

The Framework of Plant Regeneration In Duckweed (*Lemna Turonifera*) Comprises Genetic Transcript Regulation And Cyclohexane Release

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Abstract

Regeneration is essential for vegetative propagation of excellent variety, detoxification, and obtaining transgenic plant. However, plant regeneration is time-consuming. We found that duckweed regeneration could be enhanced by regenerating callus. The molecular and VOCs releasing mechanisms underlying that have been studied here. Firstly, Genetic transcript regulation has been applied to study the molecular mechanism controlling regeneration. Auxin-related genes have been significantly down-regulated in regenerating callus. Cytokinin signal pathway genes have been up-regulated in regenerating callus. Secondly, VOCs release has been analyzed by GC/MS during the stage of plant regeneration, and 11 kinds of unique VOCs in the regenerating callus were increased. Among them, cyclohexane treatment enhanced duckweed regeneration by initiating root. Moreover, Auxin signal pathway genes were down-regulated in callus treated by cyclohexane. Altogether, these results provide novel mechanistic insights into how regenerating callus promotes duckweed regeneration.

Introduction

Regeneration of entire plants from a callus *in vitro* depends on pluripotent cell mass, which provides rise to a new organ or even a whole plant (Ikeuchi *et al.*, 2016; Attila, 2019). Regeneration was widely used for vegetative propagation of excellent variety, detoxification, and the obtain of transgenic crops (Lardon *et al.*, 2020; Motte *et al.*, 2014). Many studies have focused on the molecular framework of *de novo* organ formation in *Arabidopsis thaliana*. The molecular factors of cellular pluripotency during the regeneration of plants have been investigated thoroughly. However, the regulatory modules in monocot plants were little in-depth study. With the advantages of fast reproduction, high protein content (Li *et al.*, 2004), and distinguished tolerance for a variety of toxic substances (Yao *et al.*, 2020; Yang *et al.*, 2020), Duckweed has been applied as a monocotyledons model plant for gene-expression systems. And stable transformation mediated by *Agrobacterium* depends on efficient callus regeneration protocols.

Genome and transcriptome sequencing leads to a deeper understanding of the molecular mechanism and regulatory network of duckweed. These results were of great significance to plant evolution and adaptation to the environment (Dong *et al.*, 2019). What's more, there is no study focus on the transcriptome analysis during the regeneration in duckweed. In former studies, it has been reported that the growth and development of callus were mediated by many plant hormones (Li *et al.*, 2004). The balance of Auxin and cytokinin is the basis for *Vitro* tissue culture (Shim *et al.*, 2020). Explants can be incubated to callus on auxin-rich callus-inducing medium (CIM). And on cytokinin-rich shoot-inducing medium (SIM), the vigorous callus can be induced to *novo* shoots. It is emergent to study the mechanism of duckweed regeneration via dynamic hormonal and transcriptional changes.

Jasmonates (JAs) serves as a wound signal during *de novo* root regeneration, which triggers plant regeneration (Zhang *et al.*, 2019). Moreover, JA and Methyl Jasmonate (MeJA) function as defences in nature, for example, the induced "alarm" calls of plants (Turlings *et al.*, 1990), which is allelopathy. During allelopathy, there are several volatile organic compounds (VOCs) released from the plant. For example, the allelopathic effects of VOCs of *Artemisia frigida* Willd. On the seed, germination of pasture grasses has been reported (Zhang *et al.*, 2012). Does allelopathy play a role during plant regeneration? Interestingly, we found the plant regeneration could be promoted by regeneration callus. Why? The global insight on the signal and VOCs released from regenerating callus needs to be investigated.

Here, the main objectives have been studied: (i) the molecular mechanism controlling regeneration by comprehensive transcriptomic comparison between callus and regenerating callus; (ii) which VOCs have been increased during the stage of plant regeneration; (iii) the allelopathic effects of VOCs on the inducement of callus regeneration; (iv) the transcriptome analysis on the regenerating callus which VOCs have promoted.

Materials And Methods

Plant material and *in vitro* establishment and cyclohexane treatment

Lemna turionifera used in the experiment were collected from a lake in Tianjin, China. Duckweed was cultured in the liquid medium described as Wang *et al.* and Yang *et al.* (Wang *et al.*, 1994; Yang *et al.*, 2013). The duckweed was cultured aseptically in the liquid medium. Fully expanded fronds were selected as explant for callus induction. The rhizoid was removed, and the frond was scratched for callus induction. The induction medium was B5 solid medium, which Gamborg designed for soybeans tissue culture in 1968 (Gamborg *et al.*, 1968). The induction medium contained plant hormones 15 mg/1 dicamba, 3.5 mg/1 2, 4-D, 6-BA 2mg/1 and 1.5% sucrose. The pH of the medium was adjusted to 6.2–6.4, and then it was sterilized at 121°C for 20 minutes. The tissue was cultured in an incubator with a light cycle of 23 ± 2 °C, 16 hours of light and 8 hours of darkness. After 4–5 weeks of induction, the duckweed explants developed into callus through dedifferentiation.

After 2–3 weeks of induction, calli formed. The calli were transferred to the subculture medium. Subculture medium contains B5 medium, 10 mg/L 4-chlorophenoxyacetic acid (CPA) and 2 mg/L Zip. In order to keep the callus with better morphology and activity, a new subculture medium was replaced every two weeks. Callus was transferred to the regeneration medium for duckweed regeneration. The regeneration medium contains B5 medium, 1 mM serine, and 1.5% sucrose. After 2 or 3 weeks, the callus redifferentiated and regenerated.

When three days of culture in the B5 subculture medium, the calli were cultured in B5 medium with 20 ml cyclohexane in a sizeable airtight beaker. Each day open the sealing device regularly to change the air in the beaker. And replace with a new cyclohexane every two days.

The Co-culture of regenerating callus and callus

The callus was cultured on a subculture medium for more than two weeks for subsequent experiments. Callus and regenerating callus in the same growth condition were placed in B5 medium (containing 1.5% sucrose), respectively. For fumigate, the regenerating callus and callus were put together in a closed environment for co-culture described as Fig. 1a.

VOCs Collection and analysis

Shown as Fig. 1b, the VOCs released from callus and regenerating duckweed were collected using the dynamic headspace air-circulation method described by Zuo *et al.* (Zuo *et al.*, 2018). There were three conical flasks of callus or regenerating callus for each group. The chemical composition analysis of VOCs was performed using a thermal-desorption system/ gas chromatography/ mass spectrum (TDS/GC/MS). And the GC/MS data were studied in NIST/ EPA/ NIH Mass Spectral Library (NIST 08) (National Institute of Standards and Technology, MD, USA).

RNA isolation, quantification, and sequencing

RNA degradation and contamination on 1% agarose gel were detected, and the quality of the samples was qualified. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was then assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Sequencing data filtering and transcript assembly

Image data from sequencing fragments measured by high-throughput sequencers are transformed into sequence data (reads) by CASAVA base recognition. The raw data obtained from sequencing included a small number of reads with sequencing adaptors or low sequencing quality. Our previous study's filtering contents were followed: Removed adapters; Removed reads whose proportion of N is greater than 10%; Remove low-quality reads (Yao *et al.*, 2020). The clean reads were assembled by the Trinity de novo assembly program with `min_kmer_cov` set to 2 by default. Otherwise, it was set to default (Grabherr *et al.*, 2011). Overall, a reference sequence with an average length of 1928 bp and a total length of 282527137 bp were obtained for subsequent analysis.

Data analysis

The experiment was repeated for at least triplicate independent experiments. Analysis of variance (ANOVA) method and SPSS software (IBM SPSS Statistics, Version 20) were applied to compare the statistical significances. Significant difference in experiment was indicated by asterisks (* $P < 0.05$, ** $P < 0.01$). And standard deviations were shown by the error bar. The graphs in these studies were made using Origin 9.0 (Origin Lab, USA).

Data availability

All data included in this study are available upon request by contact with the corresponding author.

Results

Promoted effect of regenerating tissue

Fronde regeneration of duckweed has been promoted when co-cultured with regenerating callus (Co). Frond formed in 14 d with Co-treatment, and duckweed regenerated at 21 days with Co-treatment (Fig. 2a). In the Co group, significant enhancement was found in the percentage of callus regeneration (77.3 %). Compared with that, the callus regeneration percentage without co-culture was 53.6% (Fig. 2b). Thus, callus regeneration has been significantly increased by Co-treatment.

Transcriptome analysis identifies Genes and Genomes (KEGG) and differentially expressed genes (DEGs) in regenerating callus

To compare the enriched pathways between regenerating callus (RG) and callus (CL), KEGG pathway analysis has been conducted (Fig. 3). The top 20 KEGG pathways with the highest representation of DEGs have been analyzed. We selected the 20 pathway items that were most significant in the enrichment process to be shown in this diagram. As shown in Fig. 3a, the "Photosynthesis antenna proteins" were the most significantly enhanced pathway in the top 20 up-regulated KEGG pathways with the highest Rich Factors of RG vs CL. This indicated that the expression of antenna protein increased after the callus developed into regenerated tissue. Antenna proteins were essential for photochemical plant reactions and could mediate the core of plant photosynthesis. The most significantly down-regulated pathway was the "Ribosome", "Pyrimidine metabolism", "Mismatch repair", "Homologous recombination", "DNA replication" and "Base excision repair", which were among the top list of enriched pathways (Fig. 3b), these were all related to the replication of DNA.

In order to understand the difference of DEGs in the regenerating callus, gene ontology enrichment analysis was conducted in RG vs CL. As shown in Fig. 3c, "cell", "cell part", and "intracellular" were in biological process with the most up-regulated and down-regulated DEGs. These were followed by "macromolecular complex" and "organelle" in the biological process category with the most up-regulated and down-regulated DEGs. "DNA integration", "pollination", and "cell recognition" were up-regulated DEGs, without down-regulated (Fig. 3).

Expression changes of genes related to Auxin and root development in regenerating callus

Novogene conducted the mRNA expression in order to study the gene that participated during callus regeneration. The course of the auxin signal pathway and related response factors have been described as Fig. 4. Transport inhibitor response 1 (TIR1) and stem cell factor (SCF), initiating subsequent signal transduction by binding of Auxin, have been down-regulated in the regenerating callus. As a transcriptional activator, the auxin response factor (ARF) could regulate auxin reaction by binding with auxin-responsive protein IAA (AUX/IAA). In this study, AUX/IAA and ARF have been down-regulated significantly, by 13.0309 and 3.0056 \log^2 Fold Change, respectively. Auxin early response factor could be divided into three categories, which were AUX/IAA, Gretchen Hagen 3 (GH3) and small auxin-up RNA (SAUR). GH3 and SAUR have been down-regulated during regeneration, as well. ETHYLENE-RESPONSIVE FACTOR3 (ERF3) and WUSCHEL-RELATED HOMEBOX 11 (WOX11), playing a role in the initiation and regulation of adventitious roots (ARs), were both down-regulated. Also, lateral roots (LRs) and root hairs (RHs) relied on zinc finger protein (ZFP) and cytochrome P450 (CYP2). The expression of ZFP was decreased by 4.0368 \log^2 Fold Change.

Expression changes of genes related to cytokinins signal pathway in regenerating callus

To obtain candidates regulating regeneration, we studied the regulation of the cytokinins signal pathway. Shown as in Fig. 5, cytokinin receptor 1 (CRE1) and cytokinin independent 1 (CKI1), as cytokinin receptors (Hwang *et al.*, 2001; Zheng *et al.*, 2003), have been up-regulated in regenerating callus. Histidine phosphate transfer protein (AHP), interacting with CRE1 and CKI1, has been up-regulated by 2.9662 log² Fold Change. Type-A ARABIDOPSIS RESPONSE REGULATORS (A-ARR) plays a role as a negative feedback regulator, which inhibit type-B activity ARABIDOPSIS RESPONSE REGULATORS (B-ARR) and form a negative feedback cycle (Liu *et al.*, 2012; Hwang *et al.*, 2012). A-ARR has been down-regulated by 4.5266 log² Fold Change. It might be lead to overall up-regulated cytokinins during the callus regenerating.

Changes of VOCs during callus regeneration

The VOCs of regenerating callus has been investigated. And the qualitative and quantitative analyses of the GC/MS data were obtained from NIST/EPA/NIH Mass Spectral Library, showed as Fig. 6. Compared to the callus, 11 kinds of unique VOCs in the regenerating callus were enhanced (Table 1). The peak area of 1, 3-dimethyl benzene in the regenerating callus was 0.84*10⁷, 3.23 times than that in the callus. And the emission of 1, 3-dimethyl benzene increased the most in the regenerating callus. Besides, the content of 4-methyl-2-pentanol and cyclohexane also have been improved. Compared with the callus' cyclohexane peak area (0.85*10⁷), the regenerating callus' cyclohexane peak area was 1.28*10⁷, 4.3*10⁶ higher than that of callus. And the peak area of 4-methyl-2-pentanol was 2.1*10⁷, 2.33 times that of callus.

Callus regeneration promoted by cyclohexane

In order to explore the effect of VOCs in callus regeneration, 1, 3-dimethyl benzene, 4-methyl-2-pentanol and cyclohexane were added to the medium of callus. As Fig. 7 showed, cyclohexane promoted the regeneration of the callus significantly. After 16 days of cyclohexane treatment, roots formed from the callus. However, 1, 3-dimethyl benzene and 4-methyl-2-pentanol groups have no apparent phenomenon of regeneration. And the callus formed compact white callus with the treatment of 1, 3-dimethyl benzene.

Transcriptome analysis identifies KEGGs and DEGs in callus treated by cyclohexane

Transcriptome analysis has been analyzed to investigate the potential functions of KEGGs and DEGs in the callus treat hydrolyzing O-glycosyl compound by cyclohexane. As shown in Fig. 8a, "RNA transport" and "glycolysis/gluconeogenesis, and galactose metabolism" were in the biological process with the most down-regulated KEGGs. "Ribosome" was the top-enriched pathway (Richfactor > 0.55). It was followed by "photosynthesis" and "oxidative phosphorylation" (Fig. 8b).

In order to understand the difference of DEGs in callus treated with cyclohexane, gene ontology enrichment analysis was conducted in callus treated by cyclohexane vs callus. As shown in Fig. 8c, "DNA integration", "ribonucleoprotein complex", and "structural molecule activity" were in biological process with the most up-regulated DEGs. These were followed by "ribosome biogenesis", "ribonucleoprotein complex", and "ribosome" in the category of the biological process with the most up-regulated DEGs. "ribonucleoprotein complex", and "structural molecule activity" were in biological process with the most down-regulated DEGs. (Fig. 8c).

Comparison of the expression of genes related to the hormone in callus treated with cyclohexane and in the regenerating callus

In order to know molecular factors underlying the participation of hormone in callus regeneration, we first checked gene expression related to the auxin signal pathway (Table 2). AUX/IAA and GH3 have been downregulated in both calli treated with cyclohexane and in the regenerating callus. A majority of SAUR have been down-regulated during regeneration and treated with cyclohexane (Fig. 9a). ERF3, cysteine-rich receptor and Zinc finger has been down-regulated as well.

Secondly, we studied the expression of genes related to CTK signal (Fig. 9b). The gene regulation in regeneration and treated with cyclohexane is different. The CRE1 has been up-regulated in the regenerating callus, which has been down-regulated in callus treated with cyclohexane (Table 3).

Thirdly, the expression of genes related to brassinosteroid signal has been investigated. In the brassinosteroid signal pathway, the expression of brassinazole-resistant1/2 (BZR1/2) has been down-regulated in callus treated with cyclohexane and the regenerating callus (Table 4). In the brassinosteroid signal pathway, the expression of BZR1/2 has been down-regulated in callus treated with cyclohexane and the regenerating callus.

Moreover, the expression of genes related to ethylene signal has been investigated (Fig. 9c). The expression of ETR and EBF1/2 has been up-regulated in callus treated with cyclohexane and the regenerating callus. Transcription factor MYC2 (MYC2), which plays a role in the jasmonic acid signal pathway, has been up-regulated in both cyclohexane treatment and regenerating callus (Fig. 9d). There is no significant difference in the gibberellin signal pathway during cyclohexane treatment (Fig. 9e).

Discussion

In line with previous studies, we established an effective way *in vivo* callus regeneration in duckweed. Interestingly, we found that one regenerating callus promoted another callus to regenerate. Genomes and transcriptome sequencing (especially plant hormones) and volatile substances were studied to reveal plant regeneration's molecule framework in duckweed.

Plant hormones played a crucial role during callus regeneration¹. Here, we compared the transcriptome of regenerating callus and callus to investigate the molecular mechanism of phytohormone (especially Auxin and cytokinins). Callus was induced by Auxin, similar to lateral root primordium (Atta *et al.*, 2009; Hirota *et al.*, 2007; Sugimoto *et al.*, 2010). In Arabidopsis, the callus tissue formed root stem cell niche by regulation the expression of root stem cell regulators, including WOX (Liu *et al.*, 2014; Akie *et al.*, 2018; Haecker *et al.*, 2004; Shimotohno *et al.*, 2018). According to our results, ARF, AUX/IAA, GH3, ARF1, SAUG and other response factor have been down-regulated significantly during the callus redifferentiation (Fig. 4). The interaction between ARF and AUX /IAA could

regulate Auxin early response's genes expression in the auxin signalling pathway. Moreover, ERF3, WOX11 and ZFP were related to the ARs, LRs and RHs of initiation in *Spirodela* (Dong *et al.*, 2019), which might lead associated with the regeneration in duckweed.

Cytokinins and Auxin have synergistic or antagonistic interactions with each other (SKOOG *et al.*, 1957). As a phytohormone, cytokinin could control critical aspects of environmental responses, such as biotic and abiotic stress responses, and regulate various developmental processes, including cell proliferation, leaf formation, and root formation growth (Karunadasa *et al.*, 2020; Romanov *et al.*, 2018). Cytokinins promoted plant regeneration by controlling the generation of somatic embryogenesis in *Fumariaceae* and Rice (Sagare *et al.*, 2001; Ram *et al.*, 1984). In this study, cytokinin receptor CRE1, CKI1, and transfer protein of histidine phosphate AHP were up-regulated, during the expression of negative feedback regulator A-ARR was down-regulated in callus regeneration (Fig. 5). And the expression of cytokinins synthesis was up-regulated, thereby promoting the differentiation of shoots. The transcriptome analysis suggested a similar result with Arabidopsis, giving evidence that Auxin and cytokinins' regulation leads to regeneration. Besides, plant regeneration has been regulated by other hormones (Ikeuchi *et al.*, 2017). Our products found that gibberellin, jasmonic acid, increased significantly, while genes related to gibberellin and brassinolide were down-regulated during callus regenerating (Fig. 9).

Plants release VOCs to the environment to affect their own or other biological life processes in plants growth and development. This phenomenon was called allelopathy (Shi *et al.*, 2020). Plants in different growing environments, such as biological stress or abiotic stress, might release other VOCs to improve their resistance to external interference (Raghava *et al.*, 2009; Loreto *et al.*, 2006; Jud *et al.*, 2016). In previous studies, VOCs have been shown to mediate cell to cell communication, thereby leading to stress responses in plants (Zuo *et al.*, 2012). In our study, 11 kinds of specific VOCs have been increased during callus regenerating. Among them, cyclohexane could significantly promote the regeneration of callus in 16 days (Fig. 7).

Here, the regulation of gene expression related to the hormone in callus treated with cyclohexane, which promoted regeneration, suggested the role of Auxin during regeneration. AUX/IAA and GH3 have been downregulated in both calli treated with cyclohexane, similar to that in the regenerating callus (Fig. 9). And adventitious root initiation and elongation has been promoted by AUX/IAA (Dong *et al.*, 2019). Interestingly, the root formation has been enhanced significantly by cyclohexane treatment (Fig. 7).

Altogether, we propose a hypothesis of how callus regenerates in duckweed. Based on the DEGs in regenerating callus, we proposed molecular regulation on plant hormone. Also, our study provides candidates for evaluating the involvement of VOCs during duckweed regeneration, especially the enhancement of regeneration by cyclohexane. It also provides a resource for comparative transcriptome analysis of plant regeneration in other species.

It was indicated that VOCs might play a crucial role in the process of plant regeneration. It also makes clear that allelopathy does affect plant growth and development.

Declarations

Declaration of interests

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables

Table 1 The main components of VOCs from regenerating callus and callus				
Designation	Chemical formula	RG Peak area $\times 10^7$	CL Peak area $\times 10^7$	Acquisition time (min)
Cyclohexane	C ₆ H ₁₂	1.28	0.85	3.06
9,12, 15-octadecarboxylic acid methyl ester	C ₂₈ H ₄₀ O ₄	0.44	0.4	3.32
10,13-octadecadiynoic acid methyl ester	C ₁₉ H ₃₀ O ₂	3.49	3.3	3.38
4-methyl-2-pentanol	C ₆ H ₁₄ O	2.1	0.9	3.81
1, 3-dimethyl benzene	C ₈ H ₁₀	0.84	0.26	5.83
1,1'-oxybis-decane	C ₂₀ H ₄₂ O	0.95	0.48	15.82
Diisobutyl phthalate	C ₂₆ H ₄₄ O ₅	1.88	1.75	17.17
Nonadecane	C ₁₉ H ₄₀	0.8	0.64	19.15
3-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-propenal	C ₁₂ H ₁₈ O	1.28	0.9	24.13
9,10-dihydro-11,12-diacetyl-9,10-ethanoanthracene	C ₂₀ H ₁₈ O ₂	2.75	1.8	31.81
Butyl 8-methylnonyl ester 1,2-benzenedicarboxylic acid	C ₂₂ H ₃₄ O ₄	1.21	0.79	34.2

Table 2 Gene expression in plant regeneration of Auxin								
Description	Gene-id	Regenerating callus vs Callus_Read_count	Cyclohexane vs Callus_Read_count	Callus_Read_count	Regenerating callus vs Callus_log2Fold Change	Cyclohexane vs Callus_log2Fold Change	pval	pac
auxin-responsive protein IAA	Cluster-6172.2761	25.79057461	/	291.8684987	-3.499	/	1.53E-20	7.2
auxin-responsive protein IAA	Cluster-6172.9506	1350.903101	/	7436.536506	-2.4616	/	8.50E-33	1.3
auxin-responsive protein IAA	Cluster-6172.9484	3097.048347	/	8093.560243	-1.3863	/	9.12E-09	8.9
auxin-responsive protein IAA	Cluster-6172.6741	115.191085	/	752.9653036	-2.7163	/	4.06E-30	5.0
auxin-responsive protein IAA	Cluster-6172.4574	329.7352314	/	2581.00597	-2.9677	/	5.94E-28	5.7
auxin-responsive protein IAA	Cluster-7966.13997	/	126.898086	427.8033881	/	-1.7564	1.96E-13	1.4
auxin-responsive protein IAA	Cluster-7966.10326	/	2912.318825	6803.456966	/	-1.2242	1.54E-20	1.9
auxin-responsive protein IAA	Cluster-7966.9984	/	757.4281355	2282.819782	/	-1.5911	5.19E-39	2.1
auxin-responsive protein IAA	Cluster-7966.7990	/	882.1458283	7136.36841	/	-3.0168	1.35E-109	9.4
auxin-responsive protein IAA	Cluster-7966.3823	/	24.87192746	135.7070458	/	-2.4536	3.10E-16	2.8
auxin-responsive protein IAA	Cluster-7966.9412	/	68.99029552	688.2924139	/	-3.3241	1.37E-77	3.2
auxin-responsive protein IAA	Cluster-7966.8499	/	2134.915379	8945.722998	/	-2.067	4.70E-93	1.8
auxin response factor	Cluster-6172.11643	642.3349812	/	5159.885188	-3.0056	/	1.02E-27	9.7
auxin response factor	Cluster-7966.6357	/	821.4127257	2005.683263	/	-1.2889	1.79E-22	2.6
auxin response factor	Cluster-7966.4925	/	2164.100171	4677.229108	/	-1.1117	8.03E-30	1.9
auxin-responsive GH3 gene family	Cluster-6172.10088	766.109379	/	5210.198946	-2.7661	/	7.72E-22	4.2
auxin-responsive GH3 gene family	Cluster-7966.4925	/	2164.100171	4677.229108	/	-1.1117	8.03E-30	1.9
SAUR family protein	Cluster-6172.1833	1482.626306	/	191.0797756	2.9556	/	1.56E-18	5.8
SAUR family protein	Cluster-6172.15713	151.8512412	/	76.03947766	1.0014	/	0.0052791	0.0
SAUR family protein	Cluster-2913.0	88.1496484	/	25.01594296	1.8182	/	1.24E-05	7.0

SAUR family protein	Cluster-3967.0	0.343464407	/	9.713352671	-4.7418	/	0.0020559	0.0
SAUR family protein	Cluster-6172.19466	95.9333365	/	263.0061674	-1.4501	/	0.00046131	0.0
SAUR family protein	Cluster-6172.1791	33.87690781	/	139.8713152	-2.0608	/	1.44E-09	1.6
SAUR family protein	Cluster-6172.18366	200.5704202	/	541.4632883	-1.4326	/	3.18E-08	2.8
SAUR family protein	Cluster-6172.17182	61.01981071	/	123.8718257	-1.034	/	0.0078308	0.0
SAUR family protein	Cluster-6172.17013	11.05396801	/	235.4106465	-4.4395	/	6.48E-30	7.9
SAUR family protein	Cluster-5374.0	11.47905177	/	33.5277143	-1.5175	/	0.016176	0.0
SAUR family protein	Cluster-6172.13654	51.66034612	/	993.507599	-4.2614	/	8.32E-34	1.4
SAUR family protein	Cluster-1875.0	/	191.2157898	56.27788869	/	1.7696	1.48E-12	1.0
SAUR family protein	Cluster-7966.1555	/	9.758739258	60.95804546	/	-2.6418	4.36E-07	1.8
SAUR family protein	Cluster-3489.0	/	26.37401935	109.0773706	/	-2.0508	1.81E-11	1.1
SAUR family protein	Cluster-7372.0	/	50.62425717	235.2504774	/	-2.2123	5.44E-15	4.6
SAUR family protein	Cluster-7966.7594	/	163.4127787	768.464197	/	-2.2365	2.29E-31	6.0
SAUR family protein	Cluster-7966.11015	/	217.5591106	523.5357639	/	-1.2668	5.94E-18	6.3
SAUR family protein	Cluster-7966.4605	/	222.9754224	490.5957546	/	-1.1396	7.22E-07	3.0
SAUR family protein	Cluster-7966.15997	/	23.92213149	206.5310611	/	-3.1169	4.05E-27	8.1
SAUR family protein	Cluster-7966.11607	/	88.77055571	876.2847193	/	-3.3056	1.04E-55	9.6
Ethylene-responsive transcription factor 3	Cluster-6172.9509	96.47590512	/	1004.215639	-3.3831	/	1.88E-29	2.1
Ethylene-responsive transcription factor 3	Cluster-6172.14530	97.2605432	/	1695.501734	-4.1228	/	1.27E-35	2.6
cysteine-rich receptor	Cluster-6172.505	11.82009373	/	80.9107182	-2.7672	/	1.41E-06	9.5
Zinc finger	Cluster-6172.2152	66.60847947	/	133.4054107	-1.0012	/	0.011746	0.0
Zinc finger	Cluster-6172.19271	48.98729315	/	12.34615377	1.9959	/	0.00093637	0.0
Zinc finger	Cluster-2307.0	24.64384028	/	90.12586254	-1.8617	/	0.00013142	0.0
Zinc finger	Cluster-2857.0	1.304040245	/	11.91316299	-3.1698	/	0.0031694	0.0

Table 3 Gene expression in plant regeneration of Cytokine							
Description	Gene-id	Regenerating callus vs Callus_Read_count	Cyclohexane vs Callus_Read_count	Callus_Read_count	Regenerating callus vs Callus_log2Fold Change	Cyclohexane vs Callus_log2Fold Change	pval
cytokinin receptor [Arabidopsis histidine kinase 2/3/4]	Cluster-6172.6079	7743.071642	/	2946.788739	1.3939	/	4.11E-13
histidine-containing phosphotransfer protein	Cluster-6172.20325	165.1914481	/	21.04093208	2.9662	/	3.08E-13
histidine-containing phosphotransfer protein	Cluster-7966.4523	/	264.877881	801.4125562	/	-1.5983	3.14E-23
histidine-containing phosphotransfer protein	Cluster-2808.0	/	3.444274279	19.24749268	/	-2.4633	0.0049514
two-component response regulator ARR-A family	Cluster-6172.12818	118.8137608	/	737.8963989	-2.6308	/	4.93E-15
two-component response regulator ARR-A family	Cluster-4229.0	14.43168456	/	54.47624248	-1.8958	/	0.0091425
Histidine kinase CKI1	Cluster-6172.4116	765.058398	/	305.7574303	1.3238	/	5.13E-10

Table 4 Gene expression in plant regeneration of Brassinosteroid								
Description	Gene-id	Regenerating callus vs Callus_Read_count	Cyclohexane vs Callus_Read_count	Callus_Read_count	Regenerating callus vs Callus_log2Fold Change	Cyclohexane vs Callus_log2 Fold Change	pval	padj
BRI1 kinase inhibitor 1	Cluster-6172.8113	291.510962	/	769.9837	-1.4001	/	3.62E-07	2.73E-06
brassinosteroid resistant 1/2	Cluster-6172.9208	243.3127962	/	545.7915	-1.1678	/	3.52E-07	2.66E-06
brassinosteroid resistant 1/2	Cluster-6401.0	/	43.03949153	146.1382	/	-1.7659	9.00E-11	5.52E-10
brassinosteroid resistant 1/2	Cluster-6172.20298	33.13147023	/	156.7584	-2.2381	/	5.50E-07	4.01E-06
cyclin D3	Cluster-6172.6746	932.3401808	/	2811.633	-1.5916	/	3.80E-21	1.91E-19

Figures

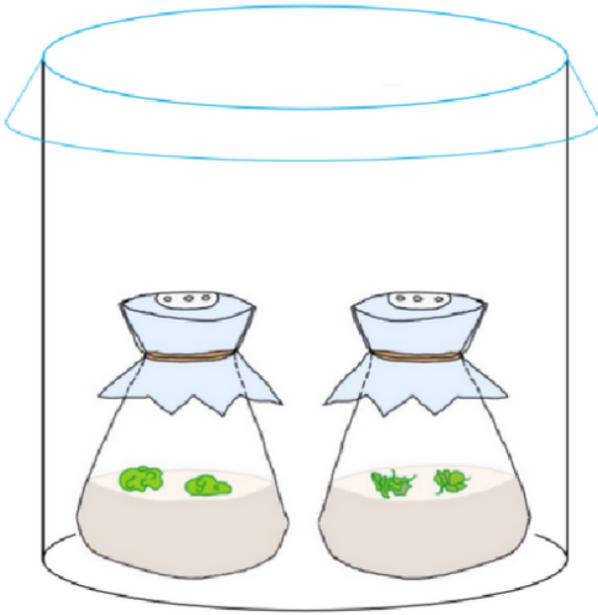
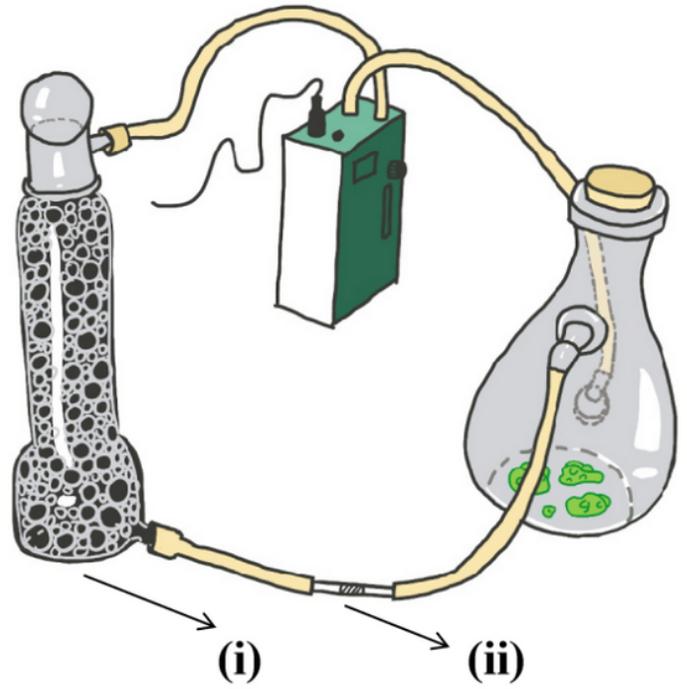
a**b**

Figure 1

System of co-culture and dynamic headspace air-circulation. a The callus and the regenerating callus of duckweed were fumigating treatment. b Collection of VOCs from plant tissue. (i) Activated carbon. (ii) Adsorption tube.

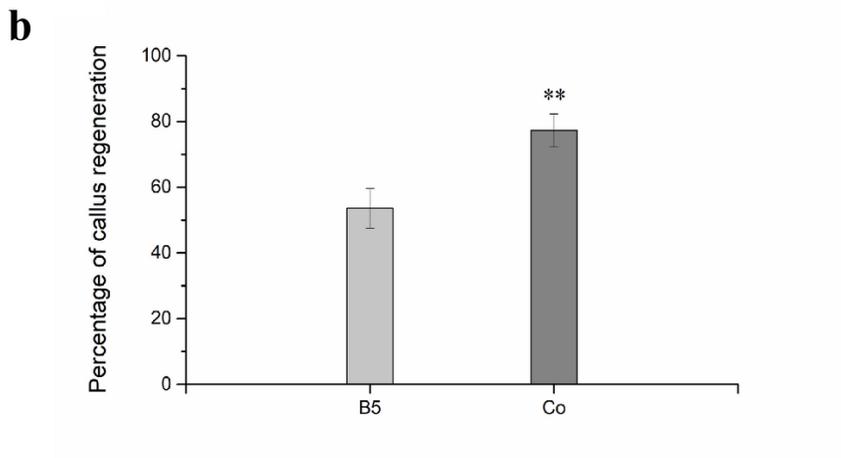
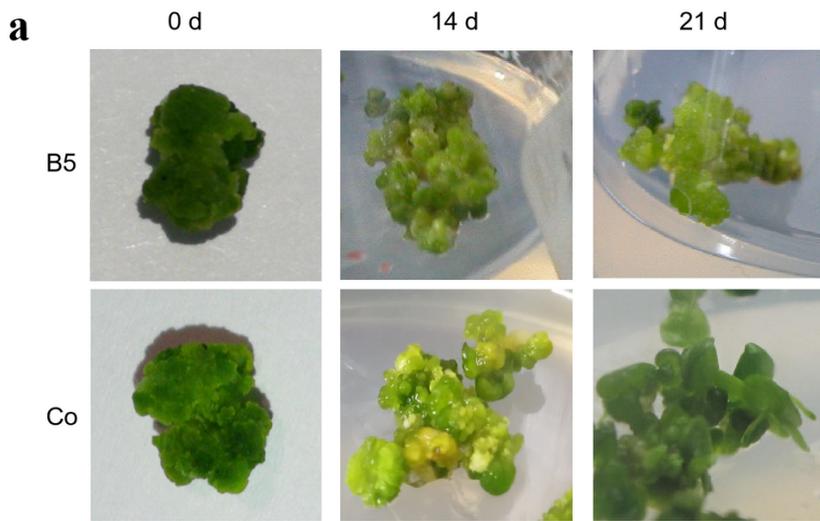


Figure 2
 The co-cultured of callus and regenerating callus. a The large beaker was sealed with plastic wrap and perforated with a sterile toothpick. b The ratio of callus regeneration between the control group (B5) and co-culture condition.

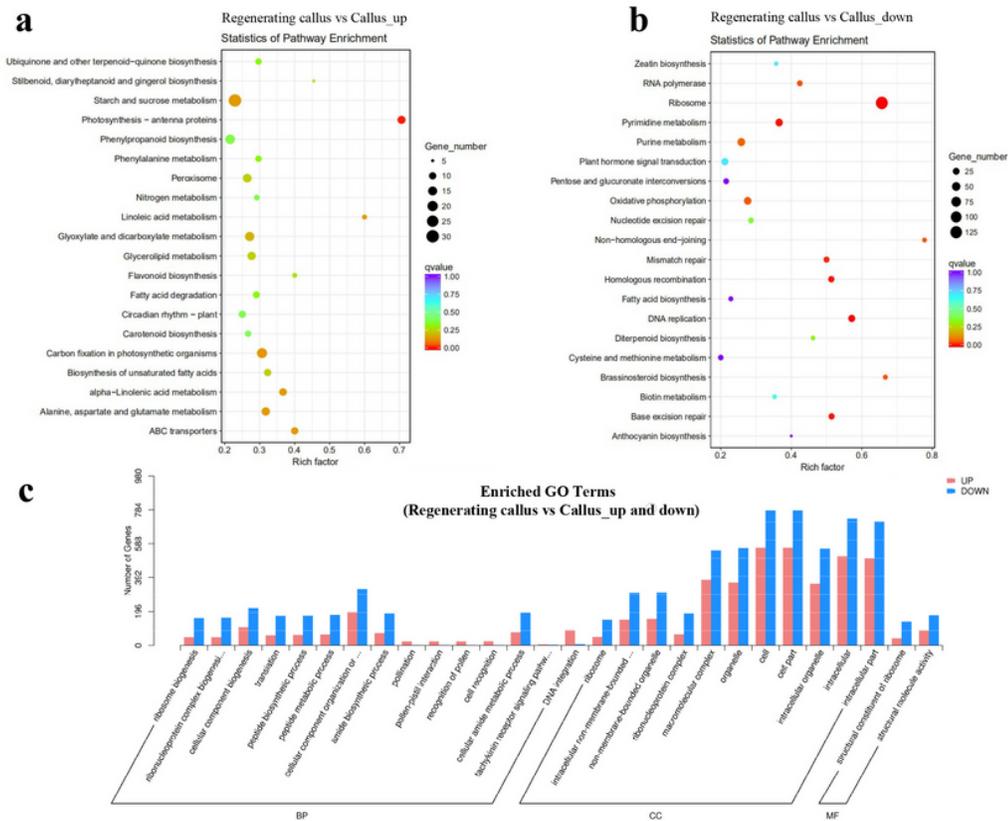


Figure 3

Statistic of KEGG pathway enrichment and the number of enriched genes in different gene ontology (GO) categories in RG vs CL. a The top 20 up KEGG pathways with the highest Rich Factors of RG vs CL, The KEGG Pathway enrichment hub diagram: The vertical axis represents pathway name, the horizontal axis represents the Rich factor corresponding to pathway, and the colour of the dots represents the size of the Q value; the smaller the Q value, the closer the colour to red; the number of different genes contained in each pathway is represented by the size of the dots, and the value range of qvalue was [0,1], and the closer to zero, the more significant the enrichment; b The top 20 down KEGG pathways with the highest Rich Factors of RG vs CL; c GO terms associated with DEGs in RG and CL. The x-coordinate was GO the next level of the three categories GO entry, and ordinate was the number of different genes commented to the entrance.

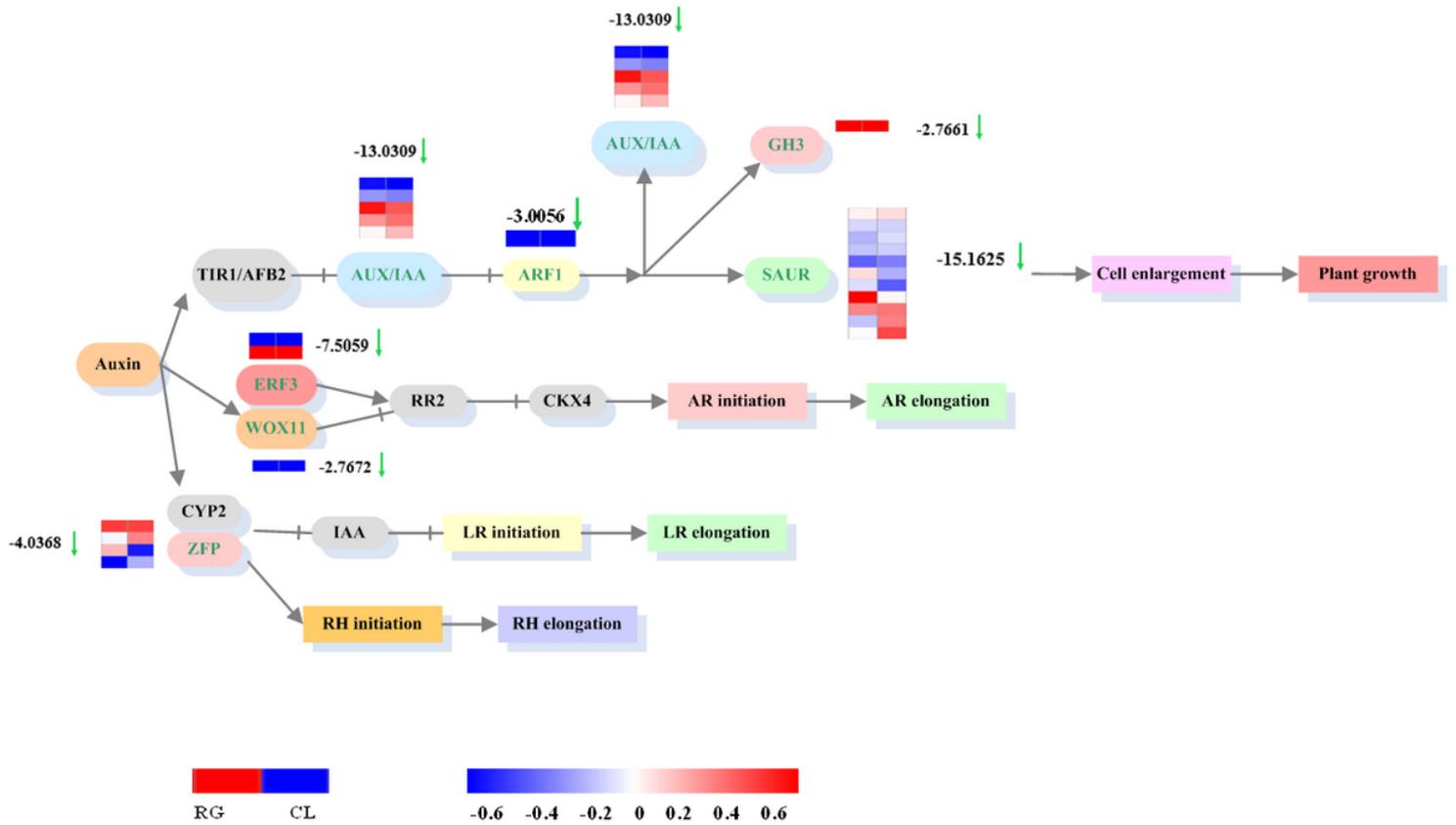


Figure 4
 The comparison between regenerating callus and callus was related to auxin metabolism response and auxin signal transduction pathway. Arrows indicated the direction of processes, while red was up, green was down. As shown in the figure was auxin signal transduction, and various response factors were down-regulated. The color in this figure legend from red to blue, which meant $\log_{10}(\text{FPKM}+1)$ from high to low. Red meant high expression, blue meant low expression.

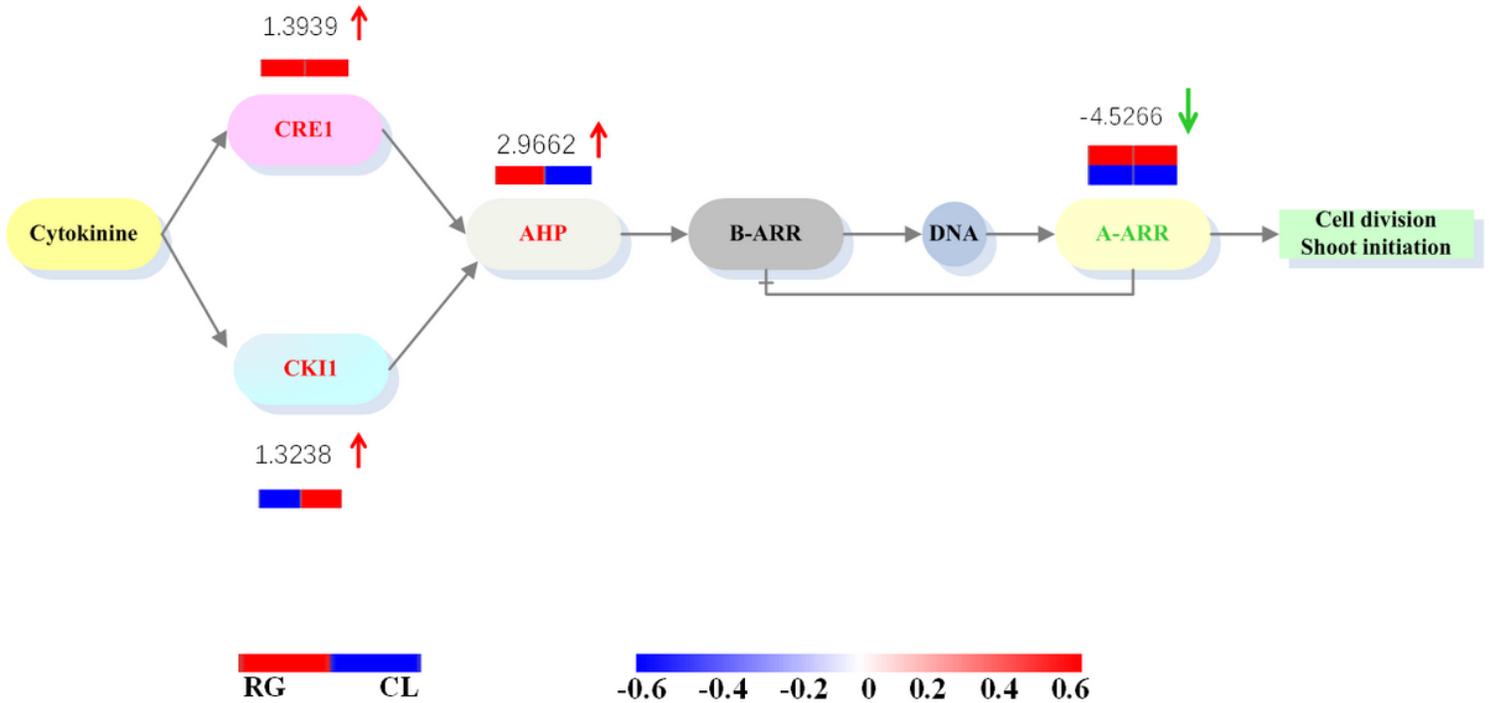


Figure 5
 Page 14/17

Comparing regenerating callus and callus was related to cytokinins metabolism response and cytokinins signal transduction pathway. Arrows indicated the direction of processes, while red was up, green was down. As shown in the figure was cytokinins signal transduction. CK11, CRE1 and AHP were up-regulated, but negative feedback regulator A-ARR was down-regulated. The colour in this figure legend from red to blue, which meant $\log_{10}(\text{FPKM}+1)$ from high to low. Red meant high expression, blue meant low expression.

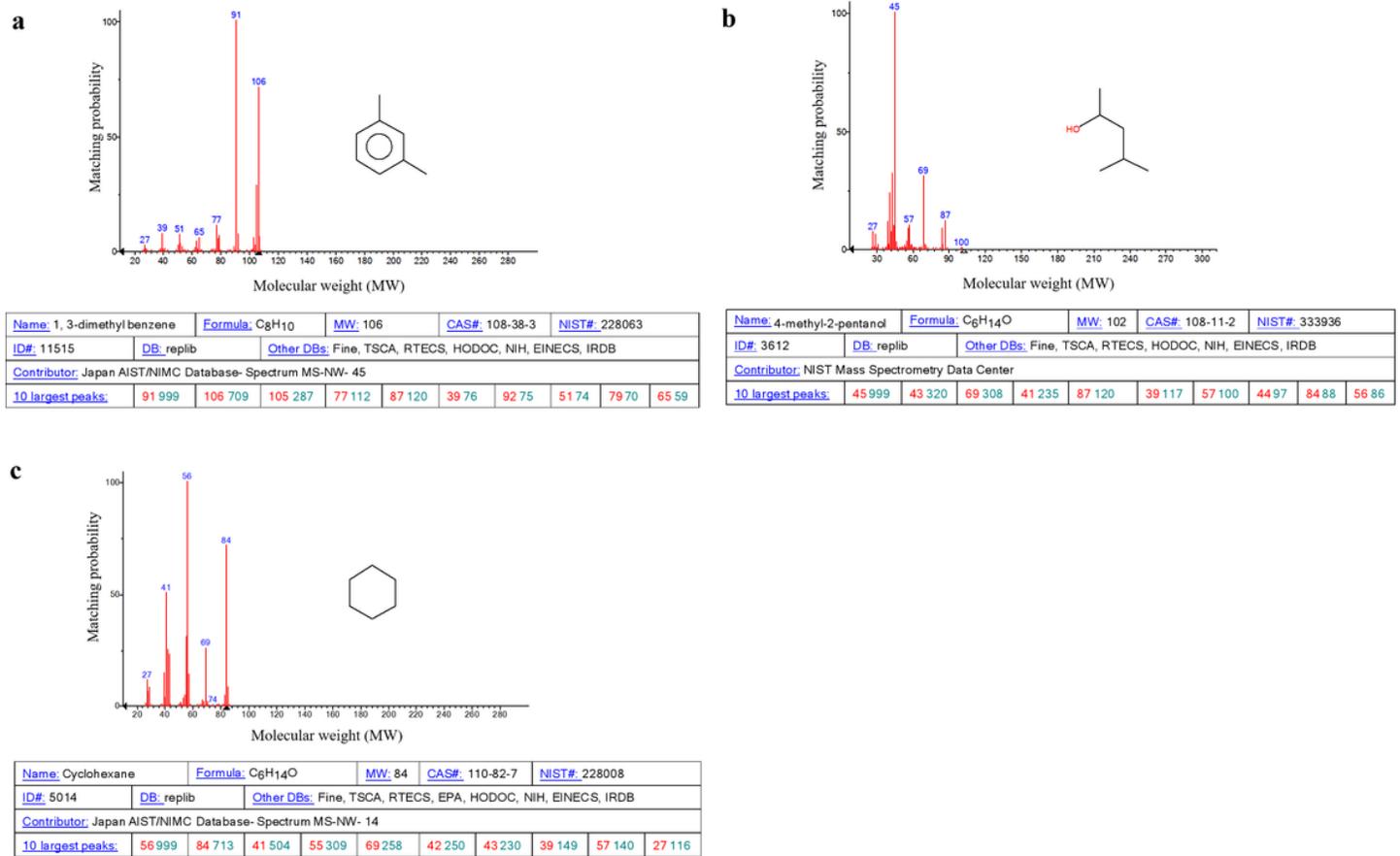


Figure 6

Three kinds of VOCs significantly up-regulated in the callus regeneration stage. The numbers in blue represented the mass-to-charge ratio (m/z) of a substance in the histogram. a Mass spectra of 1, 3-dimethyl benzene. b Mass spectra of 4-methyl-2-pentanol. c Mass spectra of cyclohexane.

Cyclohexane

4-methyl-2-pentanol

1, 3-dimethyl benzene



Figure 7

Effects of 16 days' treatment of callus by three VOCs (cyclohexane, 4-methyl-2-pentanol and 1, 3-dimethyl benzene).

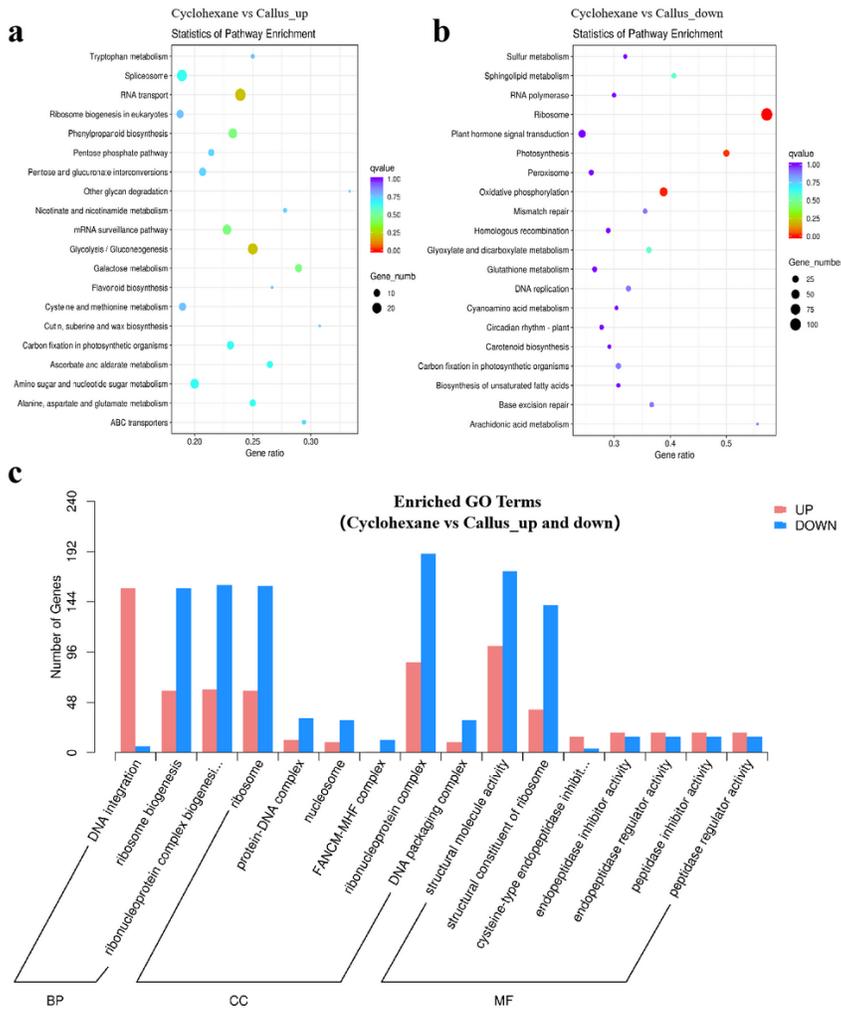


Figure 8

In the context of "Cyclohexane vs CL", the top 20 KEGG pathways of up-regulated DEGs (a) and down-regulated DEGs (b) with the highest Rich Factors. GO terms associated with DEGs in "Cyclohexane vs CL", the number of Enriched were up and down-regulated DEGs (c) in different gene ontology categories.

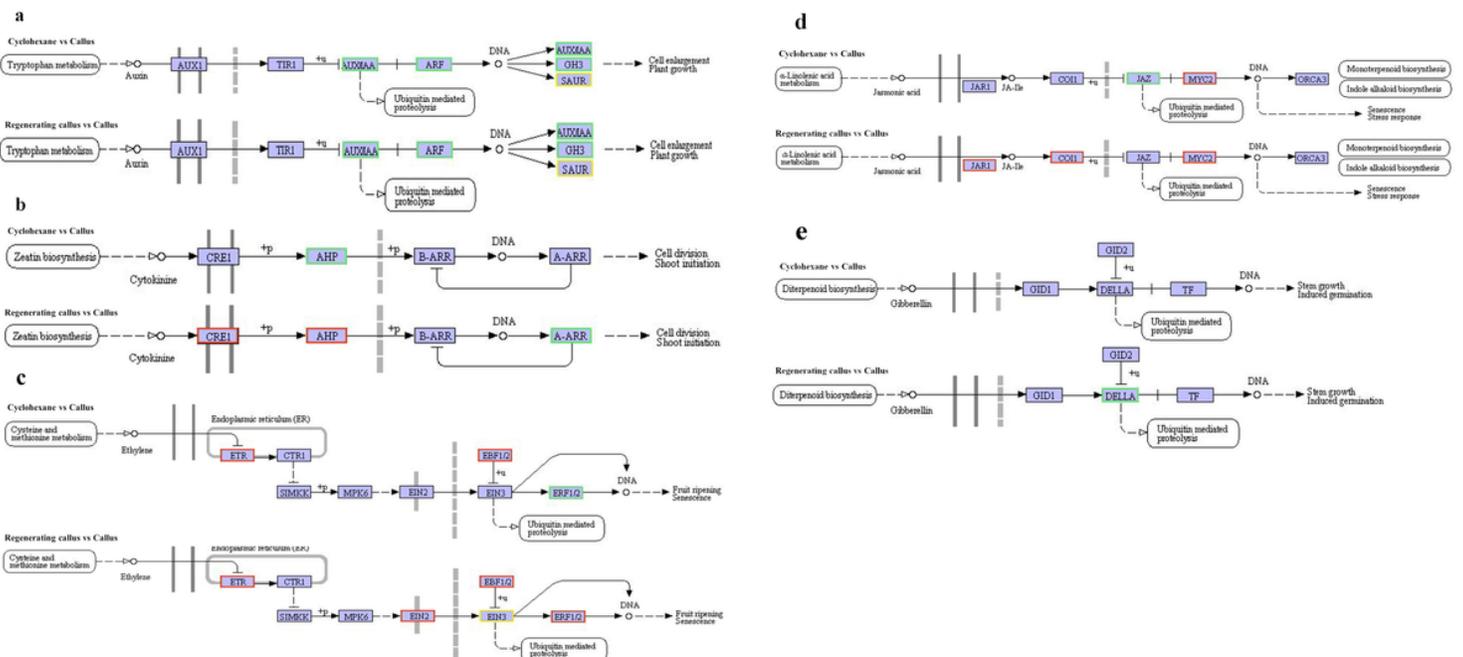


Figure 9

The pathway of biosynthesis of five types of plant hormone. Red meant high expression, and blue meant low expression. a The changes of genes in Auxin between regenerating callus and cyclohexane treatment callus. b The changes of genes in cytokinin between regenerating callus and cyclohexane treatment callus. c The differences of genes in ethylene between regenerating callus and cyclohexane treatment callus. d The changes of genes in jasmonic acid between regenerating callus and cyclohexane treatment callus. e The changes of genes in gibberellin between regenerating callus and cyclohexane treatment callus.

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