Single cell sequencing of plants: a growing story

Plant organs are composed of specialized cell communities, ensuring division of labor between various organs or their parts, and the integrity of the whole organism. Unique transcriptional programs are the basis for the diversity of cell types and thanks to single cell sequencing, it is possible to capture them, study heterogeneity among and within cell populations and get insight into the cells' microenvironment. It is possible to gain in-depth understanding of different cell types, cell states and to discover rare or transient cell populations. Single cell sequencing methods with ever increasing throughput have been developed, enabling simultaneous analysis of thousands of cells in a single run (Svensson et al., 2018).

In contrast to the well-established single cell sequencing of mammalian cells, single cell sequencing of plant tissues possesses additional challenges. Main differences are the presence of a cell wall, which hinders tissue separation and acquisition of individual cells, and the larger cell size (Bawa et al., 2022). Protoplast isolation has become the method of choice and has been applied successfully to a number of plant species and tissues (summarized *e.g.,* by Bawa et al., 2022; Shaw et al., 2021).

Arabidopsis root has been established as a popular model system and was used in pioneering studies to apply single cell sequencing technologies to plant research. Multiple publications utilized single cell solutions to characterize major cell types, rare cell populations, trajectories of cell differentiation or even transcriptional states in mutant lines (*e.g.,* Denyer et al., 2019*;* Ryu et al., 2019).

Figure 1: ***Arabidopsis thaliana*.**

Transforming plant research with single cell analysis

Single cell sequencing has the capability to transform plant research and can be applied to different fields of plant studies.

Cell type annotation and characterization

- Discover and study rare and novel cell types
- Discover cell type-specific markers
- Investigate genetic variation and heterogeneity

Plant physiology

- Unravel regulatory networks
- Study cell-cell interactions
- Characterize different cell types and cell states

Plant development

- Understand the transcriptomic regulations involved in plant development and cell differentiation
- Comparative studies between species

Environmental adaptation

- Explore the response to stress and environmental changes
- Perform studies with agriculturally relevant species

SCOPE-chip, a technology for partitioning of fragile cells

Singleron's patented SCOPE-chip® technology promotes high-throughput transcriptome profiling of single cells. The SCOPE-chip enables the partitioning of single cells and barcoding of individual mRNA molecules with capture beads. With this technology, cells are captured and partitioned by gravity. No pressurized system is necessary, making this technology particularly suitable for fragile cells, such as protoplast.

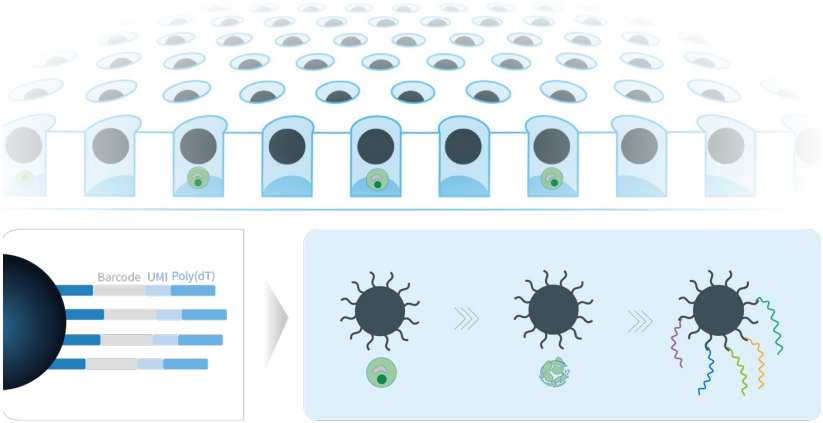

Figure 2: **SCOPE-chip technology.** The SCOPE-chip captures and partitions single cells into hundreds of thousands of microwells. Then, Barcoding Beads with attached capture oligonucleotides, carrying a cell label (Barcode), a unique molecular identifier (UMI) and a poly (T) probe, are added to the microwells. After cell lysis, the mRNA is captured by binding of the poly (A) tail to the poly (T) sequence of the capture oligonucleotides. Barcoding Beads are thereafter collected from the microwell chip and used for subsequent library generation.

The GEXSCOPE® Single Cell RNA Library Kit can be used with protoplast suspension to generate a sequencing ready NGS library that is compatible with Illumina sequencing instruments.

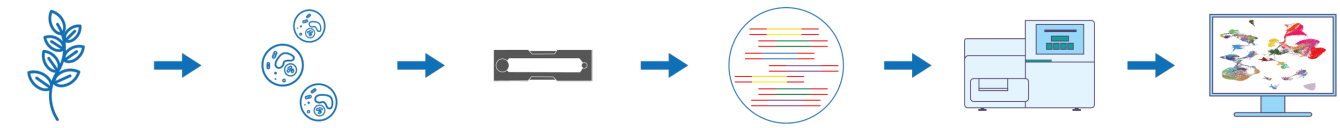

Figure 3: **Plant single cell sequencing workflow.** Protoplasts are isolated from plant tissue samples and loaded onto the SCOPE-chip for single cell partitioning, cell lysis, mRNA capture and molecular labelling. After reverse transcription of the captured mRNA, the barcoded cDNA is amplified and used to construct single cell libraries. The NGS library is sequenced and analyzed using bioinformatical tools.

Application 1: Single cell sequencing of *Arabidopsis thaliana* roots

Arabidopsis root is an ideal model system for investigating cell type-specific gene expression and cell differentiation in plants (Shahan et al., 2022) and is the most profiled plant tissue at single cell resolution (Bawa et al., 2022). Thus, it was used to demonstrate the compatibility of Singleron's technology with plant samples.

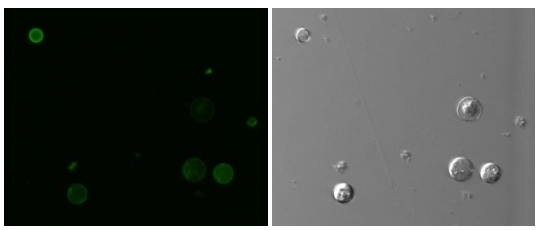

Figure 4: **Isolated root protoplast.** GFP (right) and bright field (left) channel. Image courtesy of Magda Marek (Max-Planck-Genome-Centre Cologne).

Sterilized *Arabidopsis thaliana* seeds with ubiquitous expression of green fluorescence protein (GFP) were grown on Murashige-Skoog (MS) plates under controlled conditions. Roots of eight day-old seedlings were used for protoplast isolation following the protocol by Bargmann et al. 2010. After fluorescence-activated sorting (FACS), GEXSCOPE Single Cell RNA Library Kit Cell V2 was used for single cell NGS library preparation.

Pre-processing and mapping were performed with CeleSCOPE v1.10.0, using TAIR 10 genome as a reference. Further processing was performed using R package Seurat v4.3.0 (Hao et al., 2021). A total of 9,430 cells were identified with 8,878 classified as high-quality cells. These were characterized by median of 1,761 genes per cell and median mitochondrial proportion of 0.33%.

After clustering, 19 distinct cell populations were identified. Seven clusters showed a signature of stele cells and included clearly defined populations of phloem and xylem cells representing 30% and 10% of the stele population, respectively. Additionally, a distinct population of root cap cells – characterized by expression of *ANAC033* transcription factor – was detected. Populations of trichoblasts and atrichoblasts were identified based on accumulation of *GLABRA 2 (GL2)* and *EXTENSIN 17 (EXT17)* transcripts, respectively (Figure 5).

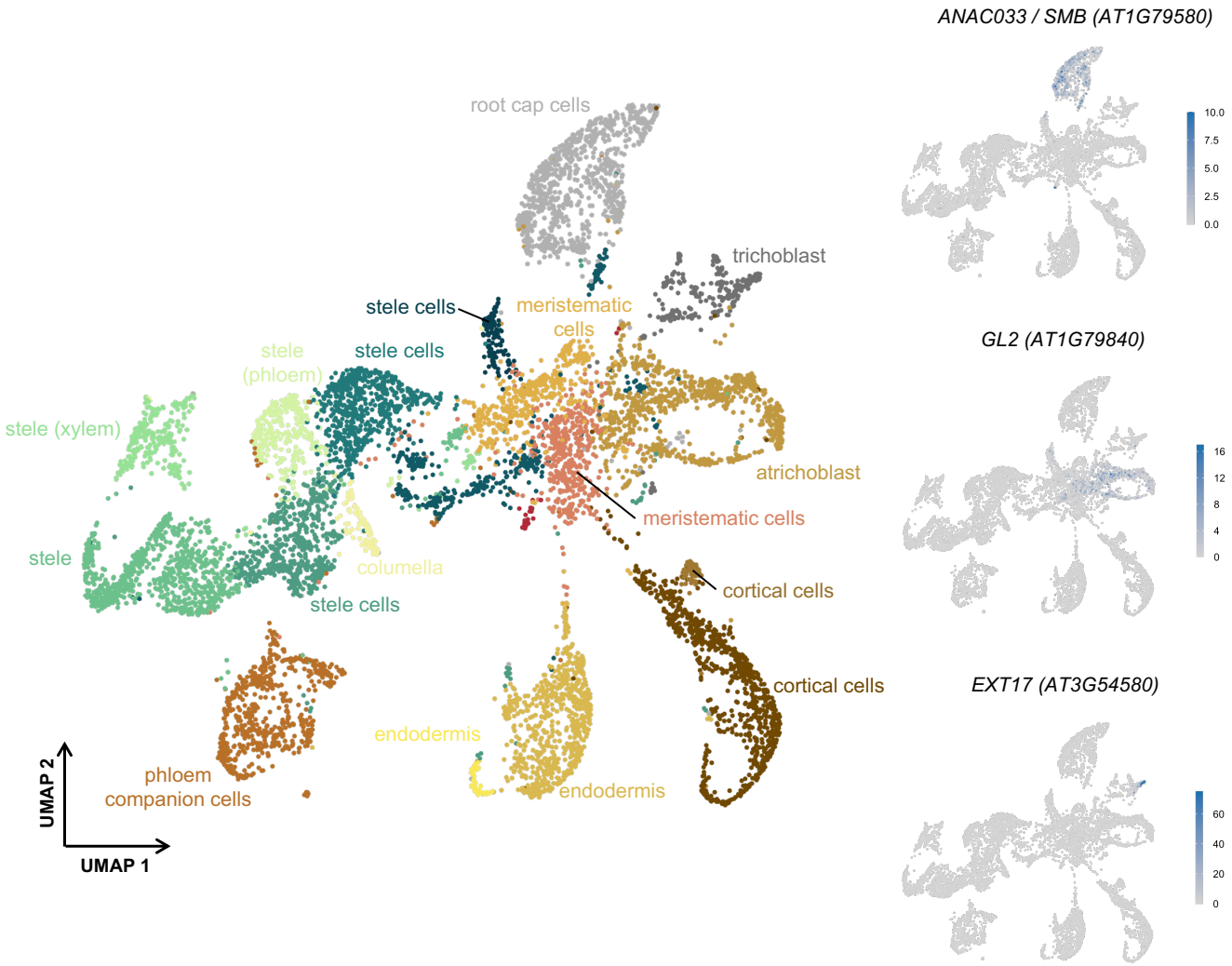

Figure 5: **UMAP with cell labels and feature plots of selected marker genes.** Major cell types were captured with RNA sequencing of 8,878 single cells. UMAP with cell-type labels (left) and expression of selected cell-type markers (right). Color scale represents log-normalized, corrected UMI counts. Transcription factor *ANAC033* is specific to root cap cells, *GLABRA 2 (GL2)* is a marker gene of atrichoblasts and *EXTENSIN 17 (EXT17)* is a marker gene of trichoblasts.

Trajectory analysis of trichoblast cells

Trajectory analysis was performed using Monocle3 (Trapnell et al., 2014). The developmental branch from meristematic cells to trichoblasts is highlighted here. Transcript levels of *AT5G62330* vanish in meristematic cells, which is followed by transcript accumulation in trichoblasts. Transcript accumulation of *METHYL ESTERASE 15 (MES15)* is restricted to a narrow time window and to the trichoblast population only.

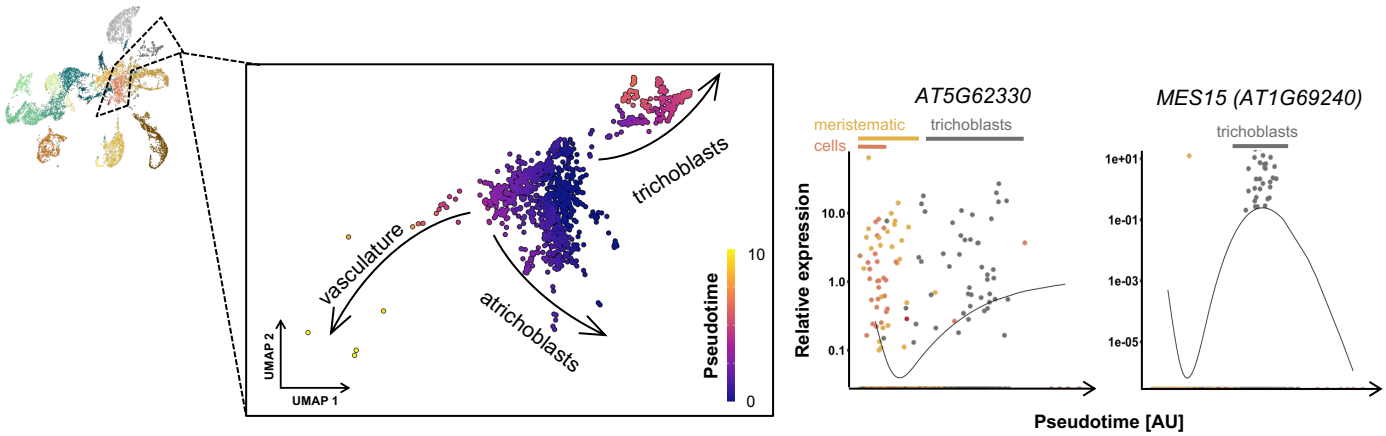

Figure 6: **Trajectory analysis and trichoblast development.** Trajectory analysis rooted in the super-cluster of meristematic cells identified three main branches of development, one of them specific to trichoblasts (left). Visualization of pseudotime-dependent gene expression predicts that *METHYL ESTERASE 15 (MES15)* expression is restricted to short duration and trichoblasts only.

Application 2: Single cell sequencing of *Arabidopsis thaliana* rosettes

Single cell sequencing of Arabidopsis rosettes shows that single cell sequencing performs well on green, chloroplast-containing tissue, indicating that the chip-based technology is robust, and that high quality data can be obtained from protoplasts purified through a mesh layer.

Sterilized seeds of *Arabidopsis thaliana* Col-0 were grown vertically on MS plates. Four weeks after sowing, rosettes of three seedlings were used for protoplast preparation. Following cell wall digestion (Apelt et al., 2022), protoplasts were collected and loaded onto the SCOPE-chip. GEXSCOPE Single Cell RNA Library Kit Cell V2 was used for preparation of single cell NGS libraries.

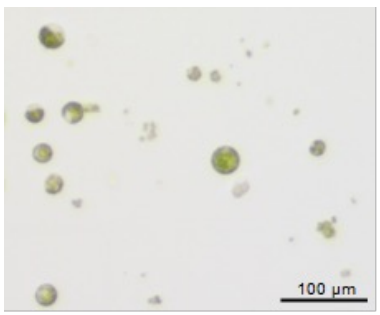

Figure 7: **Protoplasts from *Arabidopsis thaliana* rosettes.** Bright field.

Pre-processing was performed with CeleSCOPE v1.10.0 pipeline and reads were mapped to the TAIR 10 genome, leading to identification of 3,221 high-quality cells. These were characterized by a median of 3,697 UMI per cell and a median of 1,554 median genes per cell. Further processing was performed using R package Seurat v4.3.0 (Hao et al., 2021).

After clustering, populations which appeared to contain multiple cell types (based on expression of published and *de novo* identified marker genes) were further resolved. Conventional marker genes (Kim et al., 2021) were used to annotate captured cell populations.

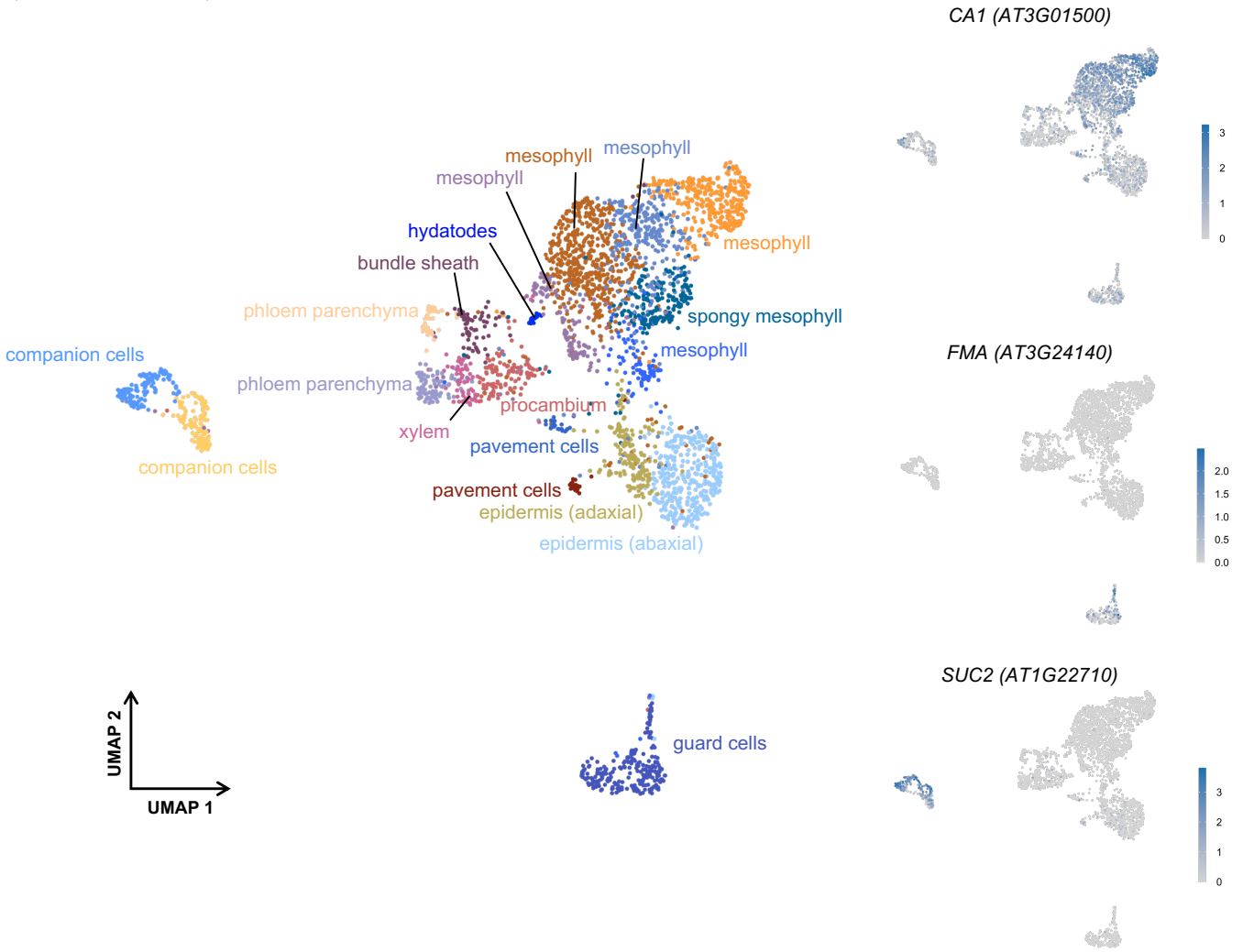

Figure 9: **Clustering and feature plot of Arabidopsis rosettes.** Major cell types were captured with RNA sequencing of 3,187 single cells. UMAP with cell-type labels (left) and expression of selected cell-type markers (right). Color scale represents log-normalized, corrected UMI counts. *CARBONIC ANHYDRASE (CA1)* is a marker gene of mesophyll cells, the transcription factor *FAMA* encoded by the *FMA* gene is a marker of guard cells and a high abundance of *SUCROSE-PROTON SYMPORTER 2 (SUC2)* transcripts is a characteristic of companion cells.

After clustering and sub-clustering steps, 19 distinct cell populations were detected, and assigned based on the expression of established marker genes (Kim et al., 2021; Figure 9). Finer annotation could be achieved by considering *de novo* identified marker genes and marker genes collated in the PlantscRNAdb v2.0 database (Chen et al., 2021). About half of all captured cells were mesophyll cells characterized by increased transcript levels of *e.g.*, *CARBONIC ANHYDRASE (CA1)*. Vasculature cells (procambium, xylem, phloem parenchyma and bundle sheath cells) comprised about 18% of the data set. Such over-representation of mesophyll sub-populations reflects the resistance of vasculature cell types to general protoplasting protocols as reported by Kim et al., 2021. Companion cells – characterized by *SUCROSE-PROTON SYMPORTER 2 (SUC2)* transcripts – formed two sub-clusters which clearly differed from all remaining vasculature cell types. Guard cells also showed unique transcriptional profiles as illustrated by accumulation of the FAMA transcription factor *(FMA)* – a regulator of stomata development.

Interestingly, two distinct sub-populations of companion cells were identified. Thus, the top ten differentially expressed genes in these two sub-populations were further explored. Genes enriched in sub-population 1, here called “companion cells 1”, are stress-induced (*e.g.*, *FBS3*), associated with response to phytohormone (*e.g.,* auxin responsive *SAUR30* and *ILL6* involved in jasmonic acid signaling) or with a role in lignin biosynthesis (*DOF4.6*). Strikingly, the “companion cells 2” sub-population was characterized by an accumulation of transcripts of photosynthesis genes (*e.g., LHCB3, LHCB6, PSAH2* or *PSAN*). Companion cells contain all organelles, including chloroplasts. They are interconnected with sieve elements and degenerate when the associated sieve elements cease to function. The possibility that a population of active and a population of aging companion cells were identified remains only a speculation. However, these results demonstrate how single cell sequencing can support formulating hypotheses for follow-up studies.

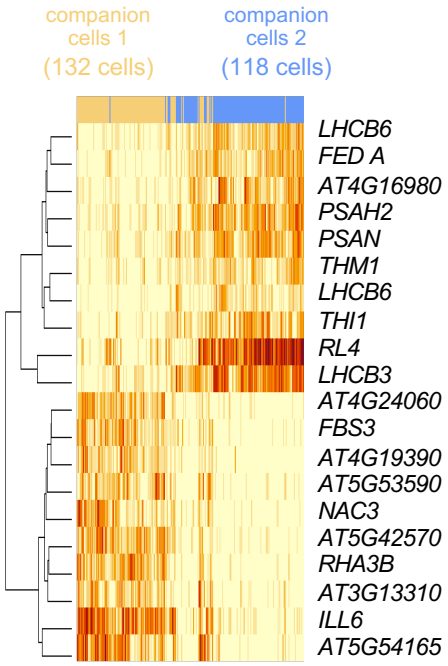

Figure 10: **Heatmap for top ten differentially expressed genes in each sub-population of companion cells.** Color scale represents log-normalized, corrected UMI counts.

References and Resources

- Svensson, V; et al., (2018). Exponential scaling of single-cell RNA-seq in the past decade. *Nat Protoc* **13**, 599–604. https://doi.org/10.1038/nprot.2017.149
- Bawa, G; et al., (2022). Single-Cell RNA Sequencing for Plant Research: Insights and Possible Benefits. *International Journal of Molecular Sciences*, *23*(9). https://doi.org/10.3390/ijms23094497
- Shaw, R; et al., (2021). Single-Cell Transcriptome Analysis in Plants: Advances and Challenges. *Molecular Plant*, *14*(1), 115–126. https://doi.org/10.1016/j.molp.2020.10.012
- Denyer, T; et al., (2019). Spatiotemporal Developmental Trajectories in the Arabidopsis Root Revealed Using High-Throughput Single-Cell RNA Sequencing. *Developmental Cell*, 48(6), 840-852.e5. https://doi.org/10.1016/j.devcel.2019.02.022
- Ryu, KH; et al., (2019). Single-cell RNA sequencing resolves molecular relationships among individual plant cells. *Plant Physiology*, 179(4), 1444–1456. https://doi.org/10.1104/pp.18.01482
- Shahan, R; et al., (2022). A single-cell Arabidopsis root atlas reveals developmental trajectories in wild-type and cell identity mutants. *Developmental Cell*, *57*(4), 543-560.e9. https://doi.org/10.1016/j.devcel.2022.01.008
- Bargmann, BOR; et al., (2010). Fluorescence Activated Cell Sorting of Plant Protoplasts. *J. Vis. Exp.* (36), e1673, doi:10.3791/1673
- Hao, Y; et al., (2021). Integrated analysis of multimodal single-cell data. *Cell*, *184*(13), 3573-3587.e29. https://doi.org/10.1016/j.cell.2021.04.048
- Trapnell, C; et al., (2014). The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat. Biotechnol.* 32, 381-386. https://doi.org/10.1038/nbt.2859
- Apelt, F; et al., (2022). Shoot and root single cell sequencing reveals tissue- And daytime-specific transcriptome profiles. *Plant Physiology*, *188*(2), 861–878. https://doi.org/10.1093/plphys/kiab537
- Kim, JY; et al., (2021). Distinct identities of leaf phloem cells revealed by single cell transcriptomics. *Plant Cell*, *33*(3), 511–530.
- Chen, H; et al., (2021). PlantscRNAdb: A database for plant single-cell RNA analysis. *Molecular Plant*, 14(6), 855–857. https://doi.org/10.1016/j.molp.2021.05.002
- CeleScope – https://github.com/singleron-RD/CeleScope [accessed 2023-01-28]
- The Arabidopsis Information Resource (TAIR) – www.arabidopsis.org [accessed 2023-01-28]

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