Shoot regeneration involves reprogramming of somatic cells and de novo organization of shoot apical meristems (SAMs). In the best-studied model system of shoot regeneration using Arabidopsis, regeneration is mediated by the auxin-responsive pluripotent callus formation from pericycle or pericycle-like tissues according to the lateral root development pathway. In contrast, shoot regeneration can be induced directly from fully differentiated epidermal cells of stem explants of Torenia fournieri (Torenia), without intervening the callus mass formation in culture with cytokinin; yet, its molecular mechanisms remain unaddressed. Here, we characterized this direct shoot regeneration by cytological observation and transcriptome analyses. The results showed that the gene expression profile rapidly changes upon culture to acquire a mixed signature of multiple organs/tissues, possibly associated with epidermal reprogramming. Comparison of transcriptomes between three different callus-inducing cultures (callus induction by auxin, callus induction by wounding and protoplast culture) of Arabidopsis and the Torenia stem culture identified genes upregulated in all the cultures as candidates of common factors of cell reprogramming. These initial changes proceeded independently of cytokinin, followed by cytokinin-dependent, transcriptional activations of nucleolar development and cell cycle. Later, SAM regulatory genes became highly expressed, leading to SAM organization in the foci of proliferating cells in the epidermal layer. Our findings revealed three distinct phases with different transcriptomic and regulatory features during direct shoot regeneration from the epidermis in Torenia, which provides a basis for further investigation of shoot regeneration in this unique culture system.

Keywords: Cytokinin • Epidermal reprogramming • Shoot apical meristem • Shoot regeneration • Torenia fournieri • Transcriptome

Introduction

Plant development is often featured by its high plasticity in contrast to the limited plasticity of animal development. The plastic nature of plant development can be seen in regeneration phenomena such as organ regeneration and somatic embryogenesis, through which many plants are able to recreate most parts of or even the entire plant body. The regeneration processes generally involve some kind of cell reprogramming and de novo organization of meristems that contain stem cells. For a representative example, shoot regeneration from mature tissues relies on reprogramming from the original, differentiated state and the subsequent generation of the shoot apical meristem (SAM) of an adventitious bud.

Since Skoog and Miller (1957) discovered that callus, shoots and roots can be artificially induced and manipulated by the application of the phytohormones auxin and cytokinin in tissue...
culture, tissue culture has become one of the main tools to study plant organogenesis including shoot regeneration. For the efficient induction of shoot regeneration, two-step culture systems, consisting of callus induction by auxin-rich culture in the first step and adventitious shoot induction by cytokinin-rich culture in the second step, have been developed for various plant species and widely used (e.g. Nishi et al. 1968, Christianson and Warnick 1983, Koornneef et al. 1987, Coleman and Ernst 1990).

A similar two-step culture system was also established in the model plant Arabidopsis thaliana (Arabidopsis) (Valvekens et al. 1988, Akama et al. 1992), which has facilitated the molecular biological analysis of shoot regeneration. In this culture system, if skipping the first step for callus induction, explants can form no or few adventitious buds, which implies that explant cells undergo reprogramming to acquire competence for shoot regeneration in the first step culture.

With the two-step culture system of Arabidopsis, transcriptome analysis was performed for the gene expression profiling of callus formation and adventitious SAM formation (Che et al. 2002, 2006, Xu et al. 2012), and spatial and temporal expression patterns of genes encoding major SAM regulatory transcription factors and phytohormone signaling factors were investigated in the process leading to SAM formation (Gordon et al. 2007, Cheng et al. 2013). The two-step shoot induction culture of Arabidopsis was used for the forward genetics of shoot regeneration as well (Yasutani et al. 1994, Tamaki et al. 2009). Recently, studies of the two-step shoot regeneration have been expanded, incorporating various new lines of research such as functional analysis of epigenetic regulation (He et al. 2012, Lee and Seo 2018, Ishihara et al. 2019) and genome-wide association analysis of natural variations (Lardon et al. 2020), which has accumulated increasing pieces of information.

One of the most important outcomes of research concerning the two-step culture of Arabidopsis over the last decade is the understanding that the callus formed in the first step is not a fully undifferentiated cell mass but a disorganized root meristem–like tissue originating from pericycle or pericycle-like tissues via the pathway of lateral root formation (Atta et al. 2009, Sugimoto et al. 2010). Furthermore, it was also shown that transcription factors regulating lateral root formation, such as LATERAL ORGAN BOUNDARIES DOMAINS (LBDs) and PLETHORAS (PLTs), participate not only in the callus formation but also in the acquisition of shoot regeneration competence (Fan et al. 2012, Kareem et al. 2015). Moreover, it was reported that lateral root primordia at certain developmental stages can be converted directly into adventitious shoot buds by the exposure to high concentrations of cytokinin (Atta et al. 2009, Rosspopoff et al. 2017). These findings suggest that auxin-induced callus shares many features with a lateral root primordium and that the root development pathway offers a mechanism of cell reprogramming during this type of callus formation. It is noted here that this reprogramming starts not from fully differentiated cells but from pericycle (or pericycle-like tissue) cells that are generally considered to remain partially meristematic (De Smet et al. 2006, Atta et al. 2009).

Arabidopsis plants can occasionally form a regenerative callus at wounded sites without hormone application. The molecular basis of this wound-induced callus formation has been also studied well for cell reprogramming, resulting in the identification of several key regulatory factors including the AP2/ERF transcription factor WOUND-INDUCED DEDIFFERENTIATION 1 (WIND1) (Hwase et al. 2011, 2017). Another good material for studying cell reprogramming is mesophyll protoplasts, which are reactivated from the quiescent state to enter the cell cycle and form regenerative callus in culture (Zelcer and Galun 1976). Transcriptome analysis of these processes revealed dynamic transcriptional changes possibly associated with cell reprogramming during wound-induced callus formation and protoplast culture (Chupeau et al. 2013, Ikekuchi et al. 2017).

In other plants, there are more diverse paths of shoot regeneration. In some cases, a preparatory callus formation step is not necessary for shoot regeneration (Hicks 1980), and the origin of regenerated shoots is not restricted to pericycle or pericycle-like cells (Chlyah and Van 1975, Creemers-Molenaar et al. 1994). In 1973, Chlyah reported a notable example of such shoot regeneration with the tissue culture of Torenia fournieri (Torenia). In this culture, adventitious bud SAMs formed directly on the surface of stem segments without intervening callus mass growth phase (Chlyah 1973, 1974a, 1974b). Importantly, histological analyses demonstrated that these adventitious bud SAMs originated exclusively from epidermal cells (Chlyah 1974a). From the 1970s to 1990s, various physiological studies were carried out with the Torenia culture system, which revealed a promotive role of cytokinin in shoot regeneration (Kamada and Harada 1979, Tanimoto and Harada 1982, 1984) and suggested wound stress as another promoting factor (Takeuchi et al. 1985). However, this culture system has never been used for molecular biological studies of shoot regeneration.

The Torenia stem culture system has three distinct features of shoot regeneration in contrast to the Arabidopsis two-step culture system: first, the entire process of shoot regeneration is triggered simply by one-step culture; second, drastic reprogramming should occur during the transformation of fully differentiated epidermal cells into meristem cells; and third, the initial process of de novo organization of SAMs takes place in a two-dimensional field of the epidermis. Because of these features, the Torenia stem culture can serve as a unique alternative experimental system for studying regeneration, particularly advantageous for cell reprogramming and SAM organization. For this reason, in the present study, we chose the Torenia stem culture to reveal the hidden aspects of shoot regeneration, which are difficult to uncover only with one model system. We performed the cytological and transcriptomic characterization of shoot regeneration with the Torenia culture system. The results obtained depict global and temporal changes in the gene expression profile that are likely to associate with each elementary process of the shoot regeneration in the Torenia stem culture, which provides a basis for further investigation of the relevant molecular mechanisms. We also compared the transcriptome data of Torenia with those reported for Arabidopsis
callus-inducing cultures to gain information of core reprogramming mechanisms common to various types of regeneration-related events and identified possible candidates for factors universally involved in the molecular network of cell reprogramming.

**Results**

**Effects of culture conditions on adventitious bud formation**

Based on previous reports (Chlyah 1974b, Tanimoto and Harada 1982) and our preliminary experiments, we set up the culture system of Torenia stem segments for effective induction of shoot regeneration, in which, the first internodes were excised from young plants at the age of 4 weeks (Fig. 1A), half-sectioned longitudinally, cut into 1.5-mm-long segments (Fig. 1B) and cultured on half-strength Murashige and Skoog (MS) medium containing 1 mg/l \(N\)-benzyladenine (BA) as cytokinin at 22°C under continuous light.

We first tested the effect of cytokinin and light on the frequency of adventitious bud formation. While the culture of stem explants in the presence of BA under continuous light induced as many as 10 adventitious buds on the epidermis of each explant on average, the culture without BA in the light and the culture with BA in the dark induced much less intensive bud formation (Fig. 1C, E, F). Particularly in the culture without BA, very few buds were formed (Fig. 1D, F). These results showed that both exogenously supplied cytokinin and light are important, with the former being more critical, for adventitious bud induction in this culture. We employed culture with BA in the light as the standard bud-inductive culture and culture without BA in the light and culture with BA in the dark as controls in the subsequent experiments.

**Early cytological events and cell division activation**

In an early report on the Torenia stem culture, it is mentioned that nuclei and nucleoli of the epidermal cells increased in the acetocarmine stainability and volume before the commencement of cell division, although without quantitative data (Chlyah 1974b). Similar changes in the nuclear and nucleolar appearance were reported in several types of dedifferentiating plant cells, implicating nuclear and nucleolar development in the preparation of cell division activation (Feldman and Torrey 1977, Williams and Jordan 1980, Paul et al. 1989, Williams et al. 2003). To verify Chlyah’s observation quantitatively and evaluate nuclear and nucleolar development in the Torenia stem culture, we stained epidermal cells of stem explants before and after culture for 2 d in three conditions with 4′,6-diamidino-2-phenylindole (DAPI) and RNAselect to visualize nuclei and nucleoli, respectively, and measured their size. In the standard

---

Fig. 1 Tissue culture system of Torenia for direct shoot regeneration from stem explants. (A) A 4-week-old plant of Torenia. Bar = 5 mm. (B) Preparation of stem segments. Bars = 1 mm. (C–E) Stem explants cultured for 14 d with 1 mg/l BA under continuous light (BA+, light), without BA under continuous light (BA-free, light) or with BA in the dark (BA+, dark). Arrowheads in the magnified image of (C) indicate adventitious buds. Scale bars = 1 mm. (F) Number of adventitious buds formed on the epidermal surface per explant. Error bars = SE (n = 50).
The stem explants cultured in the standard bud-inductive condition were inspected for cell division patterns in the epidermis. Confocal laser scanning microscopy (CLSM) after staining with propidium iodide (PI) clearly detected the appearance and development of foci of small cells, indicating local activation of cell division in the epidermal layer (Figs. 3D–F). Serial observation of the surface of cultured stem explants with a metallographic microscope showed that locally activated cell division generated the foci of proliferating cells, from which adventitious bud SAMs eventually developed (Fig. 4A–G). In all cases observed, each SAM arose from a subpopulation of dividing cells originated from more than one epidermal cells, and borderlines of these ‘presumptive SAM areas’ were inconsistent with those of original epidermal cells (Figs. 4H–M).

Global changes in gene expression during the entire course of shoot regeneration

Transcriptome data were obtained by RNA sequencing (RNA-seq) for stem explants cultured for 0, 1, 2, 4 or 8 d in the standard bud-inductive condition and stem explants cultured for 4 or 8 d in the BA-free and dark control conditions. Principal component analysis (PCA) was performed on the normalized transcription data (Supplementary Fig. S1A). As a result, the first three components explained 79.2% of the total variance among all samples. Biological replicates of each sample clustered together, confirming the reproducibility of the analysis. Among 27,137 genes of which transcripts were detected, 23,697 genes showed significant temporal changes (false discovery rate (FDR) < 0.05) in their expression level during culture under the standard bud-inductive condition. These genes were subjected to k-means clustering analysis and classified into eight clusters according to their changing patterns. Then, the Gene Ontology (GO) term enrichment analysis was performed on each of the clusters using the Blast2GO software.

The gene clusters were characterized by enrichment of different GO terms (Fig. 5). With respect to possible relationships with the cellular and morphogenetic events observed during shoot regeneration, notable GO enrichment was found in clusters 2, 4 and 5. In cluster 2, where expression levels increased in the first day and then decreased slightly, GO terms related
Fig. 3 Cell division activation during culture as influenced by BA and light. (A) Changes in the distribution of the flow cytometric measured nuclear DNA content in stem explants during culture under the standard bud-inductive condition. (B) Changes in the number of anillin blue–stained nascent cell walls in the epidermis of stem explants during culture under the standard bud-inductive condition. Error bars indicate standard errors (n = 7). (C) Comparison of the numbers of anillin blue–stained nascent cell walls in the epidermis of stem explants between standard bud-inductive, BA-free control and dark control conditions. Error bars indicate standard errors (n = 6–7). (D–F) CLSM observation of the PI-stained epidermis of the stem explants at the beginning of culture (D) and after 4 d (E) and 6 d (F) of culture in the standard bud-inductive condition. Scale bars = 100 µm.
to protein synthesis such as ‘translation’ and ‘ribosome biogenesis’ were very highly enriched. The high enrichment of these terms well corresponded to the nucleolar enlargement in the first 2 d. Genes in cluster 4 showed a gradual increase in the expression levels during the first 4 d and maintained a high expression throughout the later stage. This cluster was represented by GO terms related to the cell cycle. This seemed to reflect the cell division activation from 2 to 4 d of culture. In cluster 5, where expression levels started to increase after 2 d of culture and continued to increase thereafter, GO terms related to developmental processes were enriched. More specific GO terms such as ‘plant organ development’, ‘tissue development’, ‘shoot system development’ and ‘meristem development’ were also enriched in this cluster. Indeed, this cluster contained many SAM regulatory gene orthologs that are likely to participate in the formation of adventitious SAMs.

To look at the overall trends of how temporal changes of gene expressions are regulated by culture conditions, we also...
Cluster 1 2041 genes

| GO term                | Category | P-Value | Number of genes | Fold enrichment |
|------------------------|----------|---------|-----------------|-----------------|
| chloroplast            | CC       | 4.0E-148| 737             | 2.454           |
| plastid                | CC       | 3.6E-142| 760             | 2.344           |
| plastid stroma         | CC       | 9.2E-113| 339             | 3.777           |
| chloroplast stroma     | CC       | 1.3E-109| 329             | 3.787           |
| cytoplasm              | CC       | 1.9E-87 | 1311            | 1.417           |
| thylakoid              | CC       | 1.1E-83 | 260             | 3.711           |
| chloroplast thylakoid  | CC       | 4.7E-71 | 214             | 3.827           |
| plastid thylakoid      | CC       | 9.7E-71 | 214             | 3.812           |

Cluster 2 3154 genes

| GO term                                      | Category | P-Value | Number of genes | Fold enrichment |
|----------------------------------------------|----------|---------|-----------------|-----------------|
| ribosome biogenesis                          | BP       | 1.3E-206| 502             | 6.265           |
| ribonucleoprotein complex biogenesis         | BP       | 1.1E-293| 520             | 5.755           |
| ribosomal subunit                            | CC       | 4.9E-258| 322             | 8.545           |
| structural constituent of ribosome           | MF       | 7.3E-250| 369             | 7.088           |
| ribosome                                     | CC       | 2.8E-231| 494             | 4.776           |
| cytoplasm                                    | CC       | 8.2E-225| 2210            | 1.500           |
| translation                                  | BP       | 2.9E-223| 492             | 4.636           |
| peptide biosynthetic process                 | BP       | 7.5E-220| 492             | 4.569           |

Cluster 3 2946 genes

| GO term                                     | Category | P-Value | Number of genes | Fold enrichment |
|----------------------------------------------|----------|---------|-----------------|-----------------|
| RNA processing                               | BP       | 4.6E-122| 404             | 3.337           |
| intracellular anatomical structure           | CC       | 6.9E-113| 2246            | 1.237           |
| cytoplasm                                    | CC       | 2.4E-104| 1830            | 1.337           |
| intracellular organelle                      | CC       | 3.9E-96 | 2091            | 1.242           |
| organelle                                    | CC       | 4.1E-96 | 2092            | 1.241           |
| intracellular membrane-bounded organelle     | CC       | 1.1E-89 | 2008            | 1.247           |
| cellular anatomical entity                   | CC       | 2.2E-86 | 2435            | 1.140           |
| membrane-bounded organelle                   | CC       | 3.0E-86 | 2016            | 1.237           |

Cluster 4 3019 genes

| GO term                                      | Category | P-Value | Number of genes | Fold enrichment |
|----------------------------------------------|----------|---------|-----------------|-----------------|
| cell cycle                                   | BP       | 1.9E-94 | 334             | 3.181           |
| cell cycle process                           | BP       | 1.6E-88 | 279             | 3.485           |
| mitotic cell cycle                           | BP       | 2.6E-68 | 167             | 4.338           |
| nuclear division                             | BP       | 2.7E-66 | 154             | 4.547           |
| mitotic cell cycle process                   | BP       | 4.9E-62 | 138             | 4.732           |
| organelle fission                            | BP       | 3.9E-59 | 160             | 3.961           |
| cellular anatomical entity                   | CC       | 4.0E-57 | 2405            | 1.097           |
| microtubule cytoskeleton                     | CC       | 2.6E-55 | 171             | 3.544           |

Cluster 5 3247 genes

| GO term                                      | Category | P-Value | Number of genes | Fold enrichment |
|----------------------------------------------|----------|---------|-----------------|-----------------|
| cellular anatomical entity                   | CC       | 2.8E-32 | 2473            | 1.042           |
| biological regulation                        | BP       | 8.7E-31 | 1313            | 1.186           |
| binding                                      | MF       | 1.1E-27 | 2109            | 1.065           |
| cellular macromolecule metabolic process     | BP       | 1.5E-27 | 1324            | 1.165           |
| regulation of biological process             | BP       | 1.6E-26 | 1182            | 1.185           |
| developmental process                        | BP       | 2.9E-26 | 1074            | 1.207           |
| nucleus                                      | CC       | 8.8E-26 | 1105            | 1.196           |
| cellular process                             | BP       | 1.9E-25 | 2208            | 1.048           |

Fig. 5 (continued)
Fig. 5 Clustering of gene expression patterns during shoot regeneration in stem explants. Eight clusters of genes showing different expression patterns during the culture of stem explants in the bud-inductive condition. Expression levels of each individual gene are expressed as Z scores that were calculated from TPM values such that their mean and standard deviation over time were equal to 0 and 1, respectively. Magenta and gray lines indicate the average expression of all genes in each cluster and expressions of individual genes, respectively. Top eight GO terms enriched in each cluster are presented with a color scheme representing $\log_{10}(P\text{-value})$ as well as the number and fold enrichment of the relevant genes in it. BP, CC and MF indicate GO categories 'biological process', 'cellular component' and 'molecular function', respectively.

picked up genes that were significantly upregulated or downregulated after 4 and 8 d of culture in each of two control conditions as well as the bud-inductive condition (Supplementary Fig. S2), classified them into the above clusters and examined how they were overlapped between culture conditions (Supplementary Fig. S3). Throughout the clusters, genes significantly changed in the bud-inductive culture were overlapped more with those in the dark control than with those in the BA-free control. From the comparison among clusters, the ratio of overlap between the bud-inductive culture and the BA-free control was found to be lowest in cluster 1 and second lowest in cluster 5. In these clusters, gene expressions show a transient increase or decrease followed by a gradual reverse change, and the reverse change phase seemed to be suppressed in the BA-free control. These results showed that cytokinin has a dominant impact on the regulation of gene expressions at relatively late stages of culture while light has a minor effect.

Temporal expression patterns of SAM regulatory genes

We identified Torenia orthologs of the major SAM regulatory genes of Arabidopsis encoding transcription factors: three SHOOT MERSITEMLESS (STM) orthologs named TjSTM1, TjSTM2 and TjSTM3; two orthologs of CUP-SHAPED1 (CUC1) and CUC2 named TjCUC1/2a and TjCUC1/2b and two WUSCHEL (WUS) orthologs named TjWUS1 and TjWUS2 (Supplementary Figs. S4A–C). Expression levels of these genes in cultured stem explants and various parts of young plants were examined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

In young plants, all of the three TjSTM genes were expressed at a high level at the shoot apical region and expressed more or less in the stem as well (Supplementary Fig. S4D). Two TjCUC1/2 genes and two TjWUS genes also showed a high expression at the shoot apical region, but unlike TjSTMs, they
Expression of SAM regulatory genes during culture of stem explants. Expression of *TfSTM*s, *TfCUC1/2*s and *TfWUS*s in cultured stem explants was quantified by RT-qPCR. The left panels show expression levels in culture under standard and control conditions (green, standard bud-inductive condition; orange, BA-free control; purple, dark control), and the right panels show expression levels in the epidermal layer (light green) and inner tissue (gray) of explants cultured in the standard condition. Error bars indicate standard errors (*n* = 3). Relative expression levels were calculated with reference to the expression in the shoot apical region of young plants.

were not or only weakly expressed in the stem (Supplementary Fig. S4D).

In stem explants cultured under the standard bud-inductive condition, where visible adventitious bud formation occurred after 8 d (Fig. 1F), the expression level of *TfSTM1* increased after 4 d of culture, while the expression of *TfSTM2* showed a remarkable decrease within the first 2 d followed by a slight increase after 6 d (Fig. 6). *TfSTM3* expression increased within 2 d and remained at a relatively high level in the bud-inductive culture. Expressions of the *TfCUC1/2* and *TfWUS* genes increased dramatically after 4–6 d of culture in the bud-inductive condition. Of note, the expression levels of *TfWUS*s at day 8 were more than several times higher than those at the shoot apical region of young plants. The expression increases of *TfSTM1*, *TfSTM2*, *TfCUC1/2*s and *TfWUS2* were smaller in the dark control and not clearly observed in the BA-free control in a good correlation with the frequency of adventitious bud formation (Fig. 6). At day 8 of the bud-inductive culture, all of the SAM regulator genes tested were expressed in both inner tissues and epidermal layers with CUC1/2*s and WUS*s showing a higher expression in the epidermal layers than in the inner tissues (Fig. 6), which might be associated with SAM development from the epidermis.

**Effect of cytokinin on gene expression in the early stage of culture**

As our cytological observation detected clear differences in the nuclear and nucleolar enlargement between the standard bud-inductive culture and the BA-free control culture as early as day 2, we performed the second set of RNA-seq transcriptome analysis of stem explants focusing on cytokinin effects during the first 2 d of culture. Stem explants cultured for 0, 3, 6, 12, 24 or 48 hours in the standard bud-inductive condition and in the BA-free control condition were subjected to analysis together with the root, leaf and shoot apex of young
plants. As a result of PCA of the transcriptome data, the first three components explained 81.3% of the total variance among all samples (Supplementary Fig. S1B). According to these components, uncultured stem explants are quite distant from stem explant cultured for 3 hours or longer, indicating that there was a drastic transcriptional change in the first 3 hours of culture.

Genes differentially expressed between the standard bud-inductive condition and the BA-free condition were limited to only a few in the initial 12 hours of culture and increased drastically in the subsequent 36 hours (Table 1; Fig. 7). This result indicated that exogenous cytokinin plays a substantial regulatory role in gene expression only after 12 hours of culture, agreeing with the abovementioned trend seen in Supplementary Fig. S3.

As compared to the BA-free control culture, 868 genes and 1181 genes showed higher and lower expression levels in the standard bud-inductive culture at least at one timepoint, respectively. These two groups of genes were subjected to GO term enrichment analysis. In the lower expression group, 554 GO terms were significantly enriched (FDR < 0.05), of which top positions were largely occupied by the terms belonging to ‘response to stimuli’ including stress response–related terms (Table 2). This result implicated exogenous cytokinin in the suppression of responses to stress and/or other kinds of stimuli at the initial stage of culture.

In the higher expression group, 603 GO terms were found to be enriched, which contained many nucleolus, ribosome biogenesis or protein synthesis–related terms and their upper-order category terms (Table 3). From this result, together with the observation of nucleolar enlargement, we can reasonably speculate that nucleolar development, the increase in its activity of synthesizing ribosomes and the consequent acceleration of protein synthesis occur dependently on cytokinin in the bud-inductive culture.

Although not top-ranked, cell cycle–related GO terms such as ‘cell cycle’ (rank 21 in the category ‘biological process’, P-value = 2.34E-17, gene number = 86, fold enrichment = 2.958) and ‘cell cycle process’ (rank 77 in the category ‘biological process’, P-value = 1.36E-09, gene number = 58, fold enrichment = 2.628) were also enriched in the higher expression group, consistent with the much more active cell division in the bud-inductive culture than in the BA-free culture.

To further investigate the role of cytokinin in cell cycle progression, we compared expression levels of cell cycle phase–specific genes between the bud-inductive culture and BA-free culture. In this analysis, Torenia orthologs to the E2F target genes of Arabidopsis (Vandepoele et al. 2005) and to the Arabidopsis G2/M-specific genes (Haga et al. 2011) were used as putative indices of the G1-to-S transition phase and G2-to-M transition phase, respectively. In the standard bud-inductive condition, many of the E2F target genes showed elevation in the expression level after 24 hours of culture while the G2/M-specific genes became highly expressed after 48 hours of culture (Fig. 8). After 24 hours of culture, when cells were in the first round of cell cycle, expression levels of the E2F target genes were considerably higher in the bud-inductive culture than in the BA-free culture, suggesting a role of cytokinin in cell cycle progression from the G1 phase to the S phase (Fig. 8A). Expression of the G2/M-specific genes was also higher in the bud-inductive culture (Fig. 8B).
Expression profile of genes characteristic of various plant parts in the early stage of culture

The next analysis was performed to characterize the transcriptome of cultured stem explants with reference to gene expression profiles of various plant parts. Global gene expression data of the stem, root, leaf and shoot apex of young plants were obtained by RNA-seq. A total of 17,453 genes that showed significant differences in the expression level among plant parts were subjected to k-means clustering analysis and classified into 30 clusters according to their expression patterns. As a result, clusters of genes that were preferentially expressed in one plant part were identified as genes characterizing each part (Supplementary Fig. S5). For these genes, expression profiles in cultured stem explants were extracted from the transcriptome data of the early stage of culture (Fig. 9). In the first 3 hours of culture, the expression of genes characteristic of the stem declined while many genes characteristic of the root, leaf or shoot apex became actively expressed at the same time. These changes occurred in both the standard bud-inductive and BA-free control cultures. These results demonstrated that upon culture, regardless of the presence or absence of exogenous cytokinin, stem explants rapidly alter their gene expression profile to have signatures of multiple organs/tissues.

Comparison of transcriptome data between different culture systems of Arabidopsis and Torenia stem culture

It is of great interest how common or different the molecular networks are among various plant culture responses involving cell reprogramming. In an attempt to answer this problem, we conducted a comparative transcriptome analysis between the Torenia stem culture and three culture systems of Arabidopsis, callus induction by wounding, callus induction by auxin and mesophyll protoplast culture. First, we compared significantly upregulated genes in the cultures of Arabidopsis. Analysis of published transcriptome data (Xu et al. 2012, Ikeuchi et al. 2017) identified 4,494 genes significantly upregulated in the first 24 hours of callus induction by wounding and 351 genes significantly upregulated in the first 24 hours of callus induction by auxin. We also used 1,284 genes that were previously reported as being upregulated in the first 24 hours of mesophyll protoplast culture (Chupeau et al. 2013) in our analysis. Of these genes, 15 genes were found to be upregulated in...
Fig. 9 Expression patterns of genes characteristic of various plant parts in the early stage of culture of stem explants. (A–D) Expression patterns of genes that showed preferential expression in the stem (A), root (B), leaf (C) or shoot apex (D) of young plants in the early stage of culture of stem explants under the standard bud-inductive and BA-free control conditions. The heat map shows mean log₂ values of the fold changes relative to the expression before culture.

all three culture systems, and 860 genes were upregulated in at least two culture systems (Fig. 10).

Next, using our transcriptome data of the early stage of the Torenia stem culture, we examined expressions of Torenia orthologs to the Arabidopsis genes that were identified by the above analysis as being upregulated in at least one of the three culture systems (Fig. 10). The results clearly showed a trend that genes upregulated in common to more culture systems of Arabidopsis were also upregulated in the Torenia stem culture at a higher proportion. For nine genes out of 15 genes that were upregulated commonly in three culture systems of Arabidopsis, we found Torenia orthologs, most of which were upregulated in the Torenia stem culture under both the standard bud-inductive and BA-free control cultures (Table 4; Fig. 10). The nine genes, including an ortholog of HISTONE DEACETYLASE 3 (HDA3), an epigenetic regulator gene, were expected to be involved in the initial culture response universally beyond variations in culture protocols and plant materials.

Discussion

In this study, we revisited the stem segment culture of Torenia, which was reported nearly half a century ago to successfully induce adventitious buds from the epidermis simply in one step (Chiyah 1973), and used its modified version as a unique experimental system for the investigation of direct shoot regeneration involving cell reprogramming from the fully differentiated state. As described in the previous papers (Tanimoto and Harada 1982, 1984), cytokinin application was confirmed to be critical in this culture, and light was also found to be important. With the 1 mg/l BA-supplemented culture in the light as a standard bud-inductive culture and the BA-free culture in the light and the BA-supplemented culture in the dark as controls, we performed the cytological analysis of nuclear and nuclear development and cell cycle progression and several lines of gene-expression analysis in the course of shoot regeneration in Torenia stem explants. These analyses and further compar-
Fig. 10 Expression patterns of genes upregulated in callus induction culture systems of Arabidopsis in Torenia stem culture. (A) Venn diagram showing numbers of overlapping and non-overlapping genes among gene sets that were identified to be upregulated during callus induction by auxin, callus induction by wounding and protoplast culture of Arabidopsis. (B–H) Expression patterns in the early stage of culture of Torenia stem explants under the standard bud-inductive and BA-free control conditions for the Torenia orthologs to the Arabidopsis genes upregulated in one, two or all three of three different callus induction culture systems of Arabidopsis: callus induction by auxin, callus induction by wounding and protoplast culture. The heat maps show mean log₂ values of the fold changes relative to the expression before culture. Parenthesized numbers indicate percentages of genes that were significantly upregulated at least at one timepoint in the Torenia stem culture.
Table 2 Top 10 terms in three GO categories (biological process, cellular component and molecular function) enriched in genes expressed at a lower level in the standard bud-inductive culture than in the BA-free control culture

| Biological process          | GO term                                      | P-value | Number of genes | Fold enrichment |
|-----------------------------|----------------------------------------------|---------|-----------------|-----------------|
| Response to stimulus        | Response to stimulus                         | 1.8E-41 | 676             | 1.455           |
| Response to external stimulus| Response to external stimulus                | 3.9E-38 | 345             | 1.918           |
| Response to oxygen-containing compound | Response to oxygen-containing compound    | 9.7E-34 | 362             | 1.786           |
| Response to chemical        | Response to chemical                         | 2.9E-32 | 468             | 1.580           |
| Response to stress          | Response to stress                           | 6.0E-30 | 469             | 1.545           |
| Response to organic substance| Response to organic substance               | 5.6E-28 | 373             | 1.652           |
| Response to biotic stimulus| Response to biotic stimulus                 | 1.6E-27 | 268             | 1.887           |
| Response to external biotic stimulus | Response to external biotic stimulus       | 2.6E-27 | 267             | 1.884           |
| Response to other organisms | Response to other organisms                 | 2.5E-27 | 267             | 1.884           |
| Interspecies interaction between organisms | Interspecies interaction between organisms | 2.6E-27 | 267             | 1.884           |
| Response to stimulus        | Response to stimulus                         | 1.8E-41 | 676             | 1.455           |
| Cellular component          | Cellular component                           | 1.2E-22 | 397             | 1.517           |
| Plasma membrane             | Plasma membrane                              | 8.9E-19 | 331             | 1.528           |
| Cellular anatomical entity  | Cellular anatomical entity                   | 5.2E-18 | 968             | 1.115           |
| Membrane                    | Membrane                                    | 1.6E-15 | 608             | 1.241           |
| Anchoring junction          | Anchoring junction                           | 1.6E-08 | 183             | 1.455           |
| Cell–cell junction          | Cell–cell junction                           | 1.6E-08 | 183             | 1.455           |
| Cell junction               | Cell junction                                | 2.0E-08 | 183             | 1.450           |
| Extracellular region        | Extracellular region                         | 2.0E-08 | 177             | 1.642           |
| Plasmodesma                 | Plasmodesma                                  | 2.2E-08 | 182             | 1.450           |
| Symplast                    | Symplast                                     | 2.2E-08 | 182             | 1.450           |
| Molecular function          | Caffeoyl-CoA O-methyltransferase activity    | 2.7E-10 | 8               | 21.667          |
| Caffeoyl CoA:S-adenosyl-L-methionine O-methyltransferase activity | Caffeoyl CoA:S-adenosyl-L-methionine O-methyltransferase activity | 2.7E-10 | 8 | 21.667 |
| 4-Hydroxyferuloyl spermidine:S-adenosyl-L-methionine O-methyltransferase activity | 4-Hydroxyferuloyl spermidine:S-adenosyl-L-methionine O-methyltransferase activity | 2.7E-10 | 8 | 21.667 |
| Tricaffeoyl spermidine:S-adenosyl-L-methionine O-methyltransferase activity | Tricaffeoyl spermidine:S-adenosyl-L-methionine O-methyltransferase activity | 2.7E-10 | 8 | 21.667 |
| Trihydroxyferuloyl spermidine O-methyltransferase activity | Trihydroxyferuloyl spermidine O-methyltransferase activity | 2.7E-10 | 8 | 21.667 |
| DNA-binding transcription factor activity | DNA-binding transcription factor activity     | 3.9E-09 | 120             | 1.677           |
| Molecular function regulator | Molecular function regulator                 | 9.6E-09 | 158             | 1.522           |
| Molecular transducer activity | Molecular transducer activity                | 2.0E-07 | 36              | 2.538           |
| Protein binding             | Protein binding                              | 2.6E-07 | 492             | 1.165           |
| Oxidoreductase activity, acting on peroxide as acceptor | Oxidoreductase activity, acting on peroxide as acceptor | 2.9E-07 | 21              | 3.592           |

Table 3 Top 10 terms in three GO categories (biological process, cellular component and molecular function) enriched in genes expressed at a higher level in the standard bud-inductive culture than in the BA-free control culture

| Biological process          | GO term                                      | P-value | Number of genes | Fold enrichment |
|-----------------------------|----------------------------------------------|---------|-----------------|-----------------|
| Gene expression             | Gene expression                              | 1.5E-36 | 310             | 1.948           |
| Organic substance           | Organic substance                            | 9.1E-34 | 385             | 1.694           |
| Biosynthetic process        | Biosynthetic process                         | 3.0E-33 | 390             | 1.675           |
| Cellular biosynthetic process | Cellular biosynthetic process               | 6.2E-33 | 375             | 1.701           |
| Macromolecule biosynthetic process | Macromolecule biosynthetic process        | 7.0E-33 | 291             | 1.924           |
| Cellular process            | Cellular process                             | 3.2E-31 | 702             | 1.256           |
| Cellular nitrogen compound biosynthetic process | Cellular nitrogen compound biosynthetic process | 1.2E-30 | 291             | 1.869           |
| Ribonucleoprotein complex biogenesis | Ribonucleoprotein complex biogenesis     | 1.7E-29 | 92              | 3.888           |
| Ribosome biogenesis         | Ribosome biogenesis                          | 1.5E-26 | 82              | 3.914           |
| Gene expression             | Gene expression                              | 1.5E-36 | 310             | 1.948           |
| Cellular component          | Cellular component                           | 2.6E-42 | 233             | 2.488           |
| Non-membrane-bounded organelle | Non-membrane-bounded organelle             | 2.6E-42 | 233             | 2.488           |
| Intracellular non-membrane-bounded organelle | Intracellular non-membrane-bounded organelle | 2.6E-42 | 233             | 2.488           |
| Cellular anatomical entity  | Cellular anatomical entity                   | 1.8E-36 | 771             | 1.219           |
| Nucleolus                   | Nucleolus                                    | 9.2E-36 | 128             | 3.452           |
| Nucleus                     | Nucleus                                      | 2.9E-32 | 399             | 1.641           |
| Protein-containing complex  | Protein-containing complex                   | 3.8E-32 | 306             | 1.859           |
| Nuclear lumen               | Nuclear lumen                                | 3.6E-32 | 167             | 2.691           |
| Intracellular               | Intracellular                                | 2.4E-31 | 675             | 1.282           |
| Intracellular organelle lumen | Intracellular organelle lumen              | 6.2E-31 | 169             | 2.544           |
| Membrane-enclosed lumen     | Membrane-enclosed lumen                      | 6.2E-31 | 169             | 2.544           |
| Structural molecule activity | Structural molecule activity                 | 1.1E-24 | 77              | 3.874           |
| Structural constituent of ribosome | Structural constituent of ribosome       | 3.2E-21 | 62              | 4.083           |
| RNA binding                 | RNA binding                                  | 6.3E-21 | 124             | 2.435           |
| mRNA binding                | mRNA binding                                 | 5.0E-16 | 70              | 2.933           |
| Binding                     | Binding                                      | 8.3E-15 | 621             | 1.184           |
| Protein binding             | Protein binding                              | 4.6E-14 | 407             | 1.322           |
| Heterocyclic compound binding | Heterocyclic compound binding             | 3.3E-11 | 390             | 1.282           |
| Organic cyclic compound binding | Organic cyclic compound binding           | 3.9E-11 | 390             | 1.281           |
| DNA binding                 | DNA binding                                  | 4.6E-11 | 150             | 1.671           |
| Nucleic acid binding        | Nucleic acid binding                         | 2.0E-10 | 270             | 1.383           |

Cytokinin-dependent nucleolar development precedes cell division activation

In stem explants cultured under the standard bud-inductive condition, cell division became active after 2–3 d, which was followed by adventitious bud formation after 8 d of culture (Figs. 1, 3). Microscopic observation at day 2 revealed that, before the activation of cell division, nuclei and nucleoli were markedly enlarged in epidermal cells (Fig. 2). Similar changes in the size of nuclei and nucleoli have been reported for dedifferentiating cells provoked to divide in various plant cell/tissue cultures.
(Feldman and Torrey 1977, Williams and Jordan 1980, Paul et al. 1989, Williams et al. 2003). Taking these reports into consideration, the nuclear and nucleolar enlargement observed in the Torenia stem culture can be considered as a sign preceding cell division activation.

The nucleolus is a specialized subnuclear domain functioning as a site of ribosome biogenesis, and its development is generally linked with the stimulation of ribosome biogenesis and the resultant acceleration of protein synthesis. Indeed, consistent with nucleolar enlargement, transcriptome analysis with GO enrichment analysis indicated that expressions of genes related to ribosome biogenesis and protein synthesis were upregulated in the early stage of the culture of Torenia stem explants (Fig. 5). Upregulation of ribosome-biogenesis-related genes prior to cell proliferation was also reported from the transcriptomic characterization of Arabidopsis cultures for wound-induced callus formation (Ikeuchi et al. 2017) and mesophyll protoplast–derived callus formation (Chupeau et al. 2013). Additionally, in the mesophyll protoplast culture of tobacco, an increase in the rate of protein synthesis was observed by the [14C]Leu-feeding experiment before the start of cell division (Zelcer and Galun 1976). These previous findings together with our results suggest that nucleolar development accompanying stimulation of ribosome biogenesis and acceleration of protein synthesis is commonly involved in preparation for cell division activation in dedifferentiating plant cells.

All the above-described cytological events and gene expression changes were greatly reduced in the BA-free control culture compared to the standard bud-inductive culture. Without BA application, nuclei and nucleoli were only slightly enlarged (Fig. 2), the frequency of cell division was very low (Fig. 3), few adventitious buds were formed (Fig. 1), and nucleolus, ribosome biogenesis and protein synthesis–related genes were significantly less upregulated (Fig. 7). For a simple explanation of these results, we can hypothesize that cytokinin primarily regulates gene expression for nucleolar development accompanying stimulated ribosome biogenesis, which is a prerequisite for cell division activation and subsequent morphogenesis. In partial support of this hypothesis, previous transcriptomic studies demonstrated that ribosomal protein genes are upregulated downstream of cytokinin signaling (Brenner et al. 2005, Kiba et al. 2005).

In the dark control, cell division adventitious bud formation was reduced to about 30% and 20% of those in the standard bud-inductive culture (Figs. 1, 3), while the reduction of nucleolar enlargement was not so large (Fig. 2). It is therefore likely that light is involved not only in the nucleolar development process but also in later processes.

### Table 4 List of the Torenia orthologs to the Arabidopsis genes commonly upregulated in all three different callus-inducing culture systems of Arabidopsis

| AGI | Primary gene symbol | Torenia transcript | Significance |
|-----|---------------------|--------------------|-------------|
| AT4G44750 | HISTONE DEACETYLASE 3 (HDA3) | TF090237 | + |
| AT4G44750 | POLYOL/MONOSACCHARIDE TRANSPORTER 6 (PMT6) | TF096621 | + |
| AT4G44750 | AUXIN-INDUCED IN ROOT CULTURES 12 (AIR12) | TF043399 | + |
| AT4G44750 | PDI-LIKE 1-1 (PDIL1-1) | TF087184 | ns |
| AT4G44750 | HISTONE DEACETYLASE 3 (HDA3) | TF089190 | + |
| AT4G44750 | AUXIN-INDUCED IN ROOT CULTURES 12 (AIR12) | TB077745 | ns |
| AT4G44750 | POLYOL/MONOSACCHARIDE TRANSPORTER 6 (PMT6) | TB096621 | + |
| AT4G44750 | HISTONE DEACETYLASE 3 (HDA3) | TB087184 | + |
| AT4G44750 | AUXIN-INDUCED IN ROOT CULTURES 12 (AIR12) | TB097832 | + |
| AT4G44750 | POLYOL/MONOSACCHARIDE TRANSPORTER 6 (PMT6) | TB097832 | + |
| AT4G44750 | HISTONE DEACETYLASE 3 (HDA3) | TB100536 | + |

**Genes are listed with Arabidopsis Genome Initiative (AGI) codes in the same order as in Fig. 10H. The + sign denotes significant upregulation at least at one timepoint in the Torenia stem culture.**

**Cell cycle restarts from G1 to S progression**

As argued above, the role of cytokinin in cell division activation in the Torenia stem culture may be attributed to the cytokinin requirement of nucleolar development and stimulation of ribosome biogenesis in the preparation for cell division. Even if this is true, however, the possibility is not excluded that cytokinin more directly functions to activate cell division as well. Indeed, cytokinin is known to promote cell cycle progression in at least two pathways: one directs the G1-to-S transition via transcriptional upregulation of the D-type cyclin CYCD3 (Riou-Khamlichi et al. 1999), and the other directs the G2-to-M transition through the activation of A-type cyclin-dependent kinase (CDKA) (Zhang et al. 1996, Orchard et al. 2005).

Our transcriptome analysis of Torenia stem explants revealed that both the G1/S and G2/M genes showed a much higher expression in the bud-inductive culture than in the BA-free control (Fig. 8). As cells were mostly in the G0 or G1 phase at the beginning of culture (Fig. 3A), this result suggests that the main point of regulation of cell cycle progression by cytokinin...
is at the G1-to-S phase transition in the present culture system, aside from whether it is direct or indirect.

**De novo SAM organization occurs from cell division foci in association with SAM regulator gene expression**

Previous anatomical studies of the Torenia stem culture showed that cell division was unevenly activated in the epidermis to form local areas of active cell proliferation and that adventitious bud SAMs formed from the centers of such areas (Chlyah 1974b, Chlyah et al. 1975). Additionally, it was also pointed out that the origin of the SAM was not always a single epidermal cell (Chlyah 1974b). In agreement with these reports, our serial observation indicated that adventitious SAMs arise in cell division foci derived from more than one epidermal cell (Fig. 4). Of note, the boundary of the presumptive SAM region, which later developed into the SAM, did not correspond to the borderlines of original epidermal cells. This finding suggests that the presumptive SAM regions are determined only after several rounds of cell division (Figs. 4H–M).

There have been many papers reporting that shoot regeneration is closely associated with a high expression of SAM regulatory transcription factor genes such as STM, CUCs and WUS (Daimon et al. 2003, Zhang et al. 2017). Similarly, in Torenia stem explants cultured in the standard bud-inductive condition, the expression of most of the major SAM regulatory genes tested was found to increase greatly a few days before visible adventitious bud formation (Fig. 6). In particular, expression levels of TjCUC1/2s and TjWUS2 showed a good correlation with epidermal shoot regeneration in the following respects: lack of expression before culture, a reduced expression levels in parallel with the reduced frequencies in two control cultures and a higher expression in epidermal layers than in inner tissues. It can be inferred from these results that transcriptional activation of the SAM regulatory network is key to adventitious SAM morphogenesis.

**Gene expression profile rapidly changes upon culture likely reflecting cell reprogramming**

Transcriptome analysis of Torenia stem explants focusing on the early stage of culture revealed very rapid changes in the gene expression profile from the original state to a new state with multiple organ/tissue signatures characterized by simultaneous expression of genes that are normally expressed preferentially in the shoot apex, leaf or root. Previous studies with various organisms and various experimental systems have shown that pluripotent stem cells or cells undergoing reprogramming co-express genes that are typically associated with specific cell types (Hu et al. 1997, Buganim et al. 2012, Efroni et al. 2016, Mozgová et al. 2017, Omary et al. 2020). Recently, it has been proposed that such gene expression profile exhibiting a ‘mixed identity’ can be a common feature of the pluripotent state in either animals or plants (Moris et al. 2016, Efroni 2018). Our findings in the Torenia culture coincide with these arguments and may reflect that the explants gain pluripotency during cell reprogramming.

Comparison between genes upregulated during callus induction in three different culture systems of Arabidopsis and examination of the expression of their orthologs in the Torenia stem culture identified nine genes that were expressed in the early stage in all the four culture systems as potential candidates of genes that function in cell reprogramming commonly in any type of culture system. Interestingly, this gene list (Table 4) included an ortholog of the histone deacetylase gene HDA3, which was reported to participate in the auxin-induced callus formation (Lee et al. 2016). Functional analysis of this and other genes in the list would give a clue for a common molecular network involved in cell reprogramming.

Our analysis indicated that cytokinin has little impact on gene expression in the first 12 hours of culture (Figs. 9, 10). Accordingly, transcriptional changes in the initial phase of culture noted above must be triggered not by exogenous cytokinin but by some other stimuli during tissue culture operation. At the start of tissue culture, explants necessarily suffer wounding when excised from the mother plant, and wounding is well-known to induce a wide spectrum of cellular responses, including cell reprogramming and the resultant callus formation not only in Arabidopsis but also in various plant species (Ikeuchi et al. 2013). In Torenia stem segment culture, it was also reported that additional wounding promotes shoot regeneration (Takeuchi et al. 1985). Wounding is, therefore, one of the likely triggers of initial changes of gene expression in the Torenia culture. The wounding-triggered molecular cascade leading to cell reprogramming has been extensively studied in Arabidopsis (Ikeuchi et al. 2017, Iwase et al. 2017). However, there is also the possibility that other factors besides wounding trigger these changes. For example, explant isolation may affect the endogenous signaling molecule levels as a result of tissue disconnection, which can be a trigger of gene expression changes. Indeed, tissue disconnection by incision of the stem was shown to alter the distribution of auxin and consequently induce changes of gene expression leading to the process of tissue reunion in Arabidopsis (Asahina et al. 2011). These works would guide future investigation of the roles of wounding and tissue disconnection in shoot regeneration in the Torenia culture.

In summary of the results obtained through the present study, the whole course of shoot regeneration in the Torenia culture system is schematized in Fig. 11. Global changes of gene expression rapidly occur upon culture independently of cytokinin, possibly reflecting the epidermal cell reprogramming from the fully differentiated state to enter the pluripotent state. These initial gene expression changes include the upregulation of genes commonly implicated in various callus-inducing cultures of Arabidopsis. Then, cytokinin transcriptionally stimulates nucleolar development and ribosome biogenesis, which is followed by the activation of epidermal cell division. Finally, adventitious bud SAMs are de novo organized from cell division foci in the epidermis. Our findings depict the overall process of direct shoot regeneration in the Torenia stem culture consisting
Fig. 11 Schematic sketch of processes of shoot regeneration in stem segment culture of Torenia.

Materials and Methods

Plant material and growth conditions

All experiments were carried out using a genetically homogeneous inbred line of *Torenia fournieri* Lind. that had been developed through 11 generations of self-pollination. Surface-sterilized and stratified seeds were sown on basal medium, which was half-strength MS medium containing 2% (w/v) of sucrose, buffered with 0.05% (w/v) of 2-morpholinoethanesulfonic acid at pH 5.7 and solidified with 0.25% (w/v) of gellan gum, and plants were aseptically grown at 22°C under continuous light (60–100 µmol/s/m²).

Tissue culture

Stems were excised from the internodes between the cotyledons and the first pair of true leaves of 4-week-old plants. Each internode stem of a quadrangular prism shape with wider and narrower lateral faces was sliced longitudinally along the midline of the narrower side into two sections and then cut into 1.5-mm-long segments. The stem segments were placed on the basal medium described above or the basal medium supplemented with 1 mg/l of BA such that the sliced surface was in contact with the medium. The subsequent culture was conducted at 22°C under continuous light (60–100 µmol/s/m²) or in the dark.

Flow cytometric analysis of nuclear DNA content

To isolate nuclei, samples were chopped in CyStain UV Precise P Nuclei Extraction Buffer (Sysmex, Kobe, Hyogo, Japan) in petri dishes placed on ice and filtered through 20-µm CellTrics filter (Sysmex) after 1-minute incubation on ice. Isolated nuclei were stained with CyStain UV Precise P Staining Buffer (Sysmex), and then, the DNA content of each nucleus was quantified with SyFlow SL (Partec, Görlitz, Saxony, Germany).

Microscopic analysis

For RNAselect and DAPI staining, stripped epidermis of stem explants was fixed in methanol at −20°C for at least 10 minutes. The fixed samples were washed in phosphate buffered saline at pH 7.2 (PBS) and then stained in PBS solution containing 25% (v/v) CyStain UV Precise P Staining Buffer (Sysmex), 1 µM CYTO RNAselect Green Fluorescent Cell Stain (Invitrogen, Waltham, MA, USA) and 0.1%(w/v) TritonX-100 for 30 minutes at room temperature while being protected from light. The stained samples were washed in PBS before observation.

For detection of nascent cell walls, the epidermis of stem explants was stained with aniline blue according to the protocol described in Schenk and Schikora (2015) with minor modifications. Stripped epidermis was fixed in a 1:3 mixture of acetic acid and ethanol for at least 24 hours at room temperature. After washing in 150 mM K₂HPO₄ for 30 minutes, the samples were stained in 1% (w/v) aniline blue solution containing 150 mM K₂HPO₄ for 2.5 hours at room temperature while being protected from light. The stained samples were washed in 150 mM K₂HPO₄ before observation.

The epidermis samples stained with DAPI, RNAselect or aniline blue were observed under the Olympus BX50F4 microscope. For CLSM, explants were thinly sliced and vacuum-infiltrated with 1 µg/ml PI. Samples were then observed using a confocal laser scanning microscope (Leica, Wetzlar, Hesse, Germany; TCS SP5).

Serial observation of the surface of cultured explants was performed with a metallurgical microscope (WRAYMER, Osaka, Osaka, Japan; BM-340TL).

Transcriptome analysis

All transcriptome analyses were carried out in three biological replicates. Collected samples were immediately frozen with liquid nitrogen and stored at −80°C until use. Total RNA was isolated from the frozen samples with Directzol RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA).
For RNA-seq analysis of a set of samples consisting of stem explants cultured for 0, 2, 4, 6, and 8 d, libraries were prepared from total RNA with messenger RNA (mRNA)-seq Kit with KAPA mRNA Capture Beads (KAPA, Wilmington, MA, USA), NEBNext Multiplex Oligos for Illumina Index Primers Set 1–4 (NEB, Ipswich, MA, USA) and Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA) according to manufacturers’ protocols. The libraries were sequenced with Nextseq600 (Illumina, San Diego, CA, USA). Raw reads containing adapter sequences were trimmed using bcl2fastq (Illumina), and nucleotides with low-quality (QV < 25) were masked by N using the original script. Reads shorter than 50 bp were discarded, and the remaining reads were mapped to the Torenia cDNA database (http://dandelion.liveholonics.com/torenia/), which had been constructed from mRNAs of leaves and roots of young seedlings and floral organs of flowering plants, using Bowtie with the following parameters: “--all –best –strata” (Langmead et al. 2009). Reads were counted by transcript models.

For RNA-seq analysis of a set of samples consisting of various parts of 4-week-old plants (shoot apices, the first and second pairs of true leaves, the first internode stems and whole roots) and stem explants at the early stage of culture (explants cultured for 0, 3, 6, 12, 24 and 48 hours), libraries were prepared with mRNA HyperPrep Kit (KAPA) and Multiplex Oligos for Illumina Index Primers Set 1–4 (NEB) according to manufacturers’ protocols. The libraries were sequenced with Novaseq6000 (Illumina). Reads were mapped to the Torenia cDNA database (http://dandelion.liveholonics.com/torenia/) using Bowtie2 (Langmead and Salzberg 2012), and the expression level of each transcript was quantified with Salmon (Patro et al. 2017).

The log-transformed Transcripts Per Million (TPM) values of the RNA-seq transcriptome data were subjected to PCA using the R pcomp function.

Differential expression analysis was performed with edgeR (Robinson et al. 2009, McCarthy et al. 2012) and limma (Ritchie et al. 2015) packages of R. K-means clustering analysis was performed on Multiple Experiment Viewer platform (Saeed et al. 2003). Assignment of GO annotation to transcript sequences of Torenia was conducted with Blast2GO (Conesa and Götz 2008) based on the results of homology search against the Arabidopsis subset and Viridiplantae subset of the NCBI non-redundant database and also on the protein domains identified by InterPro domain search. GO enrichment analysis was also carried out on Blast2GO by Fisher’s exact test with cutoff at FDR <0.05.

For comparison of transcriptome data between Arabidopsis and Torenia, RNA-seq data and microarray data of Arabidopsis were obtained from the public resource. The Arabidopsis RNA-seq data were processed as described above. The microarray data were normalized by a variant of MAS5.0 with robust radius-minimax estimators (Kohl and Deigner 2010). Then, differentially expressed genes were identified with the rank products method with a cutoff at FDR <0.05 using the Rank Prod R package (Del Carratore et al. 2017).

Identification of Torenia orthologs to Arabidopsis genes

Orthologs of Torenia to the SAM regulator genes of Arabidopsis were identified by homology search against the amino-acid sequence database derived from the Torenia cDNA database (http://dandelion.liveholonics.com/torenia/) with the amino-acid sequences of Arabidopsis SAM regulators as queries followed by phylogenetic tree construction. In other cases, Torenia orthologs to a set of Arabidopsis genes of interest were identified using OrthoFinder (Emms and Kelly 2015, 2019).

RT-qPCR analysis

Total RNA was isolated with Direct-zol RNA MiniPrep Kit (Zymo Research). From each RNA preparation, potentially remaining genomic DNA was eliminated and the first-strand cDNA was synthesized using PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Kusatsu, Shiga, Japan). Then, qPCR was performed with gene-specific primers (Supplementary Table S1) using TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa) on Step One Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The data were normalized with the ubiquitin gene TjUBQ10, a Torenia ortholog of Arabidopsis UBQ10, as an internal control.

Supplementary Data

Supplementary data are available at PCP online.

Data Availability

The data underlying this article are available in the online repositories as follows. The RNA-seq sequencing data were deposited into the DNA Data Bank of Japan (https://ddbj.nig.ac.jp/DRAM/) under the accession number DRA011823. The sequences of genes used in this study can be accessed in Torenia cDNA database (http://dandelion.liveholonics.com/torenia/) with the following accession numbers: TjSTM1 (TfB072761), TjSTM2 (Tfb084476), TFB084487, TjSTM3 (Tfb080768), TjCUC1/2a (Tfb082036), TjCUC1/2b (Tfb082143), TjWUS1 (Tfb099710), TjWUS2 (Tfb094340) and TjUBQ10 (Tfb084374).

Funding

Sasakawa Scientific Research Grant (no. 29-405 to H.M.) from the Japan Science Society; Japan Science and Technology Agency ERATO (JPMJER1004 to T.H.); Japan Society for the Promotion of Science KAKENHI (JP17K07437 to M.S., JP19H04830 to M.S., JP19J13924 to H.M., JP20H04881 to M.S.).

Acknowledgements

We thank Dr. Yui Uchida for instruction of the quality check of RNA samples and cDNA libraries. We also appreciate helpful advice on transcriptomic data analysis by Dr. Misato Ohtani.

Disclosures

No conflicts of interest declared.

References

Akama, K., Shiraishi, H., Ohta, S., Nakamura, K., Okada, K. and Shimura, Y. (1992) Efficient transformation of Arabidopsis thaliana: comparison of the efficiencies with various organs, plant ecotypes and Agrobacterium strains. Plant Cell Rep. 12: 7–11.

Ashina, M., Azuma, K., Pitaksaingkarn, W., Yamazaki, T., Mitsuda, N., Ohme-Takagi, M., et al. (2011) Spatially selective hormonal control of RAP2.6L and ANAC071 transcription factors involved in tissue reunion in Arabidopsis. Proc. Natl. Acad. Sci. USA 108: 16128–16132.

Atta, R., Laurens, L., Boucheron-Dubuisson, E., Guivarch, A., Carnero, E., Giraudat-Pautot, V., et al. (2009) Pluripotency of Arabidopsis xylem pericycle underlies shoot regeneration from root and hypocotyl explants grown in vitro. Plant J. 57: 626–644.
Emerg. D.M. and Kelly, S. (2015) OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. Genome Biol. 16: 157.

Emms, D.M. and Kelly, S. (2019) OrthoFinder: phylogenetic orthology inference for comparative genomics. Genome Biol. 20: 238.

Fan, M., Xu, C., Xu, K. and Hu, Y. (2012) LATERAL ORGAN BOUNDARIES DOMAIN transcription factors direct callus formation in Arabidopsis regeneration. Cell Res. 22: 1169–1180.

Feldman, L.J. and Torrey, J.G. (1977) Nuclear changes associated with cellular dedifferentiation in pea root cortical cells cultured in vitro. J. Cell. Sci. 28: 87–105.

Gordon, S.P., Heisler, M.G., Reddy, G.V., Ohno, C., Das, P. and Meyerowitz, E.M. (2007) Pattern formation during de novo assembly of the Arabidopsis shoot meristem. Development 134: 3539–3548.

Haga, N., Kobayashi, K., Suzuki, T., Mao, K., Kubo, M., Ohtani, M., et al. (2013) Mutations in MYB3R1 and MYB3R4 cause pleiotropic developmental defects and preferential down-regulation of multiple G2/M-specific genes in Arabidopsis. Plant Physiol. 157: 706–717.

He, C., Chen, X., Huang, H. and Xu, L. (2012) Reprogramming of H3K27me3 is critical for acquisition of pluripotency from cultured Arabidopsis tissues. PLoS Genet. 8: e1002911.

Hicks, G.S. (1980) Patterns of organ development in plant tissue culture and the problem of organ determination. Bot. Rev. 46: 1–23.

Hu, M., Krause, D., Greaves, M., Shaksis, S., Dexter, M., Heyworth, C., et al. (1997) Multilineage gene expression precedes commitment in the hemopoietic system. Genes Dev. 11: 774–785.

Ikeuchi, M., Iwase, A., Rymen, B., Lambolez, A., Kojima, M., Takebayashi, Y., et al. (2017) Wounding triggers callus formation via dynamic hormonal and transcriptional changes. Plant Physiol. 175: 1158–1174.

Ikeuchi, M., Sugimoto, K. and Iwase, A. (2013) Plant callus: mechanisms of induction and repression. Plant Cell 25: 3159–3173.

Ishihara, H., Sugimoto, K., Tan, P.T., Temman, H., Kadokura, S., Inui, Y., et al. (2019) Primed histone demethylation regulates shoot regenerative competency. Nat. Commun. 10: 1–15.

Iwase, A., Harashima, H., Ikeuchi, M., Rymen, B., Ohnuma, M., Komaki, S., et al. (2017) WIND1 promotes shoot regeneration through transcriptional activation of ENHANCER OF SHOOT REGENERATION1 in Arabidopsis. Plant Cell 29: 54–69.

Iwase, A., Mitsuda, N., Koyama, T., Hiratsu, K., Kojima, M., Arai, T., et al. (2011) The AP2/ERF transcription factor WIND1 controls cell dedifferentiation in Arabidopsis. Curr. Biol. 21: 508–514.

Kamada, H. and Harada, H. (1979) Influence of several growth regulators and amino acids on in vitro organogenesis of Torenia fournieri. J. Exp. Bot. 30: 27–36.

Karen, A., Durga, K., Sugimoto, K., Du, Y., Pulinakkalak, A.J., Trivedi, Z.B., et al. (2015) PLETHORA genes control regeneration by a two-step mechanism. Curr. Biol. 25: 1–14.

Kiba, T., Naitou, T., Koizumi, N., Yamashino, T., Sakakibara, H. and Mizuno, T. (2005) Combinatorial microarray analysis revealing Arabidopsis genes implicated in cytokinin responses through the His→Asp phosphorylation circuitry. Plant Cell Physiol. 46: 339–355.

Kohl, M. and Deininger, H.P. (2010) Preprocessing of gene expression data by optimally robust estimators. BMC Bioinform. 11: 583.

Koornneef, M., Hanhart, C.J. and Martinielli, L. (1987) A genetic analysis of cell culture traits in tomato. Theor. Appli. Genet. 74: 633–641. 

Langmead, B. and Saltzman, S.L. (2012) Fast gapped-read alignment with Bowtie 2. Nat. Methods 9: 357–359.

Langmead, B., Trapnell, C., Pop, M. and Salzberg, S.L. (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10: R25.

Lardon, R., Wijnker, E., Keurentjes, J. and Geelen, D. (2020) The genetic framework of shoot regeneration in Arabidopsis comprises master regulators and conditional fine-tuning factors. Commun. Biol. 3: 549.
Lee, K., Park, O.S., Jung, S.J. and Seo, P.J. (2016) Histone deacetylation-mediated cellular dedifferentiation in Arabidopsis. *J. Plant Physiol.* 191: 95–100.

Lee, K. and Seo, P.J. (2018) Dynamic epigenetic changes during plant regeneration. *Trends Plant Sci.* 23: 235–247.

McCarthy, D.J., Chen, Y. and Smyth, G.K. (2012) Differential expression analysis of multifactor RNA-seq experiments with respect to biological variation. *Nucleic Acids Res.* 40: 4288–4297.

Moris, N., Pina, C. and Arias, A.M. (2016) Transition states and cell fate decisions in epigenetic landscapes. *Nat. Rev. Genet.* 17: 693–703.

Mozgova, I., Muñoz-Viana, R. and Hennig, L. (2017) PR2C represses hormone-induced somatic embryogenesis in vegetative tissue of *Arabidopsis thaliana*. *PloS Genet.* 13: e1006562.

Nishi, T., Yamada, Y. and Takahashi, E. (1968) Organ redifferentiation and plant restoration in rice callus. *Nature* 219: 508–509.

Ormary, M., Gil-Yarom, N., Yahav, C., Steiner, E. and Efroni, I. (2020) A conserved superlocus regulates above- and belowground root initiation. bioRxiv. 2020.11.11.377937.

Orchard, C.B., Siciliano, I., Sorrell, D.A., Marchbank, A., Rogers, H.J., Francis, D., et al. (2005) Tobacco BY-2 cells expressing fission yeast cdc25 bypass a G2/M block on the cell cycle. *Plant J.* 44: 290–299.

Paterno, R., Duggal, G., Love, M.I., Irizarry, R.A. and Kingsford, C. (2017) Salmon provides fast and bias-aware quantification of transcript expression. *Nat. Methods* 14: 417–419.

Paul, E., Harikrishna, K., Fioroni, O. and Draper, J. (1989) Dedifferentiation of *Asparagus officinalis* L. Mesophyll cells during initiation of cell cultures. *Plant Sci.* 65: 111–117.

Riou-Khamlichi, C., Huntley, R., Jacqmard, A. and Murray, J.A.H. (1999) Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. *Science* 283: 1541–1544.

Ritchie, M.E., Phipson, B., Wu, D., Hsu, Y., Law, C.W., Shi, W., et al. (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 43: e47.

Robinson, M.D., McCarthy, D.J. and Smyth, G.K. (2009) edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26: 139–140.

Rossipopoff, O., Chelysheva, L., Saffar, J., Lecorgne, L., Gey, D., Cailléux, E., et al. (2017) Direct conversion of root primordium into shoot meristem relies on timing of stem cell niche development. *Development* 144: 1187–1200.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., et al. (2003) TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* 34: 374–378.

Schenk, S. and Schikora, A. (2015) Staining of callose depositions in root and leaf tissues. *Bio-Protocol* 5: e1429.

Skog, F. and Miller, C.O. (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symp. Soc. Exp. Biol.* 11: 118–130.

Sugimoto, K., Jiao, Y. and Meyerowitz, E.M. (2010) Arabidopsis regeneration from multiple tissues occurs via a root development pathway. *Dev. Cell* 18: 463–471.

Takeuchi, N., Tanimoto, S. and Harada, H. (1985) Effects of wounding on adventitious bud formation in *Torenia* stem segments cultured in vitro. *J. Exp. Bot.* 36: 841–847.

Tamaki, H., Konishi, M., Daimon, Y., Aida, M., Tasaka, M. and Sugiyama, M. (2009) Identification of novel meristem factors involved in shoot regeneration through the analysis of temperature-sensitive mutants of *Arabidopsis*. *Plant J.* 57: 1027–1039.

Tanimoto, S. and Harada, H. (1982) Effects of cytokinin and anticytokinin on the initial stage of adventitious bud differentiation in the epidermis of *Torenia* stem segments. *Plant Cell Physiol.* 23: 1371–1376.

Tanimoto, S. and Harada, H. (1984) Roles of auxin and cytokinin in organogenesis in *Torenia* stem segments cultured in vitro. *J. Plant Physiol.* 115: 11–18.

Valvekens, D., Van Montagu, M. and Van Lijsebettens, M. (1988) Agrobacterium tumefaciens-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Natl. Acad. Sci. USA* 85: 5536–5540.

Vandepoele, K., Vlieghe, K., Florquin, K., Hennig, L., Beemster, G.T.S., Gruissem, W., et al. (2005) Genome-wide identification of potential plant E2F target genes. *Plant Physiol.* 139: 316–328.

Williams, L., Zhao, J., Morozova, N., Li, Y., Avivi, Y. and Grafi, G. (2003) Chromatin reorganization accompanying cellular dedifferentiation is associated with modifications of histone H3, redistribution of HP1, and activation of E2F-target genes. *Dev. Dyn.* 228: 113–120.

Williams, L.M. and Jordan, E.G. (1980) Nuclear and nucleolar size changes and nuclear pore frequency in cultured explants of Jerusalem artichoke tubers (*Helianthus tuberosus* L.). *J. Exp. Bot.* 31: 1613–1619.

Xu, K., Liu, J., Fan, M., Xin, W., Hu, Y. and Xu, C. (2012) A genome-wide transcriptome profiling reveals the early molecular events during callus initiation in Arabidopsis multiple organs. *Genomics* 100: 116–124.

Yasutani, I., Otsawa, S., Nishida, T., Sugiyama, M. and Komamine, A. (1994) Isolation of temperature-sensitive mutants of *Arabidopsis thaliana* that are defective in the redifferentiation of shoots. *Plant Physiol.* 105: 815–822.

Zelcer, A. and Galun, E. (1976) Culture of newly isolated tobacco protoplasts: precursor incorporation into protein, RNA and DNA. *Plant Sci. Lett.* 7: 331–336.

Zhang, K., Letham, D.S. and John, P.C.L. (1996) Cytokinin controls the cell cycle at mitosis by stimulating the tyrosine dephosphorylation and activation of p34cdc2-like H1 histone kinase. *Planta* 200: 2–12.

Zhang, T.Q., Lian, H., Zhou, C.M., Xu, L., Jiao, Y. and Wang, J.W. (2017) A two-step model for de novo activation of WUSCHEL during plant shoot regeneration. *Plant Cell* 29: 1073–1087.