in external datasets, we have now systematically evaluated label transfer stability across a range of parameter settings for the two principal parameters influencing Seurat label transfer, namely the number of principal components used and the k.anchor parameter.

Specifically, projections were repeated using 30, 40, or 50 principal components and k.anchor values of 5, 10, or 20, generating nine parameter combinations in total (**Ext. Data Fig. 5g, 6h, 7g**). Across all three external datasets examined, the proportion of ERS+ cells assigned to each cell type remained highly consistent across parameter sets (**Ext. Data Fig. 5h, 6i, 7h**). Pairwise Adjusted Rand Index (ARI) comparisons between parameter sets further demonstrated strong agreement in transferred cell type and ERS assignments (**Ext. Data Fig. 5i, 6j, 7i**), with all ARI values remaining above 0.7. Similarly, more than 75% of cells retained their baseline cell-type assignment across all parameter sets, with most conditions for most datasets close to 100% (**Ext. Data Fig. 5j, 6k, 7j**).

The slightly lower ARI values and cell-type assignment stability observed for the Farmer *et al.* dataset likely reflect the fact that this dataset is based on snRNA-seq rather than whole-cell scRNA-seq, and therefore differs more substantially from our scRNA-seq reference atlas in transcript capture characteristics and overall data structure. Nevertheless, the stability remains high despite this expected modality-related variability.

Importantly, ERS state assignment proved markedly more robust than fine cell-type assignment: across all three datasets, including the Farmer *et al.* snRNA-seq dataset, ERS+/ERS− assignments were retained for nearly 100% of projected cells across parameter sets, with only negligible proportions of cells switching ERS state (**Ext. Data Fig. 5k, 6l, 7k**). This demonstrates that the identification of the ERS dichotomy in external datasets is highly stable and does not reflect instability of the projection procedure.

Referee #2

Summary: I appreciate the authors' careful and thoughtful revisions in response to my initial review. The manuscript has improved substantially in providing evidence for the validation of the ERS:
- Validation of cell type and ERS+ marker genes by HCR
- Provide additional analysis that demonstrate that ERS+ cells are not analytical artifacts
- Characterization of the ERS in previous single cell/nuclei data sets
- The establishment of an ERS is modular and adaptable under varying nutrient (full MS or ½ MS) or stress (e.g. drought) conditions

My previous concerns and suggestions have been adequately addressed. The authors have provided new data and clear responses to all points raised in my initial review, incorporating the necessary revisions throughout the manuscript. The identification, characterization, and validation of the ERS as an additional cellular characteristic—beyond cell type lineage and developmental state—remain highly novel. The finding that drought stress can modulate the establishment of this state across multiple cell types, and that ABA biosynthesis and receptor mutants exhibit reduced numbers of ERS^{+} cells, represents a particularly interesting and significant contribution.

The source genomic data are complete, and the methods are described in adequate detail to ensure transparency and reproducibility. The provided code further enhances reproducibility. The statistical analyses are appropriate, and there are no apparent data that would require additional statistical testing. The reporting of error bars and probability values is clear and consistently presented in figure legends and relevant text sections.

The following point is an optional suggestion that could further refine a point of discussion, though it does not affect the overall novelty or conclusions of the study:

-Page 16 Line 5-8: I agree with the authors` conclusion that the underlying regulatory networks of the ERS could vary between cell types, which causes the described degree of acquisition of the ERS-state between cell types. However, as the ERS is regulated by ABA, differences in ABA sensitivity or signaling among cells within and between cell types could also offer a plausible explanation. I understand that testing this hypothesis is

beyond the scope of the present manuscript, but this argument could be added to the current statement to provide a more comprehensive view of the possible regulatory mechanisms influencing ERS establishment.
Response: We thank the referee for the very positive assessment of the revised manuscript and for recognising the substantial additional work incorporated in the revision. We are pleased that the referee considers the previous concerns to have been adequately addressed and that the novelty and significance of the ERS framework are appreciated.

We also thank the referee for this thoughtful suggestion regarding potential differences in ABA sensitivity or signalling between and within cell types as an additional factor influencing ERS establishment. We agree that this represents an important and plausible complementary mechanism to differences in the underlying regulatory networks controlling ERS acquisition. While experimentally dissecting this possibility is beyond the scope of the present study, we agree that it provides a valuable additional perspective on the potential determinants of ERS establishment.

We have therefore revised the Discussion to incorporate this possibility and now explicitly note that variation in ABA sensitivity or signalling competence between cells and cell types may also contribute to the differential propensity of cells to acquire the ERS state, as follows: "*Differences in ABA sensitivity or signaling capacity among cells within and between cell types could equally contribute to this variability, and represent an important avenue for future investigation.*"

Minor comments:

Referee 2 - point 1: Page 15 Line 9: "sRNA-seq" should be scRNA-seq
Response: Thank you, this has now been fixed.

Referee 2 - point 2: Page 32 Line 16: "Putative TF targets genes" should be "Putative TF target genes"
Response: Thank you, this has now been fixed.

Referee 2 - point 3: Figure 3 panel D: the y-axis should be "Cell state proportion"
Response: Thank you, this has now been fixed.

Referee #3

Summary: The authors have done extensive and commendable additional work in this revision, including generating new datasets, performing new experiments, and developing new analyses under specific conditions. These efforts substantially strengthen the study. However, our main concern remains whether branch 2 represents a true biological state or a technical artifact. There are some additional analyses suggested below that could be helpful and are not "major lifts". Depending on the authors' take on our concerns, we could imagine these being addressed through minor revisions & textual changes that reflect the concerns or remaining uncertainty.
Response: We thank the referee for the thoughtful summary and for recognising the substantial additional work performed in this revision. We appreciate that the central remaining question is whether branch 2/ERS− represents a *bona fide* biological state rather than a technical artefact, and we have focused this revision on addressing that concern as directly and comprehensively as possible.

In response, we have substantially strengthened the manuscript through multiple new orthogonal analyses specifically aimed at testing the biological validity of branch 2/ERS− cells. These include improved quantitative analyses addressing low-UMI/damaged-cell hypotheses, revised UMI downsampling and regression-based modelling of expected transcript loss under reduced-capture scenarios, robustness analyses across external datasets and projection parameters, and additional biological controls that exclude alternative explanations such as cell cycle and developmental-stage effects.

Most importantly, we have now added direct experimental evidence for the existence of ERS− cells *in vivo* by identifying an ERS−-enriched epidermal marker and demonstrating, through multiplex HCR and quantitative 3D fluorescence segmentation, the presence of two mutually exclusive epidermal populations corresponding to ERS+ and ERS− states. Together with the existing HCR, ABA/drought perturbation, and cross-dataset validation analyses, we believe these additions (detailed below) provide strong and convergent evidence that branch 2/ERS− represents a genuine biological cell state rather than a technical artefact.

Major comments:

Referee 3 - point 1: Low UMI / High Mitochondrial %

Branch 2 cells have low UMI counts and high mitochondrial percentages, a common issue in scRNA-seq where low-quality cells form spurious clusters. In Extended Fig. 9, Branch 2 appears to show higher mitochondrial % and chloroplastic % than Branch 1, despite the text stating they are comparable. The overplotted jitter makes it difficult to see the distribution clearly; a statistical comparison (and perhaps clearer violin or density plots) would help.

Response: Thank you for the opportunity to clarify the intent of this figure. Because branch 2 cells have lower overall UMI content, mitochondrial and chloroplastic reads represent a larger fraction of their transcriptomes, resulting in higher mitochondrial/chloroplastic percentages relative to branch 1 cells. Our intention was not to suggest that these percentages are identical between branches, but rather to show that branch 2 cells do not exhibit mitochondrial/chloroplastic proportions consistent with the levels typically used to define low-quality or damaged cells in plant scRNA-seq studies.

Specifically, only a very small minority (0.16% for mitochondrial reads and 0.19% for chloroplastic reads) of branch 2 cells exceed the 5% mitochondrial/chloroplastic percentage thresholds commonly applied as hard filtering cut-offs in published plant single-cell studies (**Ext. Data Fig. 3i-j**). Thus, the vast majority of branch 2 cells would not have been excluded based on these criteria. This analysis was included in response to concerns raised during the previous revision regarding whether branch 2 cells might represent damaged cells based on organellar read content.

Importantly, other abundant cell populations in our dataset that are not affected by the ERS dichotomy (named 'non-ERS' cells), most notably root cap cells, also display relatively low UMI content and correspondingly elevated mitochondrial/chloroplastic percentages comparable to or exceeding those of branch 2 cells (**Ext. Data Fig. 3i-j**), yet are clearly *bona fide* biological populations rather than low-quality cells. This further supports the conclusion that the observed organellar read fractions in branch 2 cells reflect lower overall transcript capture rather than cell damage.

We agree that the overplotted jitter representation was not optimal for conveying these distributions. We have therefore revised the figure to display the data using boxplots (**Ext. Data Fig. 3i-j**), which more clearly illustrate the distribution of mitochondrial and chloroplastic percentages across populations.

Referee 3 - point 2: Lack of distinct gene expression features

Most of the analyses (e.g., scRNA-seq across ecotypes, snATAC-seq) primarily strengthen evidence for Branch 1. Since the central question is whether Branch 2 is real, a more direct approach would be to identify specific genes that are up- or downregulated in Branch 2 with biological interpretability. To our reading, there are still no clear gene expression features defining Branch 2. In analogous datasets, distinct clusters are characterized by specific genes that can usually be either connected to the literature or some function in a clear, believable way (e.g. known/clear markers are best, but GO modules, though not our favorite, and the like often suffice). This facilitates gaining confidence (for authors, reviewers and readers) that a given cluster is real rather than an artifact. The authors provide some explanations in their response why distinct features were not found. We found these points to be reasonable but not entirely convincing. We recognize that this concern is not really addressable if the given reasons are valid, but it is another reason for caveating Branch 2.

Response: We agree that one of the central challenges of the framework described in this manuscript is that, unlike many discrete cell states identified in single-cell studies, ERS− cells are not characterised by a strong branch-specific transcriptional programme. Rather, ERS+ and ERS− populations share the canonical developmental and cell-type markers of their underlying lineage, with ERS+ cells distinguished primarily by the superimposition of an additional large co-expressed transcriptional programme. As a result, validation of the existence of two distinct populations has largely relied on demonstrating that 'branch 2'/ERS− cells do not represent technical artefacts.

While this overall conceptual framework remains unchanged and no broad ERS−-specific transcriptional programme has been identified, closer inspection of epidermis-specific genes revealed a small number of candidates enriched in ERS− cells. In particular, *AT3G01720* shows enrichment in ERS− epidermal cells and is expressed at levels comparable to the ERS marker *AT5G56550*, with the two genes displaying largely mutually exclusive expression patterns in the epidermis in the scRNA-seq data (**Fig. 3b-d**). Gaussian mixture modelling of their single-cell expression profiles further supported the presence of two distinct epidermal populations based on these markers (**Fig. 3e**), making them strong candidates for orthogonal experimental validation.

We therefore performed simultaneous whole-mount HCR detection of *AT3G01720* and *AT5G56550*, which revealed a clear mutually exclusive expression pattern *in vivo* (**Fig. 3f**). Segmentation and volumetric fluorescence quantification of epidermal cells across three independent roots confirmed the presence of two distinct populations, one enriched for *AT3G01720* and the other for *AT5G56550* (**Fig. 3g**), with Gaussian mixture modelling of the HCR-derived per-cell fluorescence ratios again supporting bimodality (**Fig. 3h**).

Thus, although ERS− cells are not defined by a broad alternative transcriptional programme analogous to the ERS-associated signature of ERS+ cells, these new orthogonal imaging data provide direct

experimental evidence for the existence of a distinct ERS−/branch 2 epidermal population *in vivo*, strengthening the conclusion that branch 2 reflects a *bona fide* biological cell state rather than a technical artefact.

Referee 3 - point 3: Extended Figures 9 & 10 Interpretation

We do not see how Extended Fig. 10 supports the authors' statement, which is very confusing. From Extended Figs. 9 and 10, it seems the authors have not fully ruled out the possibility that the second branch represents damaged cells with lower UMI capture (characterized by high mito% and low UMI ***which is a a common issue*** in scRNA-seq analysis. On a related point, Extended Fig. 10 is intended to show that genes enriched in Branch 1 span a similar expression range to those expressed in both branches, suggesting that Branch 1-specific genes aren't simply missed in low-UMI cells. Conceptually this makes sense, but the data are overplotted, and many Branch 1-specific genes appear lowly expressed. Two violin plots (one for "branch 1-specific" and one for "branch 1 + Branch 2-specific" genes) showing their average expression in Branch 1 might make this comparison clearer. Currently, this figure feels more qualitative than quantitative.

Response: Thank you for this suggestion. We agree that the original presentation of Extended Data Fig. 10 was not sufficiently clear and remained overly qualitative. We have therefore revised the figure and analysis to improve interpretability and to present the comparison more quantitatively.

The purpose of this analysis was to test whether the apparent absence of ERS-associated genes in ERS− cells could be explained simply by lower transcript capture in these cells, i.e. whether ERS− cells might represent damaged or partially captured counterparts of ERS+ cells in which lower-expressed ERS genes fall below the detection threshold.

As noted by the referee, genes from modules expressed in both branches are on average more highly expressed than branch 1-specific genes. However, the lower tails of the two expression distributions overlap substantially (**Ext. Data Fig. 4a,b**), indicating that both groups contain genes expressed at similarly low levels in ERS+ cells. This argues that many ERS-associated genes should remain detectable in ERS− cells if their absence were solely due to reduced transcript capture.

To test this more quantitatively, we modelled the expected ERS− expression based on the behaviour of genes expressed in both branches. Using xylem pole pericycle, the largest lineage affected by the ERS dichotomy, as a test case, we fitted a linear model describing the relationship between ERS+ and ERS− expression for genes from modules expressed in both branches. This model therefore represents the ERS− expression expected under a simple reduced-capture scenario. We then calculated residual ERS− expression values (observed minus expected expression from the fitted model) for all genes and compared the residual distributions of branch 1-specific genes and genes expressed in both branches within the shared expression range of the two categories. Branch 1-specific genes showed significantly more negative residual ERS− expression values than genes expressed in both branches (**Ext. Data Fig. 4c**), indicating that these genes are depleted in ERS− cells beyond what would be expected from reduced transcript capture alone. Together, these analyses demonstrate that the absence of ERS-associated genes in ERS− cells is inconsistent with a simple low-UMI or damaged-cell explanation.

Regarding Extended Data Fig. 9, this figure was included in response to requests during the previous review round to assess whether ERS− cells would have been excluded by conventional mitochondrial/chloroplastic filtering thresholds (**see Response to Referee 3 - point 1**). While we agree that neither Extended Data Fig. 9 nor 10 alone constitutes definitive proof of biological relevance, these analyses form part of a broader series of quality-control analyses aimed at systematically excluding technical artefact explanations.

Importantly, these computational analyses are complemented by multiple independent biological validations, including: (i) *in vivo* HCR imaging demonstrating neighbouring ERS marker-positive and -negative cells within the same tissue layers; (ii) decrease in ERS+ cell proportion in ABA-pathway mutants, consistent with the predicted regulatory associations of ERS-associated genes; (iii) strong environmentally induced increase in ERS acquisition under drought stress observed by both scRNA-seq and HCR; and (iv) most importantly, the new identification and experimental validation of an ERS−-enriched epidermal marker, enabling direct HCR-based detection of two mutually exclusive epidermal populations *in vivo* (**see Response to Referee 3 – point 2**). Together, these analyses strongly support the conclusion that the ERS− cells represent a *bona fide* biological cell population rather than damaged cells.

Referee 3 - point 4: Integration Analysis

The evidence from the integration analysis seems somewhat arbitrary. First, the first dataset (Extended Fig. 13) did not detect Branch 2. Second, the second dataset (Extended Fig. 14) was not analyzed with the same integration approach as the first. Third, it is unclear what cell filtering criteria were applied to each dataset; applying different UMI or mito% cutoffs could probably determine whether Branch 2 appears or disappears.

Response: Thank you for the opportunity to clarify this point. To avoid potential confusion with regard to referencing figure numbers in this response, we would note that in the previous manuscript version, the 'first dataset' was presented in Ext. Data Fig. 12 (the Farmer *et al.* dataset). We would first note that branch 2/ERS− cells were detected in this Farmer *et al.* dataset (**Ext. Data Fig. 5**), as evidenced by the distribution of ERS-associated module scores for each individual nucleus (**Ext. Data Fig. 5e-f)**, as well as numerous nuclei projected onto branch 2 for almost all lineages in the UMAP embeddings (**Ext. Data Fig. 5a-d**). However, because this dataset is based on snRNA-seq rather than whole-cell scRNA-seq, nuclei were isolated by tissue chopping, a process that likely under-samples the small populations of early developmental cells near the root tip. As a consequence, early branch cells and the full continuity of the branching topology are less clearly represented in the embedding, even though ERS+/ERS− populations remain detectable by module score and label transfer. We would further note that we detected branch 2/ERS− cells in all three previously published datasets: Farmer *et al.*, Wendrich *et al.*, and Shahan *et al.* (**Ext. Data Figs. 5-7**).

Regarding the integration strategy, all label transfer analyses were performed using the same integration/projection framework across the three external datasets, as described in the Methods. The only technical difference is that for the Shahan *et al.* dataset (**Extended Data Fig. 7**), we were unable to project the transferred labels directly onto our reference atlas UMAP embedding due to compatibility issues between the downloadable Seurat object version provided by that study and the Seurat version used in our analysis. We therefore retained the original Shahan *et al.* embedding for visualisation. Importantly, this does not affect the label transfer itself. Indeed, we believe that retaining their original embedding is advantageous, as their trajectory structure differs from ours in that the lineages converge more clearly to a common t = 0 before each separates into two branches, providing an independent visual representation of the same bifurcating topology.

With respect to filtering criteria, we agree that stringent filtering can strongly influence whether ERS−/branch 2 cells are retained, and indeed this was precisely the point illustrated by our reanalysis of the Wendrich *et al.* dataset (**Extended Data Fig. 6**). In that study, the authors applied a hard UMI threshold during preprocessing, which removed the ERS− population from the processed dataset. By reprocessing the raw data using our own filtering strategy, we were able to recover these cells and identify the ERS dichotomy. In contrast, the Farmer *et al.* and Shahan *et al.* datasets already retained ERS− cells in their publicly available processed objects and therefore did not require reprocessing. For the Farmer *et al*. dataset, we used the publicly available Cell Ranger output matrices without additional filtering. For the Shahan *et al*. dataset, the authors had already applied quality-control filtering using the COPILOT pipeline, including removal of low-quality cells based on mitochondrial transcript enrichment and similarity to low-quality expression profiles, exclusion of UMI-count outliers and predicted doublet.

Thus, while preprocessing choices in some cases affected whether ERS− cells were retained in the published processed data, the downstream integration and label transfer analyses were performed consistently across all three external datasets.

Referee 3 - point 5: UMI Downsampling

To test whether branch separation is driven by UMI differences, the authors downsampled cells with >1,000 UMIs to 1,000 while leaving lower-UMI cells unchanged. It's not clear why 1,000 was chosen (perhaps it corresponds to the average of Branch 2?) but ideally, downsampling would match the full UMI distribution of Branch 2 rather than applying a fixed cutoff. Even after downsampling, Branch 2 remains slightly lower and retains a pronounced low-UMI tail not seen in Branch 1.

Response: Thank you for this helpful suggestion. We agree that matching the full UMI distribution of branch 2/ERS− cells provides a more appropriate test of whether the observed bifurcation could be driven by differences in transcript capture alone.

Accordingly, we have now revised our downsampling strategy such that branch 1/ERS+ cells are no longer downsampled to a fixed UMI threshold. Instead, branch 1/ERS+ cells are now randomly downsampled to match the full UMI distribution of their branch 2/ERS− counterparts within each lineage. This procedure was repeated independently three times.

Importantly, the conclusions remain unchanged. In all three downsampling replicates, the two branch populations remain clearly separable, and cells with the highest meristematic scores consistently localise between the two branches, maintaining the inferred t = 0 position and supporting the bifurcating trajectory topology (**Extended Data Fig. 4d-g**).

These results further demonstrate that the ERS-associated branching structure cannot be explained solely by differences in UMI content between branch 1 and branch 2 cells.

Referee 3 - point 6: Cell Cycle Effects (Extended Fig. 6b)

The text states that branch separation is not explained by cell cycle stage, which may be true, but there appears to be a consistent difference in the proportion of G1 cells between branches. Although the two branches remain after regressing out cell cycle effects, regression may not fully remove all related variation?

Response: We agree that a modest difference in cell-cycle phase proportions between branches is visible in some lineages. However, if the observed bifurcation were primarily driven by cell-cycle state, a substantially stronger segregation by cell-cycle phase would be expected. Importantly, regression of cell-cycle scores does not abolish the branching structure, indicating that cell-cycle-associated transcriptional variation is not sufficient to explain the observed dichotomy.

More broadly, we consider a cell-cycle-based explanation unlikely for biological reasons. First, if ERS+ and ERS− populations primarily reflected different cell-cycle stages, clear differences in cell size and morphology would be expected in the HCR images between ERS marker-positive and -negative cells. No such systematic differences are observed.

Second, previous studies using mitotic/cell division markers have shown that cell division in the Arabidopsis primary root is largely confined to the meristematic zone near the root tip and becomes rare once cells enter elongation and differentiation zones, where the ERS+/ERS− dichotomy is most prominently observed[1,2]. Thus, the spatial position of the ERS-associated branching is inconsistent with the expected localisation of proliferative cell-cycle effects.

Together, these observations strongly suggest that while minor cell-cycle differences may contribute modestly to transcriptional variation between branches, they do not account for the ERS-associated bifurcation observed in our data.

Referee 3 - point 7: HCR Validation (Fig. 2e)

The HCR experiments validating Branch 1–specific genes are among the most promising evidence. We suggest adding quantitative analyses and results, rather than only highlighting selected examples through visualization, as this would provide stronger support.

Response: Thank you for the positive comment regarding the HCR validation. We agree that quantitative analysis provides stronger support than representative optical sections alone.

We initially did not include segmentation-based quantification because the thin, elongated cells located in the inner tissues of the root are very difficult to segment reliably in three dimensions. However, for the markers shown in Fig. 2 (now Fig. 3 in the revised manuscript), the relevant positive and negative cells are located in the outer root layers (epidermis, cortex, and endodermis), where cell boundaries are larger and more readily segmented.

We have therefore now performed 3D segmentation of these outer tissue layers in the roots presented in Fig. 3 of the revised manuscript, and quantified volumetric fluorescence intensity on a per-cell basis. In the revised figure, segmented cells are colour-coded by mean fluorescence intensity, providing quantitative support for the positive and negative cells highlighted in the representative images (**Fig. 3a**). For all four ERS-associated markers, the cells visually identified as positive and negative show clear and substantial differences in fluorescence intensity, with cells within the same layer differing by up to 20 fold-change in signal intensity (**Fig. 3a**). Furthermore, we have also performed 3D segmentation and per-cell quantification of volumetric fluorescence intensity for the new HCR experiments comparing the ERS+ (*AT5G56550*) and ERS- (*AT3G01720*) markers in epidermal cells (**Fig. 3f-h**).

These quantitative analyses confirm that the heterogeneous HCR patterns highlighted in the representative images reflect robust and reproducible differences in transcript abundance between neighbouring cells, providing strong orthogonal support for the existence of ERS-associated transcriptional heterogeneity *in vivo*.

Response references:

1. Verbelen, J.-P., De Cnodder, T., Le, J., Vissenberg, K. & Baluska, F. The root apex of Arabidopsis thaliana consists of four distinct zones of growth activities: Meristematic zone, transition zone, fast elongation zone and growth terminating zone. *Plant Signal. Behav.* **1**, 296–304 (2006).
2. Lavrekha, V. V., Pasternak, T., Ivanov, V. B., Palme, K. & Mironova, V. V. 3D analysis of mitosis distribution highlights the longitudinal zonation and diarch symmetry in proliferation activity of the Arabidopsis thaliana root meristem. *Plant J.* **92**, 834–845 (2017).

Point by point response to comments on Nature manuscript 2022-06-09447C

We extend our sincere thanks for the constructive feedback and time and effort in considering our manuscript. We believe we have addressed all comments and requested changes, which has helped us improve our study considerably. Below we provide a point-by-point response to all comments and suggestions.

Referee #1

Summary: This round of review responds to the authors' rebuttal and the new data provided; several points are well addressed, but my principal concern — the naming of the state (point 8) — remains unresolved, and the rebuttal in some respects reinforces it. My remaining concerns are largely interpretive: the wording in several places asserts function, causation, and responsiveness that the data, which are transcriptional-state correlations, do not yet establish, together with one methodological point about the HCR validation that bears on a central claim.

Response: We thank the Referee for their careful evaluation of our revised manuscript and for acknowledging the extensive revisions and additional analyses performed in response to the previous round of review. We also appreciate the remaining comments, which have helped us further clarify the interpretation of our findings and ensure that our conclusions remain closely aligned with the evidence presented. In response, we have revised the manuscript to remove conceptual ambiguities, adopt more precise terminology, and further temper several interpretations. We have also performed additional analyses, including per-cell quantification of HCR fluorescence under water-deficit conditions, analyses explicitly accounting for the increased transcript abundance observed under water deficit, and revised analyses and presentation of the epidermal dual-marker HCR experiments. Collectively, we believe these revisions substantially improve the clarity and interpretation of the manuscript while providing stronger support for our conclusions.

To comply with Nature's revised length requirements, we have also streamlined the manuscript where appropriate. For ease of re-review, we have provided both a clean PDF of the revised manuscript and a Word version with tracked changes. The tracked-changes version highlights all modifications relevant to the Referee's comments, while purely editorial revisions made to meet the formatting requirements have been accepted.

Major points:

Referee 1 - point 1: The recurring inference of function and responsiveness from a transcriptional state need to be toned down (Abstract, p.2; Results, p.3). Several framing statements claim more than the data show.

– Abstract: "…genes involved in responses to environmental stimuli, including those activating the abscisic acid (ABA) pathway." It is unclear whether these genes are activating the ABA pathway or responding to it; the distinction matters because it is the difference between a causal and a correlative claim, and the present data support the latter.

– Abstract: "…a highly active and environmentally responsive cell state." Is this accurate? Activity (UMI/gene number) is measured; responsiveness of individual cells to environmental signals is not directly demonstrated.

– p.3: "…cell types in the root are in fact composed of subsets of cells whose function is modulated in response to environmental information." What is demonstrated is a difference in gene expression state, not a difference in function. I would recommend the authors substitute "gene expression state" for "function" here and edit comparable statements throughout.

Response: We thank the Referee for this helpful comment and agree that several statements in the previous version of the manuscript could be interpreted as implying functional consequences or intrinsic environmental responsiveness beyond what is directly supported by our data. Our intention was to describe a transcriptional state characterised by coordinated expression of genes associated with environmental responses, rather than to imply that ERS+ cells are intrinsically more responsive to environmental stimuli or that functional differences between ERS+ and ERS− cells have been demonstrated.

To address this concern, we have carefully revised the wording throughout the manuscript to distinguish more clearly between transcriptional state, transcriptional activity, and cellular function, and to avoid language implying causality or experimentally demonstrated responsiveness where this has not been directly tested.

Specifically, for the examples highlighted by the Referee:

- **Abstract:** We have replaced *"genes involved in responses to environmental stimuli, including those activating the abscisic acid (ABA) pathway"* with *"genes involved in responses to environmental stimuli, including the abscisic acid (ABA) pathway"* to avoid implying activation of the ABA pathway.
- **Abstract:** We have replaced *"a highly active and environmentally responsive cell state"* with *"a highly transcriptionally active cell state enriched for genes associated with environmental responses"* to more accurately reflect the evidence provided by the transcriptomic analyses.
- **Introduction:** The statement highlighted by the referee has been removed following revision of the Introduction.

Overall, we have carefully reviewed the manuscript and revised similar wording wherever appropriate to ensure that our conclusions remain closely aligned with the evidence presented. The revised manuscript consistently distinguishes between the transcriptional characteristics of the ERS and experimentally demonstrated cellular behaviour, while preserving our central conclusion that the prevalence of this transcriptional state is modulated by environmental conditions.

Referee 1 - point 2: The transcriptional asymmetry between the branches, and what defines ERS− (p.8) needs revision. The statement that "branch 1 is substantially more transcriptionally active than branch 2" is not accurate as supported. The identification of branch-1-specific genes alone does not indicate that branch 2 cells are less transcriptionally active. The lack of a defined branch-2 expression program does support the asymmetry, as does the UMI/gene-count comparison the authors cite — but the sentence as written rests the claim on the module/gene identification, which is not strong support; the wording should be tightened to reflect what actually establishes the difference. This connects to a broader question about ERS−: ERS+ is well defined by co-expression modules and ABA/environmental-response signatures, but ERS− remains largely defined by absence of the ERS program. Is this interpretation correct, or are there programs enriched in ERS− cells? The possibility that ERS− represents a lower-transcriptional or dormant state (see point 6) should be addressed directly rather than left implicit.

Response: We thank the referee for this thoughtful comment. We agree that the previous wording did not clearly distinguish the evidence supporting the transcriptional asymmetry between the two branches from the identification of branch-specific gene modules. We also appreciate the opportunity to clarify the interpretation of the ERS− branch.

Regarding the first point, we have revised the relevant text to clarify that the conclusion that the ERS+ branch is more transcriptionally active is supported by its higher transcript abundance, rather than solely by the identification of branch-specific genes. The revised wording no longer attributes this conclusion to the module analysis alone.

Regarding the identity of the ERS− branch, we agree that it is primarily defined by the lack of activation of the coordinated ERS transcriptional programme while retaining its canonical cell-type identity. We also agree that ERS− cells exhibit lower overall transcript abundance than ERS+ cells. However, our analyses show that lower transcript abundance alone does not explain the lack of activation of the ERS transcriptional programme. As shown in **Extended Data Fig. 4a–c**, ERS-associated genes remain specifically depleted in ERS− cells even after controlling for their expression level in ERS+ cells, demonstrating that their reduced expression in ERS− cells cannot be explained solely by lower transcript abundance or reduced transcript detection. Furthermore, UMI downsampling analyses (**Extended Data Fig. 4d–g**) show that reducing transcript abundance in ERS+ cells does not abolish the bifurcating trajectories, further supporting that lower transcript abundance alone cannot explain the observed transcriptional asymmetry.

We agree that our data do not exclude the possibility that ERS− cells represent a less transcriptionally active state. However, our analyses indicate that this lower transcriptional activity is accompanied by a specific lack of activation of the coordinated ERS transcriptional programme, rather than simply reflecting reduced detection of ERS-associated transcripts. We have revised the manuscript accordingly to clarify both the basis for the transcriptional asymmetry between the two branches and the interpretation of the ERS− population.

Referee 1 - point 3: The position of the bifurcation differs between datasets (cross-dataset validation). The authors validated the presence of two branches using the Shahan et al. dataset and demonstrated the specific expression of Modules 20 and 21 in that dataset. However, the branching/bifurcation appears to be very different between the two datasets: in the authors' atlas the ERS+/ERS− bifurcation is found almost immediately following their defined developmental origin, whereas in the Shahan et al. dataset it appears to occur much later (likely the differentiating cells) along the developmental axis. It would be worthwhile to perform a cross-mapping between the two datasets and examine what could be the cause of this discrepancy.

Response: We thank the Referee for this insightful comment and agree that the apparent difference in the position of the bifurcation between our atlas and the Shahan *et al.* dataset warrants further consideration.

In the manuscript, we acknowledge that the earliest stages of the bifurcation are incompletely represented outside the epidermis in our atlas, stating:

> *"Only the epidermis contained cells preceding this bifurcation and a clearly identifiable meristematic population. In most other lineages, branching occurred directly from t=0, likely reflecting the difficulty of capturing the rarer inner-layer meristematic cells during protoplasting."*

We agree that projecting our data onto the Shahan *et al.* embedding would have been an informative way to determine whether the apparent difference reflects differences in sampling of early developmental stages or differences in trajectory inference. We therefore attempted this analysis. Unfortunately, we were unable to project our data onto the published embedding because of incompatibilities between the Seurat version used for the original analysis and current versions, together with differences in how the published embedding was generated.

Nonetheless, the apparent difference in bifurcation position is consistent with our interpretation that differences in sampling of early developmental stages contribute to the inferred trajectories. As shown in **Fig. 2b**, genes associated with meristematic and early developmental states (Module 23) are expressed across a much larger proportion of epidermal cells than cells from other lineages, consistent with our observation that early inner meristematic populations are incompletely represented outside the epidermis in our atlas. Consequently, the precise position of the inferred bifurcation may differ between datasets without altering the underlying biological interpretation.

Referee 1 - point 4: The in vivo HCR demonstration of cell-to-cell heterogeneity is not yet convincing (pp.10–11).

– The claim that canonical cell-type markers “remained detectable in negative cells, ruling out differences in HCR efficiency” (Ext. Data Fig. 10c) requires quantitation. Those canonical markers should show less heterogeneity between cells than the ERS markers do; otherwise the observed variability is not specifically attributable to the ERS. This can be addressed with the existing imaging data by quantifying the cell-to-cell variance of the canonical markers against that of the ERS markers; if such a comparison is not feasible, the claim should be tempered accordingly.

– The complementary-expression result in Fig. 3e–h is mainly showing hair vs. non-hair differences in expression. As presented it is not a convincing demonstration of the branch 1 / branch 2 dichotomy independent of the known hair/non-hair distinction, and the text should either show the effect within a single epidermal class or soften the claim.

Response: We thank the Referee for these helpful comments and agree that both the interpretation of the canonical marker analysis and the presentation of the epidermal HCR experiment could be improved.

Regarding the first point, we agree that the presence of canonical cell-type marker expression in cells lacking ERS marker expression does not, by itself, rule out differences in HCR efficiency. We have therefore tempered this statement in the revised manuscript to avoid overinterpretation and now state only that the continued detection of canonical markers in ERS-negative cells makes a general failure of HCR detection unlikely, rather than ruling it out.

Regarding the second point, we agree that the representative HCR image in the original manuscript could give the impression that the complementary expression pattern primarily reflects the well-established alternation of hair and non-hair cell files. To clarify this point, we have revised both the figure and the accompanying analyses. Specifically, the Gaussian mixture analysis now shows the proportion of hair and non-hair cells within each of the two Gaussian components (**Fig. 3e**), demonstrating that both epidermal cell types are represented in both populations. We have also replaced the representative HCR image (**Fig. 3f**) with one showing complementary expression of the two markers within the same epidermal cell file (**Response Figure 1**).

Because intact roots were imaged without flattening to avoid the image distortion that it would cause, it is difficult to obtain a single optical section containing the entire epidermis in focus simultaneously. The image used in the original manuscript was selected because it contained the largest number of epidermal cells within a single focal plane. However, to avoid the possibility that readers might interpret this image as reflecting the canonical hair/non-hair cell alternation, we have replaced it with one that better illustrates complementary marker expression within the same epidermal cell file.

Although the HCR expression pattern of these markers appears to show some organisation into longitudinal cell files in some images, the combined evidence from the scRNA-seq analysis and the HCR data indicates that this organisation cannot be attributed to the canonical hair/non-hair cell pattern. The revised analyses and representative image therefore better reflect the conclusions of the study.



Response Figure 1 / Figure 3f: Optical section showing transcript detection of *AT3G01720* ('branch 2' marker) and *AT5G56550* ('branch 1' marker) in the epidermal cells (asterisks). The dotted yellow line delineates two adjacent cells within the same epidermal cell file that alternately express the 'branch 1' and 'branch 2' markers.

Referee 1 - point 5: The scRNA-seq and HCR proportions are not the same quantity, so the claimed correspondence is not established (p.16). The statement that "the positive and negative populations detected by HCR correspond to the ERS+ and ERS− branches… as their relative proportions vary similarly across both independent experimental approaches" is not accurate as an inference. The scRNA-seq data are characterized by the proportion of cells with expression, whereas the HCR data are shown as the proportion of cell area with positive signal. Similar trends in two different metrics do not demonstrate correspondence of the underlying populations. Expression needs to be quantified on a per-cell basis, and the distribution of per-cell expression examined across these conditions, before this correspondence can be claimed.

Response: We thank the Referee for this important comment and agree that our original comparison was based on two different quantities. In the original manuscript, the HCR analysis for the water-deficit experiment quantified overall fluorescence intensity and the area occupied by positive cytoplasmic signal across the tissue, whereas the scRNA-seq analysis measured the proportion of individual cells assigned to the ERS+ and ERS− states. We agree that these measurements are not directly comparable and that similar trends alone do not establish correspondence between the underlying cellular populations.

To address this, we have now complemented the tissue-level measurements with a per-cell quantification of HCR fluorescence (**Fig. 5k**). Rather than imposing a threshold to classify cells into discrete positive and negative populations, we examined the empirical cumulative distribution of fluorescence intensities across individual cells under control and water-deficit conditions. This analysis reveals a clear global shift towards higher ERS-marker expression under water deficit for both markers, indicating that a greater proportion of cells express higher levels of these ERS-associated genes.

Importantly, we have deliberately avoided categorising HCR-labelled cells into ERS+ and ERS− populations. ERS assignment in the scRNA-seq data is based on the coordinated expression of the whole transcriptome, whereas HCR measures the abundance of a single transcript. Moreover, the two techniques differ in sensitivity and dynamic range, and fluorescence intensity is inherently continuous rather than binary. Applying an arbitrary threshold to define HCR-positive and HCR-negative cells would therefore imply a level of equivalence between the two approaches that is not supported by the data.

Accordingly, we have revised both the figure and the manuscript text. We no longer state that HCR-positive and HCR-negative populations correspond to the ERS+ and ERS− states. Instead, we conclude that the per-cell HCR measurements independently demonstrate that more cells express ERS markers under water-deficit conditions, consistent with the increased prevalence of ERS+ cells observed by scRNA-seq.

Referee 1 - point 6: The drought-induced increase in ERS+ may reflect global transcriptional activation rather than specific induction (p.18). Because ERS+ cells have higher UMI/gene counts and water deficit increases overall transcript abundance, the increase in ERS+ proportion under drought could partly reflect global transcriptional activation rather than specific induction of the ERS+ program. This also raises the possibility that ERS− cells represent a lower-transcriptional or dormant state (see point 2). The authors should comment on whether this is a valid alternative hypothesis and, if so, include mention of it in the manuscript. Relatedly, the framing of the result ("the extensive reassignment of cells into the ERS under drought… can be strongly modulated by environmental inputs") does not distinguish a difference in the strength of the ERS signature (and therefore the threshold for calling a cell ERS+) from a difference in the number of cells in versus out of

the state (a more binary switch). If the existing data can distinguish these, the result should say which is occurring; if not, the framing should be qualified. The authors already raise the closely related question in the Discussion — whether the ERS+/ERS− proportions reflect probabilities of switching between states — and I would ask that the same caveat be carried back into the Results so the drought interpretation is not stated more strongly there than in the Discussion.

Response: We thank the Referee for this important comment and agree that these are key points for the interpretation of our results. We agree that the increased prevalence of ERS+ cells under water-deficit conditions could potentially reflect the higher overall transcript abundance observed under stress rather than a specific induction of the ERS transcriptional programme. We also agree that, with the current data, it is important to distinguish between changes in transcriptional output, changes in the prevalence of the ERS state, and the underlying dynamics governing entry into and maintenance of this state.

To address the possibility that the increase in ERS+ cells simply reflects global transcriptional activation, we performed several additional analyses that explicitly account for transcript abundance. First, we compared ERS module scores between cells in the control and water-deficit conditions that have comparable transcript abundance and found that ERS module activity remained significantly higher under water-deficit conditions (**Fig. 5g**). Second, we modelled the probability of a cell being classified as ERS+ using logistic regression while adjusting for log-transformed UMI counts. As expected, accounting for transcript abundance reduced the estimated effect of water-deficit, as higher transcript abundance is an intrinsic characteristic of the ERS+ state and therefore explains part of the increased probability of ERS+ classification. Importantly, however, the adjusted odds ratio remained significantly greater than one and highly significant (**Fig. 5h**), demonstrating that the increased prevalence of ERS+ cells cannot be explained solely by higher transcriptional output.

We have also expanded the discussion of this point in both the Results and the Discussion. Specifically, we now clarify that the present data cannot distinguish whether this reflects an increased probability of cells entering the ERS+ state, increased persistence within that state, or a strengthening of the ERS transcriptional programme resulting in more cells being classified as ERS+. Likewise, as discussed in our response to Point 2, we do not exclude the possibility that ERS− represents a less transcriptionally active state. However, our additional analyses indicate that lower transcript abundance alone does not account for the lack of activation of the coordinated ERS transcriptional programme. We have therefore revised both the Results and the Discussion to reflect these distinctions and avoid overstating the interpretation of the drought response.

Referee 1 - point 7: “Functionally regulated rather than stochastic” — the basis is unclear (p.17). The authors claim that the consistency of the two populations and their modules “strongly supports the hypothesis that the ERS reflects a functionally regulated transcriptional program rather than stochastic variability”? Reproducibility across datasets does not by itself exclude a stochastic but consistently-proportioned process (this bears directly on the naming question, point 8). The logic connecting the evidence to this conclusion should be made explicit, or the conclusion softened, especially as it relates to function.

Response: We thank the Referee for this thoughtful comment and agree that reproducibility alone does not exclude a stochastic process. Our intention was not to imply that the reproducibility of the ERS across datasets is, by itself, evidence of functional regulation.

Rather, our interpretation is based on the observation that the ERS comprises a coordinated transcriptional programme that is consistently detected across independent datasets, ecotypes, and experimental platforms, and whose prevalence is reproducibly modulated by defined environmental and genetic perturbations, including nutrient availability, water deficit, and ABA signalling. We therefore interpret that the combination of coordinated gene expression and predictable modulation across multiple independent biological contexts is more consistent with a regulated transcriptional programme than with stochastic transcriptional variability alone.

Nevertheless, we agree that the original wording overstated this inference. We have therefore softened the corresponding statement in the manuscript to make clear that our data are consistent with, rather than definitive proof of, a regulated transcriptional programme, and have avoided implying that stochastic contributions can be excluded.

Referee 1 - point 8: The name “Environmentally Responsive State” remains unresolved.

I appreciate the authors’ detailed response and the new data provided. However, the core concern remains unresolved: the name “Environmentally Responsive State” still implies per-cell sensing and response. The authors’ rebuttal helpfully clarifies what the data do and do not show, but in doing so it further undermines the name. First, the authors state: “ERS+ and ERS− cells are not defined as cells that are intrinsically more or less

capable of responding to environmental stimuli." If the cells are not defined by per-cell responsiveness, they should not be named for it. Second, Response Figure 1 shows ERS+ cells exhibit lower transcriptional responses to water deficit than ERS− cells (UMI fold-change: 1.32 vs. 1.50), making it harder still to justify calling the state "environmentally responsive."

The authors have already made the revision in one place: the Discussion now reads "This additional transcriptional state, whose prevalence is modulated by environmental conditions." This phrasing is more accurate, and the name should reflect this description. The unknown per-cell determinant of this state is equally consistent with stochastic gene expression tuning the probability of entering the state. Therefore, ERS may represent a stochastically-accessed transcriptional state whose threshold or probability is environmentally modulated, consistent with a bet-hedging strategy. In addition, reproducibility across datasets excludes technical noise but does not rule out biologically stochastic switching, which is itself a regulated and consistent process. The revised Discussion's own definition of the ERS, "an additional layer of identity regulated by ABA," is a cell-intrinsic explanation that would be better reflected in the name than what "environmentally responsive" implies. Suggested alternative: "Environment-modulated state" or "environment-regulated state," the latter preserving the ERS acronym. This requires no new experiments.
Response: We agree with the Referee that the term *Environmentally Responsive State* could be interpreted as implying that ERS+ cells are intrinsically more responsive to environmental stimuli at the single-cell level, which is not supported by our data. To avoid this implication and better reflect our observations, we have adopted the referee's suggestion and renamed the ERS throughout the manuscript as the *Environment-Regulated State*. We believe this terminology more accurately captures our findings that the prevalence of this transcriptional state is regulated by environmental conditions, while remaining agnostic regarding the underlying mechanisms governing entry into or maintenance of the state.

Minor points:

Referee 1 - point 9: "cells acquire the ERS" should be adjusted to use more neutral phrasing. Phrases like "cells acquire the ERS" imply an active switch that has not been directly observed. "Cells in the ERS" or "ERS-enriched cells" are more neutral.
Response: We agree with the Referee and have revised the terminology throughout the manuscript to avoid implying an active transition into the ERS. References to cells "acquiring" the ERS have been replaced with more neutral phrasing throughout.

Referee 1 - point 10: "upwards" should be replaced with "shootward" (p.3). The field-standard directional term for displacement away from the tip is "shootward" (vs. "rootward"); "upwards" is orientation-dependent.
Response: We have replaced "upwards" with the field-standard directional term "shootward" throughout the manuscript where appropriate.

Referee 1 - point 11: "border cells" should be replaced with "border-like cells" (p.5). Arabidopsis produces border-like cells (BLCs), which remain attached in sheets, rather than the individually detaching border cells of other species. The term should be corrected.
Response: We have replaced "border cells" with "border-like cells" throughout the manuscript.

Referee 1 - point 12: "drought" should be replaced with "water deficit" in most cases. The system is a hard-agar / reduced-water plate model; "drought" overstates it. I would refer to it as water deficit or a model of water-limitation stress and reserve "drought" for the qualified term the authors use elsewhere.
Response: We have revised the terminology throughout the manuscript to use "water deficit" where appropriate, reserving the term "drought" for references to the experimental model or in contexts where it is explicitly qualified.