Single-cell analysis opens a goldmine for plant functional studies

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Abstract

Functional studies in biology require the identification of genes, regulatory elements, and networks, followed by a deep understanding of how they orchestrate to specify cell types, mediate signaling, and respond to internal and external cues over evolutionary timescales. Advances in single-cell analysis have enabled biologists to tackle these questions at the resolution of the individual cell. Here, we highlight recent studies in plants that have embraced single-cell analyses to facilitate functional studies. This review will provide guidance and perspectives for incorporating these advanced approaches in plant research for the coming decades.

Introduction

Single-cell analysis has boosted plant biology research in recent years by building single-cell gene expression atlases to answer diverse biological questions (Table 1). Many excellent review papers have summarized the step-by-step experimental and analytic workflows, and discussed the technical challenges [1–4]. After generating a single-cell atlas, critical steps such as mRNA *in situ* hybridization and reporter line imaging are used to validate its robustness (Figure 1). However, the ultimate validation of single-cell results is in functional studies, such as mutant characterization. This can inspire additional biological questions that can be further addressed by additional single-cell analysis (Figure 1). In this review, we will primarily discuss recent plant single-cell publications that incorporate functional analysis (Table 1), and highlight how plant scientists tackle biological questions in the single-cell era. The topics we will cover include: 1) identification of new cell types, cell type-specific markers, and developmental trajectories; 2) co-expression and gene regulation; 3) differential expression in mutant cell types and cell type-specific responses to internal and external cues; and 4) future perspectives on single-cell analysis in cell-to-cell communication, cross-species cell type evolution, and cell-type-specific *cis*-regulatory elements (Figure 1; Table 1). These cases are just the early signs of opening a goldmine for the coming decades of plant functional studies.

Cell types and Cell type-specific markers.

Development is driven largely by genes expressed in specific cell types or developmental domains. In the past decades, plant developmental biologists mainly used mutant analysis to identify such genes. However, this approach is severely limited by gene redundancy. For example, in arabidopsis, less than 10% of single-gene T-DNA insertion mutant lines produce an obvious phenotype [5]. Plant biologists have adopted technologies such as Fluorescence-Activated Cell Sorting [6,7], Laser-Capture Microdissection [8], Isolation of Nuclei in Tagged Cell Types [9], and Translating Ribosome Affinity Purification followed by RNA sequencing [10] to identify candidate developmental genes. However, these approaches can be limited by the need to construct transgenic reporter lines, low throughput, and need for prior knowledge of specific cell types or developmental domains. Single-cell technology opened the gates to profile all cell types and

developmental domains in a single experiment, and can identify novel cell types. For example, in recent single-cell profiling of arabidopsis lateral roots, Serrano-Ron et al. [11] identified five novel cell types and built a high-resolution cellular model for lateral root primordium patterning. The authors next characterized mutants of *C-REPEAT BINDING FACTOR 3* (*CBF3*), a marker gene of one novel endodermis/quiescent center-transitioning stem cell type, and found abnormal lateral root formation. These data suggest that this newly identified cell type is important in lateral root development. A parallel arabidopsis lateral root single-cell study during gravistimulation also identified a new group of lateral root primordia (LRP) cells derived from xylem pole pericycle cells [12]. Cell-type-specific repression of marker genes for this LRP cell type, including two *AUXIN RESPONSE FACTORS*, three *HISTONE DEACETYLASES*, and two *RECEPTOR FOR ACTIVATED C KINASES*, found that all three groups of genes controlled lateral root initiation. As such, identification and functional characterization of cell types and cell type-specific markers has become a trend in the recent plant single-cell literature (Table 1). These studies span different species (arabidopsis [11–17], maize [18,19], and rice [20]) and tissue types (root [11,18], leaf [13,14,17], vegetative shoot apex [16,19], and inflorescence [20]). Of note, Ortiz-Ramirez et al. [18] found that maize *SHORT-ROOT* (*SHR*) orthologs are expressed specifically in endodermis cell clusters, rather than in the stele, where *SHR* is expressed in arabidopsis. *shr* mutants in both maize and setaria have fewer cortex cell layers, suggesting that the novel expression of SHR orthologs in monocots, along with an enhanced range of cell-to-cell movement of SHR protein, is responsible for expansion of cortex tissues [18]. Overall, the many cell type-specific markers across different plant single-cell studies could be prioritized for functional studies. However, challenges remain in capturing some rare cell types, such as the central zone and organizing center cells in shoot meristem single-cell studies [16,21], likely due to their relatively low abundance.

Developmental trajectory analysis identifies key regulators along cell lineages.

Single-cell analysis offers an opportunity to order cells into a lineage or pseudotime trajectory during development. Roots [22–25] and stomata [26] have been characterized by trajectory analysis, because of their relatively simple cell differentiation lineage. For instance, trajectory analysis of tomato shoot-borne root initiation found a novel transition progenitor cell cluster that produces root cap and stem cells [22]. A root-specific transcription factor SHOOT BORNE ROOTLESS (SBRL) was identified as a key regulator of this transition state, and *sbrl* mutants lack shoot-borne roots. Subsequent phylogenetic and reverse genetic analysis of *SBRL* orthologs in different species indicate that regulation of shoot-borne root initiation is deeply conserved in angiosperms [22]. Single-cell trajectory analysis could also gain new insights into the already well-studied arabidopsis root. For example, Roszak et al. [23] captured distinct developmental phases along the root protophloem sieve element trajectory. The authors found that the protophloem sieve element bifurcates into procambial and metaphloem cells early in development. A group of protophloem sieve element markers including Rho family guanosine triphosphatase *RHO OF PLANTS 9* (*ROP9*) and *ROP GUANINE NUCLEOTIDE EXCHANGE FACTORS* (*ROPGEFS*) are highly expressed preceding and during this bifurcation. Further functional analysis of inducible *ROP9* lines and ectopic expressing *ROPGEFS* confirmed their role in cell division orientation and protophloem sieve element bifurcation [23]. In another study, Lopez-Anido et al. [26] performed trajectory analysis of the well-studied stomatal lineage cells, and found that many novel cell-cycle regulators, such as CYCLIN D7;1 (CYCD7;1) and WEE family kinase 1 (WEE1), peaked at the stomatal guard mother cell stage. Mutant analysis of *cycd7;1* and *wee1* found an unexpectedly opposing role in regulating stomatal guard mother cell sizes. These results suggest that *CYCD7;1* and *WEE1* play antagonistic roles in stomatal guard mother cell fate progression. Surprisingly, Lopez-Anido et al. [26] also found that expression of the well-known stomatal regulator *SPEECHLESS* (*SPCH*) is extended to the late stomatal guard mother cell stage. This was confirmed by time-lapse imaging of a *SPCH* reporter using a longer promoter than had been used previously. Silencing of late-stage *SPCH* expression indeed diverted cell identities from guard cell fate, suggesting that SPCH

has previously unappreciated roles in the completion of the guard cell fate trajectory [26]. In summary, single-cell developmental trajectories not only identify novel regulators along cell lineages, but also reveal new functions for known regulators.

Single-cell gene co-expression networks.

Gene co-expression network analysis evaluates similarity in gene expression patterns, and can infer gene function. A recent co-expression analysis in developing maize ear inflorescences found that functionally redundant *ZmTREHALOSE PHOSPHATE PHOSPHATASE* genes were highly co-expressed at the single-cell level [21]. Thus, single-cell co-expression analysis could be used to predict functional redundancy, which is common in most plants [27]. In another study involving regeneration of arabidopsis from callus [15], single-cell co-expression analysis of the callus middle cell layer marker gene *WUSCHEL-RELATED HOMEOBOX 5* and its interactor *PLETHORA* identified a common target, *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1* (*TAA1*). *TAA1* is an auxin biosynthesis gene, and mutants of *TAA1* and its paralog are defective in formation of shoots and roots from callus. Similarly, in the arabidopsis root [28], Yang et al. built a single-cell network to identify genes that are co-expressed with the cytokinin biosynthesis gene *LONELY GUY4*. They identified and characterized a candidate *BETA-GLUCOSIDASE 44* (*BGLU44*) and found that it had a role in protoxylem differentiation [28]. Single-cell co-expression analysis can also facilitate functional studies of non-model plants, such as in the identification of UDP-glucuronosyltransferase genes that control key metabolites for the unique taste of tea leaves [29], and candidate genes for benzylacetone biosynthesis in production of tobacco scents [30].

Gene regulatory networks in the single-cell era

Gene regulatory networks help in understanding the intiricacies of transcription factor control of gene expression, and could inform functional studies and crop improvement. Single-cell analysis can be used to build cell-type-specific gene regulatory networks. In a recent study of cotton fiber cells [31], Qin et al. built a gene regulatory network along several stages of development, and identified the MYB25-like transcription factor (*GhMYB25-like*) as a key player in the network. They confirmed this by making *GhMYB25-like* mutants, which failed to initiate fiber cells. In another study of arabidopsis leaf development [14], Liu et al constructed a cell type-specific gene regulatory networks for phloem parenchyma, companion cells, and xylem parenchyma cells. They found that *REPRESSOR OF ga1-3* (*RGA*) genes were core transcription factors in these cell types, and *rga* quintuple mutants were defective in vein development [14]. A recent analysis [21] also demonstrated the power of integrating transcription factor binding site datasets and single-cell RNA-seq to predict the direct targets of transcription factors. The same data was used to validate a computational tool that infers such cell-type-specific gene regulatory networks in plants [32].

Single-cell mutant analysis

Single-cell analysis of mutants was initially used for validating cell type annotation [33,34]. However, in more recent studies, comparative mutant studies have examined changes in tissue composition and cell identity, and identified differentially expressed genes underlying distinct cell types [19,35,36]. For instance, Shahan et al. [35] conducted single-cell analysis of arabidopsis *shr* and *scarecrow* (*scr*) mutant roots, and confirmed these mutants only have a single cell layer in place of cortex and endodermis. In *shr*, these mutant layer cells showed cortex-like attributes, but in *scr* the mutant layer cells were cortex-like early in development, and acquired endodermal attributes later on [35]. In another study, Graeff et al. [36] performed 3D single-cell shape analysis of brassinosteroid receptor triple mutants, *brassinosteroid insensitive 1 (bri1)*;*bri1-like 1 (brl1)*;*brl3*. They found that brassinosteroids affect the timing of anisotropic cell expansion. They next performed single-cell RNA-seq of the triple mutant roots, and found that genes encoding arabinogalactan proteins and peptides (AGPs) are expressed at a lower level across almost all

mutant cell types. They experimentally confirmed the functions of AGPs by measuring the cellular anisotropy of distinct cell types upon AGP inhibitor treatment [36]. There are many plant mutants that could be examined by single-cell analysis to understand plant development and growth at unprecedented resolution.

Response to internal and external cues at the single-cell level

As a sessile organism, plants have a unique aptitude to respond to diverse internal cues and to external harsh environments. Recent plant single-cell studies indicate that these responses are largely cell-type-specific. For example, single-cell profiling of the arabidopsis root after cytokinin treatment found that *CYTOKININ OXIDASE3* (*CKX3*) is preferentially upregulated in vascular procambium cells [28]. Indeed, double mutant analysis of *CKX3* and its paralog *CKX5* suggested that these two genes modulate cytokinin levels for normal vascular development [28]. In another study, Nolan et al. [37] performed time series single-cell RNA-seq with brassinosteroid treatment of arabidopsis roots. They found that elongating cortex cells are responsive to brassinosteroid. They confirmed this result by single-cell profiling of *bri1;brl1;brl3* triple mutants. After additional developmental trajectory and gene regulatory network analysis, the authors identified and functionally characterized key regulators of the brassinosteroid response in elongating cortex cells. These regulators included HOMEOBOX-LEUCINE ZIPPER protein HAT7 (HAT7) and TRIHELIX Transcription factor GT2-like 1 (GTL1) that control cell wall organization or biogenesis genes [37]. Cell-type-specific responses in response to external cues have also been reported. For example, Wendrich et al. [38] found that arabidopsis root epidermal cells respond to limiting phosphate conditions. Functional analysis showed that this response is dependent on the TARGET OF MONOPTEROS 5/LONESOME HIGHWAY (TMO5/LHW) transcription factor complex. TMO5/LHW complex is expressed in inner layer vascular cells. Under limiting phosphate conditions, TMO5/LHW expression and downstream local cytokinin biosynthesis are induced. Cytokinin then moves from inner vascular cells to outer epidermal cells to direct both the length and identity of epidermal cells, and improve the efficiency of root to forage phosphate from soil.

Conclusions and future perspectives

High-throughput single-cell sequencing [1–4] has propelled the plant biology field into a new era (Figure 1). The different aspects of single-cell analysis illustrated in this review will guide plant biologists to adopt these cutting-edge toolkits into functional studies. A combination of one or more single-cell approaches followed by functional validation will be a trend in future plant single-cell research.

Functional studies inspired by single-cell analyses will continue to bloom in three aspects, including cell-to-cell communication, cross-species cell-type evolution, and characterization of cell-type specific *cis*-regulatory elements (Table 1). Cell-to-cell communication has been explored in single-cell analysis of roots, such as the cytokinin-mediated cross-talk between vascular and epidermal cells in arabidopsis [38] and SHR protein-mediated signaling between endodermis and cortex cells in maize [18]. Single-cell profiling of the developing maize ear inflorescence supported the idea that *KNOTTED1* mRNA can move from the inner layers to the epidermal layer in shoot meristems [39]. Future functional studies of plant cell-to-cell communication could also be validated by spatial transcriptomics through either untargeted [40] or targeted approaches [41,42]. At a cross-species level, studies comparing either rice and Arabidopsis [24,43] or maize and rice [44] identified conserved and divergent cell types. Functional validation of orthologous genes underlying these differences will be critical to understanding cell type evolution at higher resolution. Finally, cross-modality single-cell analysis can discover cell type-specific *cis*-regulatory elements in addition to gene expression [45–47]. These *cis*-regulatory elements could be used to fine-tune gene expression using promoter editing technology [48] at cell type-specific levels to improve crop performance.

Overall, single-cell analysis is opening a new horizon of plant functional studies to address a broad spectrum of biological questions in both fundamental and applied research.

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have been highlighted as:

• of special interest

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Figure-1 Single-cell analysis facilitates functional studies to answer biological questions.

A schematic diagram showing representative biological questions (top panel) can be answered by different aspects of single-cell analysis (left panel) followed by expression validation (bottom panel) and functional studies (right panel). The new biological questions can be inspired by single-cell analysis-based functional studies to be tackled by further single-cell research (red arrow).

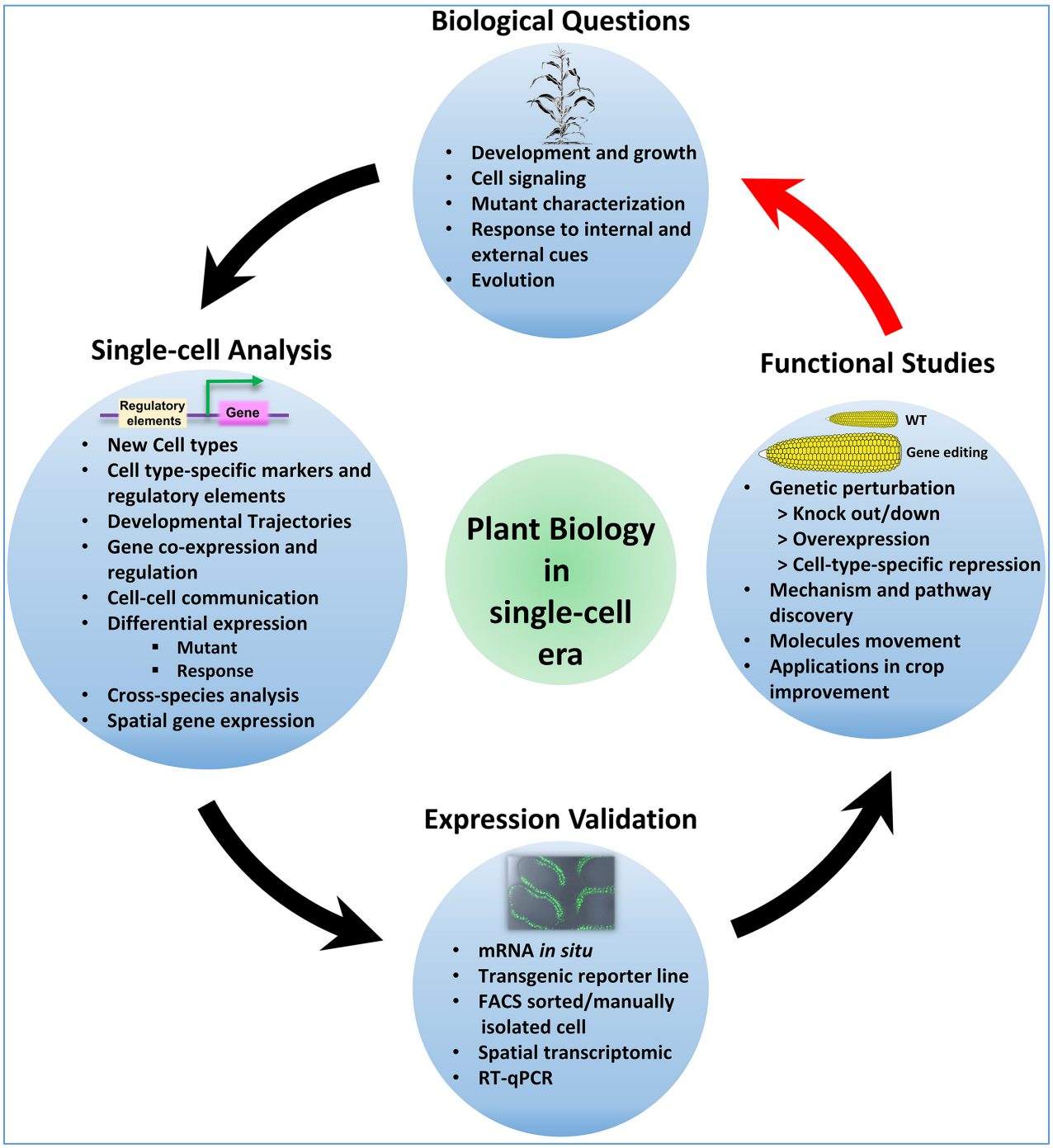

Table 1. Summary of recent plant single-cell studies

See attached.

Table 1. Summary of recent plant single-cell studies

Single-cell analysis	Organism	Tissue	Cell type/Developmental domain	Functionally characterized genes	Reference
New cell type	Arabidopsis	Lateral root	Lateral root initiating primordial cells, endodermis/quiescent center -transitioning stem cells, quiescent center-transitioning cells, vascular-like cells.	*CBF3*	[11]
	Arabidopsis	Lateral root	Lateral root primordia cells.	*ARF7, 9; HDA3, HD2B,* and *HDT4; RACK1B* and *RACK1C*	[12]
Cell type-specific markers	Arabidopsis	Lateral root	Lateral root initiating primordial cells, endodermis/quiescent center -transitioning stem cells, quiescent center-transitioning cells, vascular-like cells.	*CBF3*	[11]
	Arabidopsis	Lateral root	Lateral root primordia cells.	*ARF7, 9; HDA3, HD2B,* and *HDT4; RACK1B* and *RACK1C*	[12]
	Arabidopsis	Leaf	Pavement cells and guard cells	*PIF1, 3, 4, 5*	[13]
	Arabidopsis	Leaf	Phloem parenchyma	*bZIP9*	[14]
	Arabidopsis	Callus	Middle cell layers	*WOX5, 7*	[15]
	Arabidopsis	Vegetative shoot apex	Endodermis cells	*SGR6*	[16]
	Arabidopsis	Leaf	Stomatal meristemoid mother cells	*WRKY33*	[17]
	Maize	Root	Endodermis cells	*ZmSHR1, 2, 2-h*	[18]
	Maize	Vegetative shoot apex	Core region cells	*ZmGRAS33, 32*	[19]
	Rice	Inflorescence	Meristem cells	*DWT1*	[20]
Developmental trajectories	Arabidopsis	Root	Cortex cells	*HAT7* and *GTL1*	[37]
	Tomato	Shoot-borne root	Transition state cells of root meristem initiation	*SBRL*	[22]
	Arabidopsis	Root	Protophloem cells	*GTPase*, *ROPGEF*, *PLETHORA*, *PEAR*, and *APL*	[23]
	Rice	Root	Ground tissue and vascular cells	*OsGATA6*	[24]
	Arabidopsis	Root	Lateral root cap cells	*ARR1, 10, 12*	[25]
	Arabidopsis	Leaf	Stomatal guard mother cells	*CYCD7;1* and *WEE1*	[26]
	Arabidopsis	Vegetative shoot apex	Xylem cells	*ARF* gene *At2G04850*	[16]
Gene co-expression network	Arabidopsis	Root	Protoxylem cells	*BGLU44*	[28]
	Tee tree	Leaf	Mesophyll cells	*UGT7B23*	[29]
	Wild tobacco	Corolla limbs and throat cups	Corolla cells	*Na4CL1, NaPKS2, NaAER1,* and *NaIFR3*	[30]
	Arabidopsis	Callus	Middle cell layers	*TAA1*	[15]
	Maize	Ear inflorescence	Meristem cells	*RA3, ZmTPP4, ZmTPP12, ZmVOZs*	[21]
Gene regulatory network	Arabidopsis	Root	Cortex cells	*HAT7* and *GTL1*	[37]
	Cotton	Cotton ovule outer integument	Fiber cells	*GhMYB25-like* and *GhHOX3*	[31]
	Arabidopsis	Leaf	Phloem parenchyma, companion cell, and xylem parenchyma cells	*RGA*	[14]
	Arabidopsis	Leaf	Dividing stomatal guard mother cells	*SPCH*	[26]
Differential expression (mutant)	Arabidopsis	*bri1;brl1;brl3* and *gtl1;df1* mutants root	Cortex cells	*HAT7* and *GTL1*	[37]
	Arabidopsis	*shr* mutants root	Endodermis cells	NA	[33]
	Arabidopsis	*rhd6* and *gl2* mutants root	Root hair and non-hair cells	NA	[34]
	Arabidopsis	*scr* and *shr* mutants root	Cortex and endodermis cells	*MYB36*	[35]
	Arabidopsis	*bri1;brl1;brl3* mutants root	All cell types	*AGPs*	[36]
	Maize	*Kn1-O*/+ mutants vegetative shoot apex	Determinate cells	*ZmGA2OX1*	[19]
Differential expression (response to internal and external cues)	Arabidopsis	Root response to brassinosteroid	Cortex cells	*HAT7* and *GTL1*	[37]
	Arabidopsis	Root response to cytokinin	Procambium cells	*CKX3*	[28]
	Arabidopsis	Root	Xylem cells and hair cells	*TMO5* and *TMO5L1; LOG3, LOG4,* and *LOG7*	[38]
Cell-to-cell communication	Arabidopsis	Root	Xylem cells to hair cells	*TMO5* and *TMO5L1; LOG3, LOG4,* and *LOG7*	[38]
	Maize	Root	Endoermis to cortex cells	*ZmSHR1, 2, 2-h*	[18]
	Arabidopsis	Root	Xylem cells to procambium cells	*TMO5/LHW; LOG4; BGLU44; SHR; CKX3*	[27]
	Maize	Ear inflorescence	Meristem L2 to L1 epidermis cells	*KN1*	[39]
Spatial gene expression	Arabidopsis	Leaf	Untargeted for all cell types	NA	[40]
	Arabidopsis	Root	Targeted for selected cell types	NA	[41]
	Maize	Vegetative shoot apex	Targeted for selected cell types	NA	[42]
Cross-species analysis	Rice and Arabidopsis	Root	Root hair, xylem, and phloem	NA	[24]
	Rice and Arabidopsis	Root	Root hair, cortex	NA	[43]
	Maize and rice	Root	Root hair, endodermis and phloem	NA	[44]

Single-cell analysis	Organism	Tissue	Cell type/Developmental domain	Functionally characterized genes	Reference
Cell type-specific *cis*-regulatory elements	Maize	Axillary buds, tassel inflorescence, ear inflorescence, whole seedling, embryonic root tips, and post-embryonic crown roots	All cell types	NA	[45]
	Arabidopsis	Root	All cell types	NA	[46]
	Arabidopsis	Root	All cell types	NA	[47]

Gene Abbreviations:

C-REPEAT BINDING FACTOR 3 (CBF3)
AUXIN RESPONSE FACTOR7, 9 (ARF7, 9)
HISTONE DEACETYLASE 3 (HDA3)
HISTONE DEACETYLASE 2B (HD2B)
HISTONE DEACETYLASE 13 (HDT4)
RECEPTOR FOR ACTIVATED C KINASE 1B (RACK1B)
RECEPTOR FOR ACTIVATED C KINASE 1C (RACK1C)
PHYTOCHROME INTERACTING FACTOR1, 3, 4, 5 (PIF1, 3, 4, 5)
BASIC LEUCINE ZIPPER 9 (bZIP9)
DWARF TILLER1 (DWT1)
ZmSHORT-ROOT1, 2, 2-h (ZmSHR1, 2, 2-h)
WUSCHEL-RELATED HOMEOBOX 5, 7 (WOX5, 7)
SHOOT GRAVITROPISM6 (SGR6)
ZmGRAS family transcription factor 33, 32 (ZmGRAS33, 32)
WRKY family transcription factor 33 (WRKY33)
HOMEOBOX-LEUCINE ZIPPER protein HAT7 (HAT7)
TRIHELIX Transcription factor GT2-like 1 (GTL1)
SHOOTBORNE-ROOTLESS (SBRL)
RHO-RELATED GUANOSINE TRIPHOSPHATASE (GTPASE)
PLETHORA (PLT)
PHLOEM EARLY DOF 1 (PEAR1)
ALTERED PHLOEM DEVELOPMENT (APL)
PRONE-TYPE ROP GUANINE NUCLEOTIDE EXCHANGE FACTORS (ROPGEF)
OsGATA family transcription factor 6 (OsGATA6)
CYCLIN D7;1 (CYCD7;1)
WEE family kinase 1 (WEE1)
ARABIDOPSIS THALIANA RESPONSE REGULATOR1, 10, 12 (ARR1, 10, 12)
URIDINE DIPHOSPHATE-DEPENDENT GLYCOSYLTRANSFERASE7B23 (UGT7B23)
N. attenuate 4CL/CNL-like gene (Na4CL1)
N. attenuate POLYKETIDE SYNTHASE2 (NaPKS2)
N. attenuata 2-ALKENAL REDUCTASE1 (NaAER1)
N. attenuata INTERFERON REGULATORY FACTOR3 (NaIFR3)
Beta-GLUCOSIDASE 44 (BGLU44)
TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1)
RAMOSA3 (RA3)
ZmTREHALOSE PHOSPHATE PHOSPHATASE 4 (ZmTPP4)
ZmVASCULAR PLANT ONE-ZINC-FINGER1, 2, 4, 5 (ZmVOZ1, 2, 4, 5)
GhMYB family transcription factor 25-like (GhMYB25-like)
Gh HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP) transcription factor HOX3 (GhHOX3)

REPRESSOR OF GA (RGA)
SPEECHLESS (SPCH)
ARABIDOPSIS THALIANA MERISTEM LAYER1 (ATML1)
TARGET OF MONOPTEROS 5, 5L1 (TMO5, 5L1)
LONELY GUY3, 4, 7 (LOG3, 4, 7)
CYTOKININ OXIDASE3 (CKX3)
KNOTTED1 (KN1)
MYB family transcription factor 36 (MYB36)
ARABINOGALACTAN PROTEINS AND PEPTIDE (AGPs)
ZmGIBBERELLIN (GA) 2-OXIDASE (ZmGA2OX1)