

Differential expression and co-localization of transcription factors during the indirect de novo shoot organogenesis in the fern *Ceratopteris richardii*

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Abstract

Background Regeneration can be achieved through various approaches in flowering plants and different sets of transcription factors are involved in those processes. *Ceratopteris richardii* is one of the water ferns and can obtain intact plantlets from the callus, thus providing an opportunity for comprehensively understanding plant regeneration.

Results The callus could be induced from the aerial part and the putative embedded root primordia in emergent leaves within this fern, with formation of shoot pro-meristems and meristems verifying an indirect *de novo* shoot organogenesis. Differentially expressed genes identified by RNA-seq indicated that photosynthesis including both light and dark reaction, response to stimuli, and transmembrane signaling were immediately altered upon transition of the callus from proliferation to differentiation. Moreover, genes concerning hormones and transcription factors were up- or down-regulated, with a suggestion that mechanisms controlling this transition was partly conserved across green plants. Three *STM*-, *ANT*-, and *ESE3*-like transcription factors differentially expressed were all localized at the presumptive vascular-initial-like cells but not in the other callus tissues as indicated by *in situ* hybridization, thus identifying a putative mechanism underlying callus development.

Conclusions This work provided a basis for studying regeneration in ferns and generated a hypothesis related to callus development.

Background

Plants have a striking regeneration capacity and many types of cells including the haploidy pollens and the isolated protoplasts can dedifferentiate or reprogram and then re-differentiate to perform an autonomous life. From a callus which itself is induced from explants by the application of exogenous auxin and cytokinin with a balanced ratio, shoots can be produced under low auxin-to-cytokinin ratio while roots emerge when this ratio is high, and this is called the indirect *de novo* organogenesis [1, 2]. In contrast, the direct organogenesis relies on the parental cells and can be exemplified by the cutting propagation of plants. A whole plant can also be regenerated via direct or indirect somatic embryogenesis which performs like zygotic embryogenesis, whereas there is also tissue repair such as meristem reconstruction after micro-surgical excision and vascular reformation in debarking/grafting [2].

The callus emerges due to a series of cell division of the pericycle cells opposite to the xylem poles within the vasculature of explants [3, 4]. The callus aged about 10 days is organized by the same cell layers as those establishing a lateral root primordium (LRP), for an instance a non-hair epidermal cell marker firstly vanishes away upon callus induction but later recurs to the callus as to roots [3, 5]. A single-cell RNA sequencing of the 6-day hypocotyl-derived callus identifies the layer of epidermis-like cells and lateral-root-cap-like cells, the middle layer of QC-like cells, and the inner layer of vascular-initial-like cells [6]. From one or multiple types of callus cells which should be pluripotent, progenitor cells of shoots will arise,

followed by the formation of the shoot pro-meristem and meristem respectively, and shoot outgrowth in the indirect *de novo* shoot organogenesis (IDNSO) [7, 8].

The *PLETHORA3* (*PLT3*), *PLT5*, and *PLT7* transcription factors (TFs) are upregulated during callus induction, and they can activate either *PLT1*, *PLT2* to produce shoot progenitor cells or *CUPSHAPED COTYLEDON2* (*CUC2*) to complete shoot formation via a two-step mechanism [9]. Both *CUC1* and *CUC2* are ubiquitously distributed in the proliferating callus, but when the callus begins to differentiate, they are gradually restricted to the progenitor cells and finally the pro-meristem located within the low cytokinin response domain, while *WUSCHEL* (*WUS*) is simultaneously expressed in the high cytokinin response domain subtending the pro-meristem [7, 10]. In contrast, *SHOOT MERISTEMLESS* (*STM*) is involved in the formation of shoot meristems [7, 11]. *WUSCHEL RELATED HOMEODOMAIN11* (*WOX11*) and *WOX12* participate in callus formation too, through activating *LATERAL ORGAN BOUNDARIES DOMAIN16* (*LBD16*) that is specifically expressed in the newly formed callus but immediately ceases to express itself when the callus is induced to produce shoots [12–14]. Meanwhile, *LBDs* can form a complex with *BASIC REGION/LEUCINE ZIPPER MOTIF59* (*bZIP59*) and be bound at the promoter by *MYB94/96* [15, 16], and act downstream of *AUXIN RESPONSE FACTOR7* (*ARF7*) and *ARF19* [17]. Thus, *PLTs*, *WOXs*, and *ARFs* have initiated three cascades of gene expression critical to callus development; however, they probably converge on a more general mechanism which has functioned for root development [18].

Ferns are the sister group of seed plants, and the two taxa have diverged from each other for about 380 million years [19]. *Ceratopteris richardii* is a water fern that is frequently employed in research, and it can regenerate itself not only indirectly from the *in vitro* callus but directly from leaves or gametophytes *in planta* [20, 21]. Additionally, the callus is induced from an intact plant rather than an explant, and the employed media are somewhat different. However, details are short on callus development and plant regeneration in *C. richardii*. Here, it was shown that regeneration had occurred through IDNSO in *C. richardii*, and that TFs had been differentially expressed between callus proliferation and differentiation, with an intendency of co-localizing to the presumptive vascular-initial-like cells.

Results

Morphology and histology analysis of the callus in *C. richardii*

The callus displayed an emerald color in the BA medium (Fig. 1a), and it was still in this color after 4-day culturing in the MS medium (Fig. 1b). The callus acquired a green color after it had been maintained in the MS medium for 8 d (Fig. 1c), and it had not further changed in color since then (Fig. 1d). Meanwhile, the callus possessed a glossy surface when maintained in the BA medium and in the MS medium for 4 d but became rough and dark thereafter. The callus always developed rod-like structures accompanied by numerous white trichomes, but it was in the callus after 19-d culturing in the MS medium that lamina leaves were observed (Fig. 1e). Some rosette leaves were outstanding while the other was under-developed or seemingly degenerated in the callus maintained in the MS medium for 26 d (Fig. 1f).

The callus arose from the explant at the back of the assumed fourth leaves, with both the aerial part and the basal locations corresponding to root primordia generating more than one block of callus (Fig. 2a). The callus grew well during subculture in the BA medium and could produce protrusions which might initiate the next generation of the callus; moreover, cells were characteristic of larger nuclei and relatively dense cytoplasm in the outermost layer of the callus, which might constitute the assumed protoderm (Fig. 2b). Protrusions continued to form and the protoderm kept intact in the callus cultured in the MS medium for 4 d, but there were more newly born cells characterizing by small volume and nuclei in the underneath layer of the protoderm and in the interior of the callus (Fig. 2c). A few pro-meristems of shoots occurred to the callus differentiated for 8 d, which was characteristic of an apex based on a high base possibly derived from protrusions (Fig. 2d). Meanwhile, the protoderm disappeared. The dome-shaped shoot meristems shaped after another 4-d differentiation for the callus, with the base being faded away (Fig. 2e). This transition was not synchronically and both pro-meristems and meristems could be observed in the same phase of the callus, and the latter remained after nearly one-month differentiation for the callus where leaf had established (Fig. 2f). Additionally, the callus finally began to deteriorate, producing leaks in its interior. Occasionally, shoot meristems could be observed accompanied by leaf primordia or vice versa (see thereafter).

Functional prediction of differentially expressed genes (DEGs)

Transcriptomes were compared between the proliferating callus that was cultivated in the BA medium and the differentiating callus that had been cultivated in the MS medium for 4 d. For each of the three proliferating callus specimens, there were ~42 million clean reads amounting to ~6 Gb, and the situation was similar for the differentiating callus specimens (Table 1). Most of the reads could be mapped to the genome and more than a half to genes.

There were 375 DEGs, with 126 DEGs being upregulated in the proliferating callus and 249 DEGs in the differentiating callus. 255 DEGs (68%) could be given a GO term, producing 72 enriched GO terms before REVIGO reduction. KEGG terms could be assigned for 171 DEGs, but only ko00196 (photosynthesis - antenna proteins), ko00710 (carbon fixation in photosynthetic organisms), and ko00965 (betalain biosynthesis) were significantly enriched. The enriched terms could share some DEGs, and thus they were clustered into four groups (Fig. 3). Group I contained terms related to photosynthesis light reaction, and within the representative GO: 0019684 (light reaction) all genes were upregulated in the differentiating callus [see Additional file 1]. A subset represented by GO: 0009628 (response to abiotic stimulus) might be different since the involved genes were not related to photosynthesis but encoded putative copper transporters and auxin carriers etc. Group II was dominated by terms related to carbon fixation within photosynthesis, and nearly all ko00710 genes were upregulated in the differentiating callus except for an aspartate aminotransferase gene. Group III appeared participating in transmembrane signaling, and most involved genes were upregulated in the differentiating callus as indicated by GO:0005576 (extracellular region). Group IV was overall a pool of insignificant terms.

Identification of DEGs related to hormones and TFs

There were a handful of DEGs associated with plant hormones, for example three type-A *ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) genes were all upregulated in the proliferating callus (Fig. 4a). An auxin-induced gene was also upregulated in the proliferating callus, while an auxin-responsive gene and two auxin efflux *PIN-FORMED* (*PIN*) genes were upregulated in the differentiating callus. A gibberellin-regulated gene was also expressed more in the differentiating callus.

There were 17 upregulated and 13 downregulated TFs in the differentiating callus relative to the proliferating callus, concerning various families (Table 2). They all had a homolog in *A. thaliana*, and in several cases, they were orthologous to genes playing a role in callus development [see Additional file 2]. For example, *STM* has been found participating in IDNSO and here 04G064800 was orthologous to it. 19G069000 was also orthologous to the important *WOX11* though there were more paralogs in each side. In terms of upregulation or downregulation, 38G014600 and 25G000100 could be considered as equivalent to *LBDs* involved in IDNSO. Moreover, two TFs were verified differentially expressed between the proliferating and the differentiating callus by an independent analysis (student t-test, $p < 0.05$) (Fig. 4b).

RNA *in situ* tissue hybridization of three TFs

04G064800 a *STM*-like gene, produced serpentine distribution of staining signal in the proliferating callus maintained in the BA medium (Fig. 5a). There are vascular-initial-like cells in the callus, and they are possibly organized into the serpentine vascular. Thus, the serpentine staining signal probably came from the vascular-initial-like cells. The same signal distribution occurred in the callus differentiating for 4 d in the MS medium (Fig. 5b). After differentiating for 8 d, the staining became localized but still existed in the presumptive vascular-initial-like cells (Fig. 5c). The situation was the same in the callus that had differentiated for 12 d, with the staining looking serpentine and distant from emergent shoot meristems/leaves (Fig. 5d).

31G036600 an *AINTEGUMENTA* (*ANT*)-like gene, was also expressed in the presumptive vascular-initial-like cells in the proliferating callus (Fig. 5e), and the serpentine distribution of the staining signal was also apparent in the callus differentiating for 4 days (Fig. 5f). The signal distribution became less continuous in more differentiated callus (Fig. 5g); however, there were apparent trails of staining in the callus tissue underneath the regenerated leaves (Fig. 5h).

11G046500 an AP2-EREBP gene with the closest relationship to *ESE3*, was also expressed in the presumptive vascular-initial-like cells in the proliferating callus (Fig. 5i). The three sections were consecutive (Fig. 5a,e,i), and they showed a few common staining signals at the same position, revealing the three genes being expressed at the same tissue for instance the presumptive vascular-initial-like cells. Consistently, 11G046500 displayed a serpentine staining in the callus starting to differentiate (Fig. 5j), and its staining distribution appeared like that of the other two genes in the more differentiated callus (Fig. 5k,l).

Discussion

The callus is emerald when it is proliferating, whereas the color changes into green after it has differentiated for about one week in *C. richardii*. Callus greening also occurs in *A. thaliana* [31], and it is a discrete event there [32]. The callus has a relatively smooth and bright surface during proliferation and early differentiation in *C. richardii*; correspondingly, there are only protrusions possibly for generation new callus and the protoderm keeps intact. Further differentiation may firstly give birth to shoot progenitor cells within protrusions, and then shoot pro-meristems in the shape of an apex appear on an apparent plateau, and finally the dome-like shoot meristems shape, indicating main processes of IDNSO in flowering plants [7, 8]. The callus can be produced on the aerial part and at the basal locations corresponding to root primordia at the same time from the presumptive fourth leaves in *C. richardii* [33], supporting the callus being derived from pericycle cells opposite to xylem poles within vasculature and being constructed as root tips [3–5]. The callus is induced from an intact plant in *C. richardii*, and the callus-inducing medium contains only cytokinin hormone while the medium without any hormones is used for shoot organogenesis [21, 34]. However, it is explants and different combinations of various hormones that are used for IDNSO in flowering plants [35]. So, whether there are taxon-specific factors especially different levels of auxin and/or cytokinin controlling IDNSO is intriguing in *C. richardii* [7].

Both photosynthetic light reaction and dark reaction are significantly different between the proliferating and the differentiating callus in *C. richardii*, and the concerned genes are nearly all upregulated in the latter which indicates an enhancement of photosynthesis. It is obvious that photosynthesis shall be strengthened during IDNSO; however, photosynthesis is promoted very early here since the callus has just been transferred to the MS medium for 4 d and it has not apparently changed in tissue and color. The hallmark change of photosynthesis promotion stands for the alteration of response to stimuli including copper and auxin and that of extracellular region during the transition from callus proliferation to differentiation in *C. richardii*. Moreover, there are hormone-dependent regulatory elements in the promoter of copper transporter genes [36], auxin must change in concentration and distribution from callus proliferation to differentiation for the morphogen role in plant development [37], and extracellular region is found capable of reprogramming cell fate recently [38]. Thus, there are three main functional changes during the callus proliferation to differentiation transition in *C. richardii*.

It is interesting that three type A *ARR* homologs are all upregulated in the proliferating callus in *C. richardii*, and coincidentally that their overexpression possibly represses callus greening and shoot formation in flowering plants [39]. Similarly, two *PIN* are simultaneously upregulated in the differentiating callus, recollecting their critical role in establishing auxin maxima and minima for morphogenesis across green plants [40, 41]. Among differentially expressed TFs, a *STM*-like gene and a *WOX11*-like gene can be validated by highly supported two-to-two orthology and many-to-many orthology, respectively. Two *LBD*-like genes reduce in expression level after the callus has been shifted onto the differentiating medium, which is consistent with the specific expression of *LBD16* in the newly formed callus while immediate reduced expression when the callus begins to differentiate [12]. Thus, it is possible that there is a conserved mechanism for callus proliferation to differentiation transition across plant kingdom.

When explants are incubated by the callus-inducing-medium (CIM), pericycle cells opposite the xylem can produce the LRP-like callus [3]. This type of callus is considered consisting of the outer layer of epidermis- and lateral-root-cap-like cells, the middle layer of QC-like cells, and the inner layer of vascular-initial-like cells [3, 6]. QC-like cells have not concentrated together in the callus but composed a subepidermal layer in *A. thaliana* [5], whereas vascular-initial-like cells possibly have been organized into vasculature, which looks serpentine. Then, it is safe to interpret that 04G064800 is expressed in the vascular-initial-like cells in the proliferating callus (Fig. 5a). As the callus differentiates, its vascular-initial-like cells are gradually exhausted, causing a serpentine but restricted expression for 04G064800 (Fig. 5b–d). It is a surprise that all the three TFs are similarly expressed in the callus e.g., simultaneously at the presumptive vascular-initial-like cells. We propose that vascular-initial-like cells have played an eminent role in the transition from callus proliferation to callus differentiation in IDNSO.

Conclusions

The callus emerges on the back of the putative forth leaf and its root primordia, and an apex of shoot pro-meristem and the dome-like shoot meristem can be distinguished from each other, indicating IDNSO has occurred to the water fern *C. richardii*. RNA-seq has been employed and there are biological replicates for statistic analysis, identifying up- and down-regulated genes and enriched functional terms. The hallmark change of photosynthesis enhancement and conserved function of hormones and PIN proteins in morphogenesis further support the differential gene expression between callus proliferation and differentiation during IDNSO. Thus, the involved TFs must have played a role in this transition, particularly for those orthologous to genes with similar functions. Three different TFs are co-localized to the presumptive vascular-initial-like cells, suggesting a role for the latter in this transition. Despite IDNSO has hardly been studied in non-flowering plants, this type of regeneration can at least occur in ferns. Genes responsible for the early stage of callus proliferation to differentiation transition have been identified on purpose, which is beneficial to understanding maintenance of cell pluripotency at molecular level. The vascular-initial-like cells possibly express multiple different TFs up- or down-regulated during callus proliferation to differentiation transition, which suggest a factor other than the middle layer of QC-like cells important to organogenesis.

Methods

Plant materials

The water fern *Ceratopteris richardii* was employed, and the callus was induced from its aseptically young sporophytes with the application of 5 μ M 6-benzylaminopurine (BA) and 16-hour illumination under 25 $^{\circ}$ C. The sub-cultured callus was similarly maintained or allowed for regeneration by supplying with the Murashige and Skoog (MS) medium without any hormones. The proliferating callus and the callus differentiating for 4, 8, 12, 19, and 26 days were photographed with the Leica M165 C stereo microscope, and they were also observed in 5 μ m sections stained by toluidine blue using the Leica DM3000 transmission light microscope. The proliferating callus and the callus differentiating for 4 days were also

frozen using liquid nitrogen, stored under -80°C , and then shipped in dry ice for transcriptome analysis. There were three biological replicates for each of the two samples, and thus a total of six sequencing libraries were constructed.

Transcriptome sequencing

The total RNA was extracted with a CTAB method [22], and then mRNA was purified using beads covered with polyA oligonucleotides. Ion cation was employed in fragmenting mRNA and the random N6 primer was used in retro-transcription. The double-strand cDNA was added with an adapter on both ends to be amplified. The single-strand DNA was produced by heating denaturation and amplified via circle replication using a splint oligo for generating tandem repeats. The produced DNA “balls” were separately loaded into individual sequencing holes, and two sequencing reactions were set from the 5′ and the 3′ end respectively, using the sequencing-by-synthesis method to produce 150 bp reads. The DNBSEQ sequencing platform ran by BGI (Shenzhen, China). Clean reads were available in the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) with the accession number GSE212819.

Differential expression analysis

Reads were filtered to get rid of DNA contamination and low quality using SOAPnuke v1.4.0 software with the setting of “-l 15 -q 0.2 -n 0.1” [23]. Clean reads were mapped to a draft genome (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/020/310/875/GCA_020310875.1_C.richardii_v2/) using HISAT v2.1.0 with the setting of “-dta -phred64 unstranded -new-summary -x index -1 read_r1 -2 read_r2” [24], which will identify both novel transcripts and novel genes. Clean reads were also mapped to genes using Bowtie2 v2.2.5 with the setting of “-q -phred64 -sensitive -dpad 0 -gbar 99999999 -mp 1,1 -np 1 -score-min L,0,-0.1 -p 16 -k 200” [25], laying a foundation for estimating gene expression level through Fragments Per Kilobase of exon model per Million mapped fragments (FPKM) using the program RSEM. Differential expression was determined by comparing read counts of genes using DEseq2 [26]. Gene function annotation had taken use of Blast search (<http://www.ncbi.nlm.nih.gov>), and databases of Gene Ontology (GO) (<http://www.geneontology.org>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg>). GO terms were enriched using phyper function (<http://www.r-project.org/>) and *P*-values were adjusted using FDR, and REVIGO was used in reducing size of the enriched terms [27]. Finally, a matrix was computed on how much genes were shared among GO and/or KEGG terms, which was fed to hclust function to produce a dendrogram for demonstration.

Construction of phylogenetic trees

Blastp program was used and an empirical *E* value of $1\text{e-}005$ was employed in collecting similar sequences with queries of the investigated fern genes, and CLUSTAL W was used for multi-alignment [28]. FastTree program was used in constructing phylogenetic trees [29]. Trees of selected genes were displayed using iTOL v6 (<https://itol.embl.de/>).

Conduction of RT-qPCR

cDNA was synthesized by employing RNA remained in the construction of transcriptome libraries, with the use of PrimeScript II reverse transcriptase (TAKARA, Beijing, China) and polyT primer. TB Green Advantage qPCR Premix (TAKARA) was used, and a 10 µL reaction system was established using 1.8 µL water, 5 µL 2× Premix, 0.2 µL ROX dye, 2 µL pairs of primers (1 µM for each), and 1 µL template cDNA. PCR procedure consisted of an initial denaturation at 95 °C for 7 min, a subsequent 45 cycles of 95 °C for 5 s, 60 °C 30 s, and finally a dissociation. The instrument was a PikoReal fluorescence RT-qPCR (Thermal Fisher Scientific, Shanghai, China). Threshold Cq (Ct) was subtracted for tested genes in reference to an actin gene, and relative expression was estimated with the $2^{-\Delta Ct}$ method [30]. RT-qPCR used the same three biological replicates of the proliferating and the differentiating callus.

RNA in situ tissue hybridization

Materials were fixed in medium size and free of superfluous tissues with FAA constituted by 50% alcohol, 10% formaldehyde, and 5% acetic acid for about 12 h. Materials were then cleared by xylene after dehydration and subsequently sectioned into 8 µm after wax embedment. The rehydrated sections were firstly digested by 2 µg/mL proteinase K for 30 min under 37 °C, and secondly post-fixed by FAA for 10 min. RNA probes labeled by digoxigenin were denatured and then dropped onto sections in a 100 µL volume for an overnight hybridization under 50 °C and with coverslips. Probe templates were obtained with specific primers circumventing amplification of conserved regions, consisting of 31G03F279 (5'-TGCTTCGCTGTGTTTCAATC-3'), 31G03R939 (5'-CATGTGCGAATTTGTTGGAC-3'), 11G046F496 (5'-TGGCAATCTTCCTCATCCTC-3'), 11G046R1009 (5'-GACAACGTAGCCGGATCAAT-3'), 04G06F60 (5'-CTACGCCGAGGGACAAGTAG-3'), and 04G06R507 (5'-CAGACTGTCCACTGCTGGAA-3'). Templates were firstly cloned into pGEM-T Easy vector (Promega, Madison, USA) and then amplified by the forward primer of genes and a primer located upstream of the SP6 or T7 RNA polymerase promoter in the vector. These PCR products were purified with phenol and chloroform and re-dissolved in the diethyl pyrocarbonate (DEPC) water, from which 7 µL was used in synthesizing probes under a 20 µL reaction system. DNase I treatment, alkaline hydrolysis, and alcohol precipitation had been performed in probe preparation. After hybridization probes were washed away by submerging sections in 0.2× SSC under 55 °C for 1 h for two times. Sections were further washed with TBS containing 1% BSA and 0.3% Triton X-100 for 1 h before antibodies were added onto them for an incubation of 2 h. After antibodies had been stripped by three washings using TBS, the NBT/BCIP solution was employed for a pink or blue staining.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the Gene Expression Omnibus (GEO) repository, [GSE212819].

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

YX and GL conceived the study, performed the experiment, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

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Research involving plants

The authors confirm that all the experimental methods and plants complied with relevant institutional, national, and international guidelines and legislation. The plants have been taken from the lab by using the germinated spores which are also collected in the lab.

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Tables

Table 1 Parameters of transcriptome sequencing

specimen	clean data			genome mapping (%)		gene mapping (%)	
	reads (M)	bases (Gb)	ratio (%)	total	unique	total	unique
BA-1	42.66	6.40	97.36	96.09	73.03	65.68	57.87
BA-2	42.32	6.35	96.58	92.20	67.54	64.54	57.31
BA-3	42.64	6.40	97.32	96.19	73.63	66.01	57.65
MS-1	42.47	6.37	96.92	93.23	69.05	65.25	58.01
MS-2	42.62	6.39	97.26	90.01	64.17	64.93	57.37
MS-3	42.42	6.36	96.79	95.98	72.91	65.34	57.54

Table 2 TFs among DEGs

name ^a	MS ^b	6BA ^c	log2FC	q ^d	family	<i>Arabidopsis</i> homologs
03G102500	19.36	3.71	-2.04	0.00	bHLH	<i>NAI1</i>
07G094900	23.38	7.16	-1.45	0.00	MADS	<i>AGL15/AGL18</i>
25G010400	9.25	3.15	-1.36	0.03	bHLH	<i>MYC4</i>
04G070700	37.58	15.61	-1.23	0.00	C2C2-GATA	<i>HAN</i>
31G036600	33.32	13.99	-1.22	0.00	AP2-EREBP	<i>ANT/AIL1</i>
35G000900	24.24	9.80	-1.21	0.01	zf-HD	<i>HB23</i>
23G046300	18.16	7.31	-1.19	0.04	bZIP	<i>TGA2/PAN</i>
15G016900	27.26	12.03	-1.09	0.00	bHLH	<i>NAI1</i>
03G003600	31.81	17.41	-0.84	0.01	TUB	<i>TLP8</i>
01G010300	20.10	11.34	-0.82	0.00	C2H2	<i>SGR5</i>
11G073300	39.76	23.75	-0.75	0.00	SBP	<i>SPL8</i>
11G046500	36.96	22.21	-0.70	0.04	AP2-EREBP	<i>ESE3</i>
04G064800	97.91	62.23	-0.64	0.02	HB	<i>STM/KNAT1</i>
06G073900	52.07	33.88	-0.62	0.01	bHLH	<i>TM05/ABS5</i>
17G083000	115.74	74.66	-0.62	0.01	HD-ZIP	<i>CAN/ATHB8</i>
24G079900	24.86	16.53	-0.56	0.04	LIM	<i>DAR1-7</i>
17G002100	84.24	58.15	-0.53	0.04	C3H	<i>SOM</i>
19G069000	13.20	20.93	0.65	0.00	WOX	<i>WOX12</i>
38G014600	60.53	99.69	0.66	0.02	LOB	<i>LBD25</i>
11G039200	21.06	34.20	0.69	0.00	AP2-EREBP	<i>AT1G50680</i>
18G086200	16.09	26.46	0.70	0.02	bHLH	<i>RHD6</i>
24G046500	18.17	30.60	0.75	0.03	bHLH	<i>AIB</i>
15G043400	30.38	51.78	0.76	0.04	AP2-EREBP	<i>RAP2.3</i>
01G011000	28.23	53.69	0.90	0.00	Alfin-like	<i>AL2</i>
03G038600	13.32	27.40	0.99	0.04	WRKY	<i>WRKY3</i>
08G068500	42.89	89.73	1.00	0.02	WRKY	<i>WRKY71</i>
04G000400	14.90	33.99	1.12	0.03	HD-ZIP	<i>HAT22</i>
25G000100	4.41	10.74	1.20	0.02	LOB	<i>SCP</i>

08G068100	3.30	8.13	1.24	0.01	bHLH	<i>NAI1</i>
24G032800	1.69	5.57	1.51	0.03	WRKY	<i>WRKY40</i>

^a*Ceratopteris* genes, ^bthe average FPKM of the MS samples, ^cthe average FPKM of the BA samples, ^dadjusted *p*-value

Figures

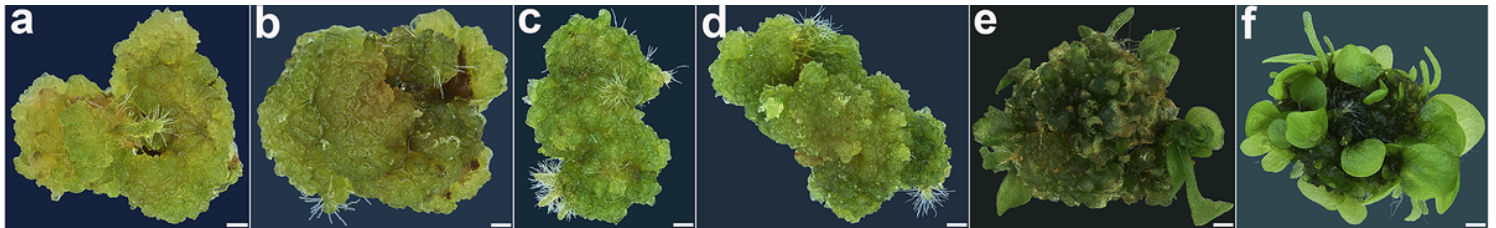


Figure 1

Morphology of the callus at different stages. (a) In the BA medium. (b) In the MS medium for 4 d. (c) In the MS medium for 8 d. (d) In the MS medium for 12 d. (e) In the MS medium for 19 d. (f) In the MS medium for 26 d. Bar = 1 mm.

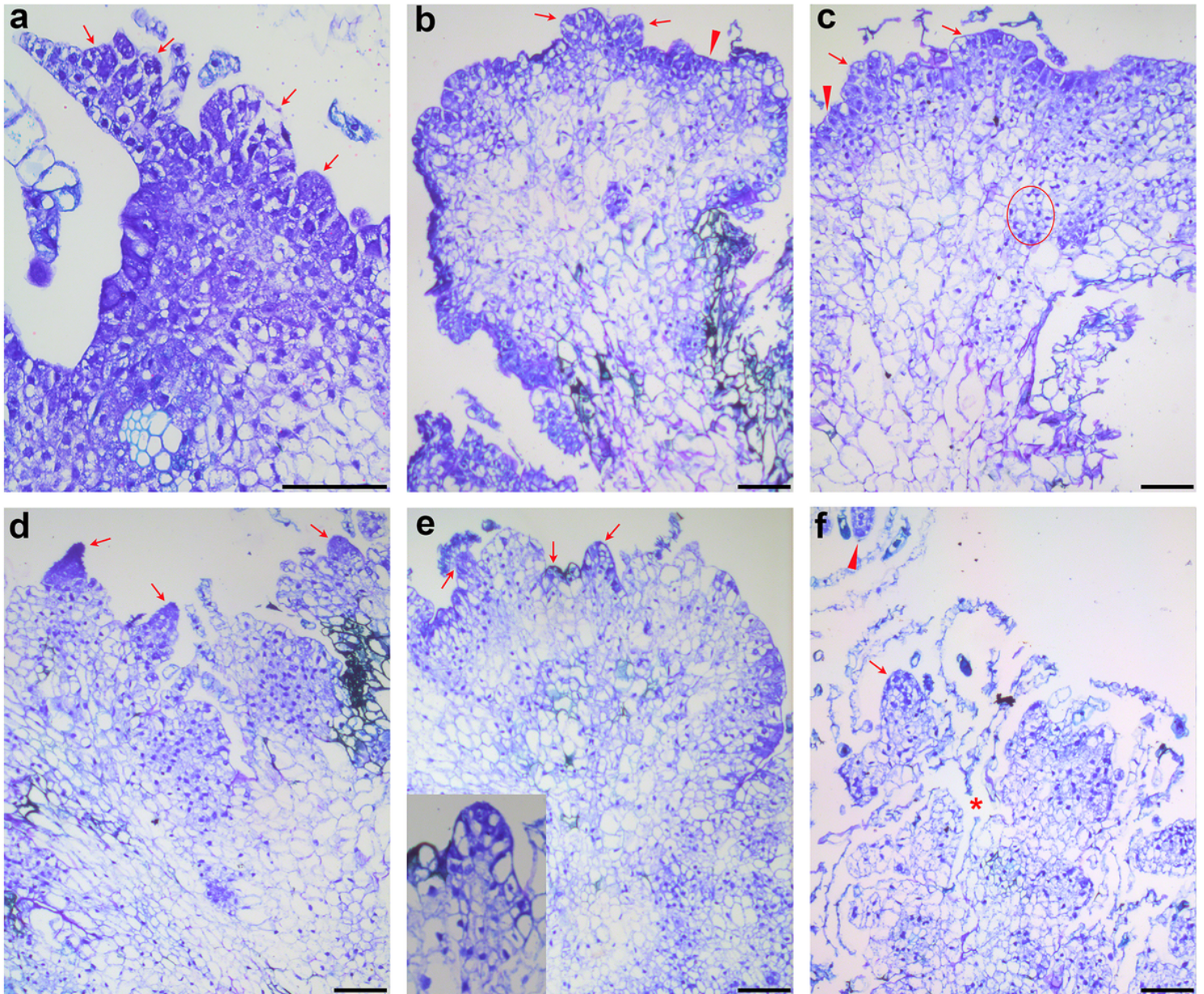


Figure 2

Histology of the callus at different stages. (a) In the explant. Arrows indicated individual callus on the aerial part and in the embedded roots of a young leaf. (b) In the BA medium. Arrows indicated protrusions and arrowheads indicated protoderm. (c) In the MS medium for 4 d. Arrows indicated protrusions and arrowheads indicated protoderm, and the circle indicated dynamic cell divisions. (d) In the MS medium for 8 d. Arrows indicated shoot pro-meristems. (e) In the MS medium for 12 d. Arrows indicated shoot meristems, and the insert showed the dome-shape of shoot meristems. (f) In the MS medium for 26 d. The arrow indicated a shoot meristem, the arrowhead indicated one leaf, and the asteroid indicated the deteriorated leaks. Bar = 100 μm .

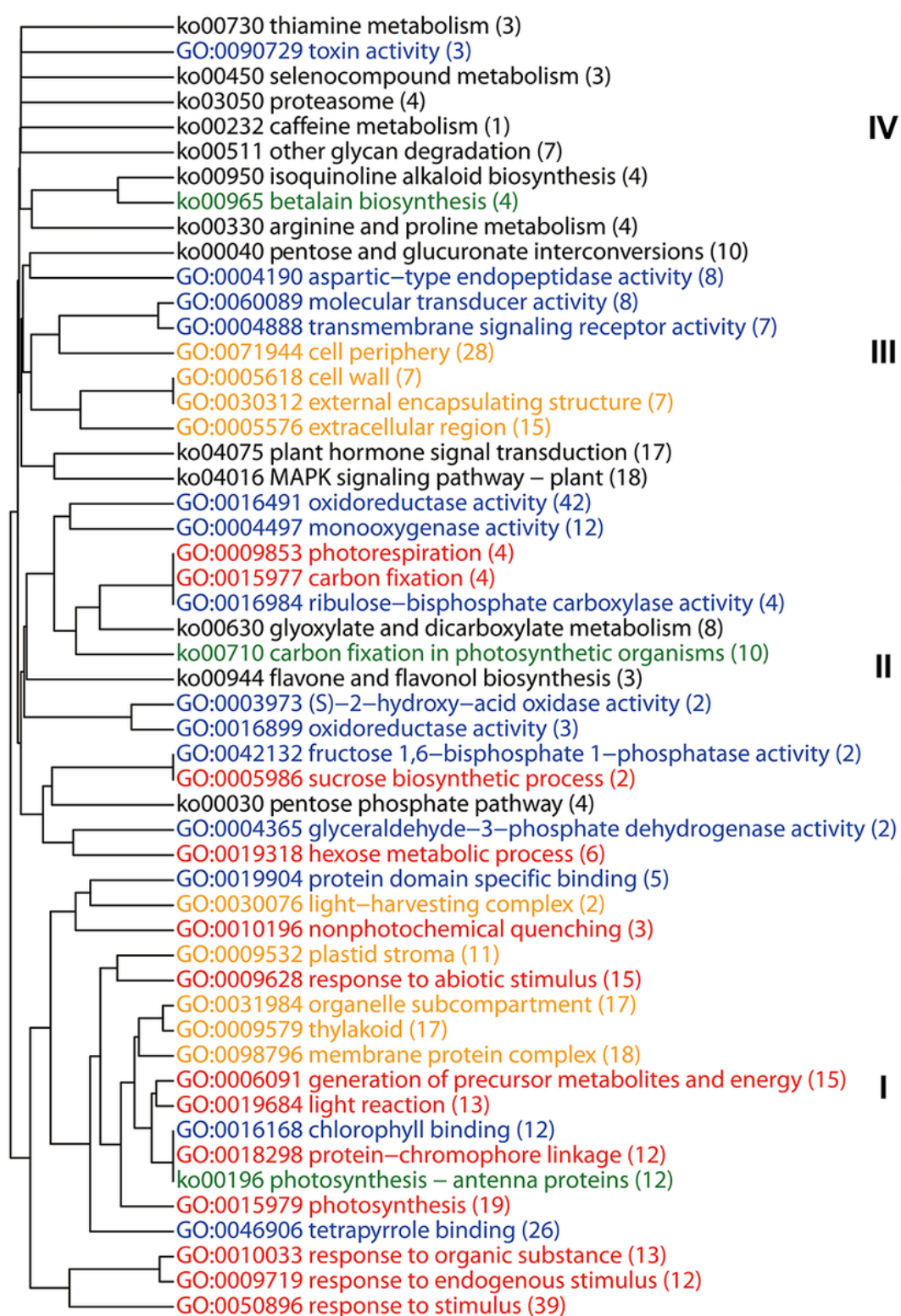


Figure 3

Clustering of enriched GO and KEGG terms. Four groups were indicated by black vertical bars and letters. Numbers of genes in terms were shown in brackets. Terms were classified by colors, namely biological process terms in red, cellular component terms in orange, molecular function terms in blue, and significant KEGG terms in green and insignificant in black.

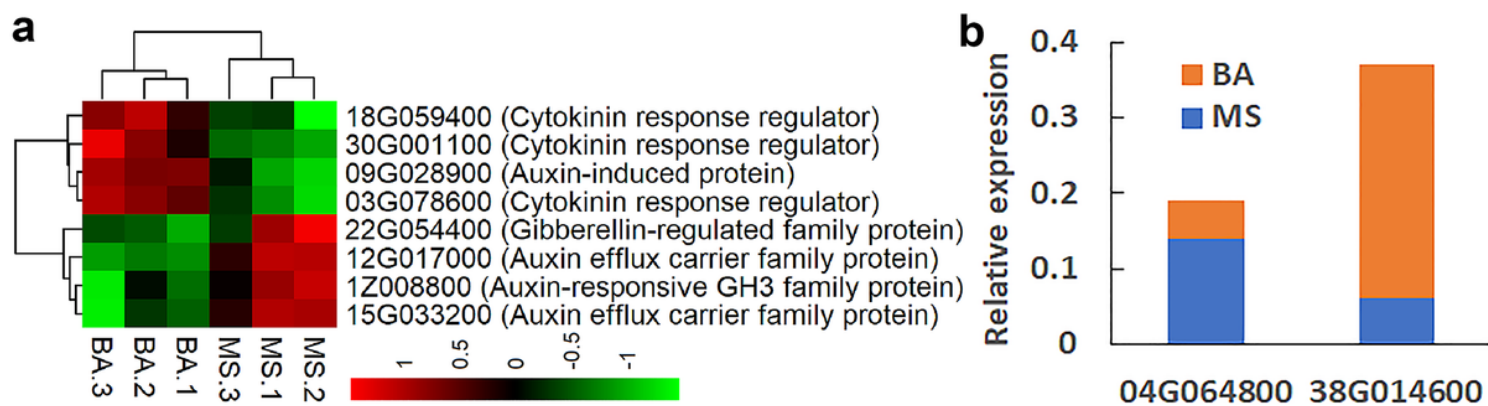


Figure 4

Representation of gene expression level. (a) Expression level of genes concerning hormones. Genes were described referring to *Arabidopsis* homologs (in brackets). (b) RT-qPCR of two TFs.

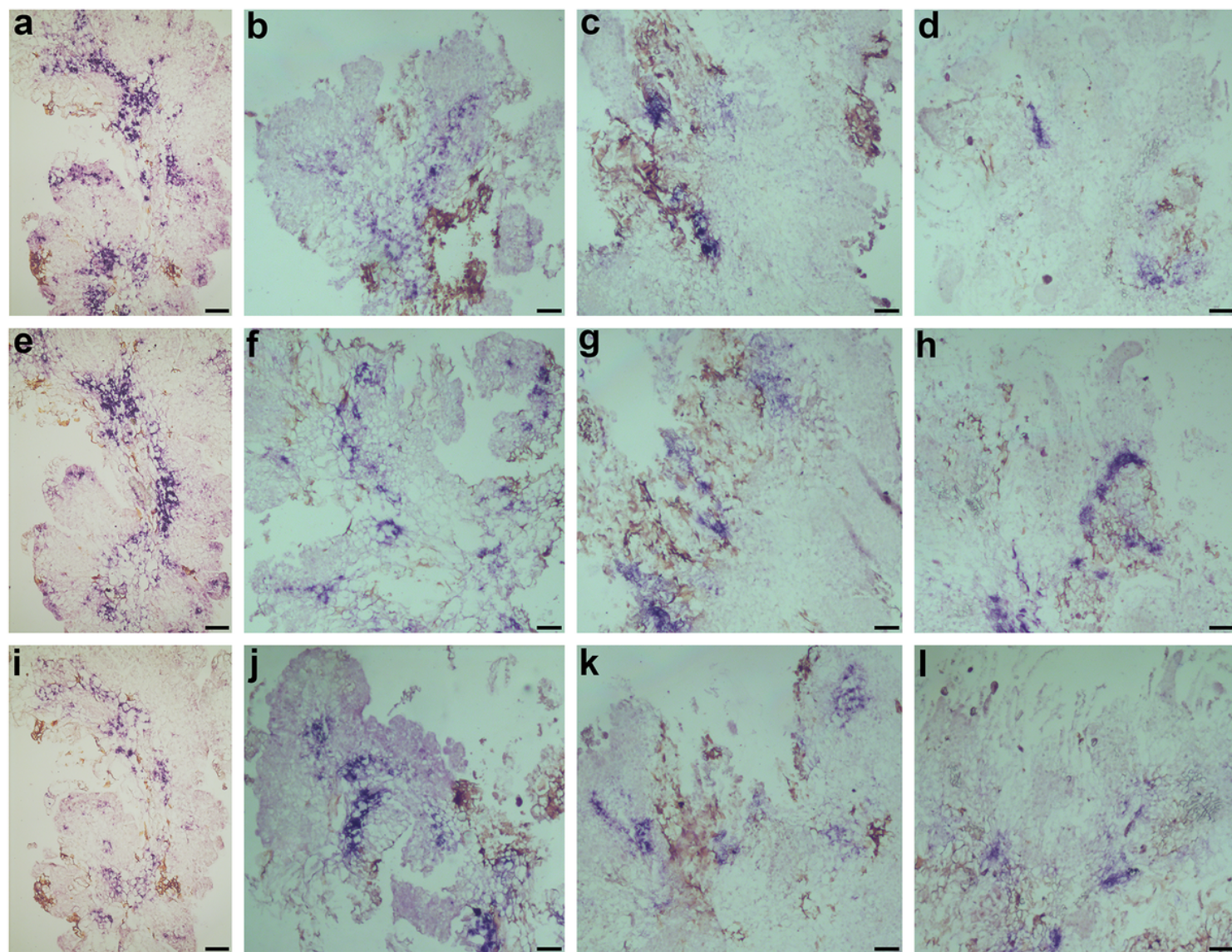


Figure 5

In situ hybridization. Expression pattern of 04G064800 (a–d). (a) The proliferating callus. (b) The callus differentiating for 4 days. (c) The callus differentiating for 8 days. (d) The callus differentiating for 12 days. Expression pattern of 31G036600 (e–h). Expression pattern of 11G046500 (i–l). Bar = 100 μ m.

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