The framework of plant regeneration in duckweed (*Lemna turonifera*) comprises genetic transcript regulation and cyclohexane release

Lin Yang[1¶], Jinge Sun[1¶], Jie Yao[1¶], Yaya Wang[1&], Congyu Yan[1&], Junyi Wu[1&], Qiuting Ren[1&], Ling Zhao[2&], Jinsheng Sun*[1]

1 Tianjin Key Laboratory of Animal and Plant Resistance, College of Life Sciences, Tianjin Normal University, 300387, Tianjin, China

2 College of Life Sciences, Department of Plant Biology and Ecology, Nankai University, 300071, Tianjin, China

* Corresponding author

E-mail: skysjs@tjnu.edu.cn (Jinsheng Sun)

Acknowledgements

Present research has been supported by National Natural Science Foundation of China (No. 32071620), Tianjin Science and technology project (19ZYPTSN00030).

Abstract

Regeneration is important for vegetative propagation of excellent variety, detoxification and the obtain of transgenic plant, but plant regeneration is time-consuming. Here, we found that duckweed regeneration could be enhanced by regenerating callus. 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, volatile organic compounds release has been analysised by gas chromatography/ mass spectrum during the stage of plant regeneration, and 11 kinds of unique volatile organic compounds 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. All together, these results provide novel mechanistic insights into how regenerating callus promotes duckweed regeneration.

Introduction

Regeneration of entire plants from callus in vitro depends on pluripotent cell mass, which provides rise to a new organ or even an entire plant[1-2]. Regeneration was widely used for vegetative propagation of excellent variety, detoxification and the obtain of transgenic crops[3-4]. A large number of 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. Duckweed, with the advantages of fast reproduction, high protein content[5], and distinguished tolerance for a variety of toxic substances[6-7], has been applied as a monocotylous modle plant for gene-expression systems. In duckweed, stable transformation mediate by *Agrobacterium* depends on efficient callus regeneration protocols.

Here, we use transcriptome sequencing technology to explore the molecular mechanism of plant hormones regulating callus regeneration[8]. Nevertheless, 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 was mediated by many plant hormones[5]. The balance of auxin and cytokinin is the basis for vitro tissue culture[9]. Explants can be incubate to callus on auxin-rich callus-inducing medium (CIM). And on cytokinin-rich shoot inducing medium (SIM), the vigorous callus can be induce to novo shoots. It is emergent to study the mechanism of duckweed regeneration via dynamic hormonal and transcriptional changes.

The volatile organic compounds (VOCs) could be produced to defense against herbivores, and it may also play a secondary role in attracting natural enemies, which is allelopathy[10-11]. For example, the VOCs of *Artemisia frigida Willd* play an allelopathic role on the seed germination of pasture grasses[12]. 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 has 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 has been promoted by VOCs.

Results

Promoted effect of regenerating tissue

Frond 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 with Co treatment (Fig 1a). In 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 1b). Thus, the callus regeneration has been significantly increased by Co treatment.

Fig. 1 The co-cultured of callus and regenerating callus.

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. 2). 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. 2a, the "Photosynthesis antenna proteins" was 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 very important for plant photochemical reactions and could mediate the core of plant photosynthesis. The most significantly down-regulated pathway was the “Ribosome”, “Pyrimidine metabolism”, “Mismatch repaire”, “Homologous recombination”, “DNA replication” and “Base excision repair”, which were among the top list of enriched pathways (Fig. 2b), 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. 2c, “cell” “cellpart” 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 category of biological process with the most up-regulated and down regulated DEGs. “DNA integration”, “pollination”, and “cell recognition” were up-regulated DEGs, without down-regulated (Fig.2).

Fig. 2 Statistic of KEGG pathway enrichment and the number of enriched genes in different gene ontology (GO) categories in RG vs CL.

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

The mRNA expression was conducted by Novogene in order to study the gene that participated during callus regeneration. The course of auxin signal pathway and related response factors have been described as Fig. 3. 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, 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, respectivly. 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 HOMEOBOX 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) were rely on zinc finger protein (ZFP) and cytochrome P450 (CYP2). The expression of ZFP was decreased by 4.0368 $\log^2$ Fold Change.

Fig. 3 The comparison between regenerating callus and callus was related to auxin metabolism response and auxin signal transduction pathway.

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

To obtain candidates regulating regeneration, we studied the regulation of cytokinins signal pathway. Shown as Fig.4, cytokinin receptor 1(CRE1) and cytokinin independent 1(CKI1), as cytokinin receptors[13-14], have been up-regulated in regenerating callus. Histidine phosphate transfer protein (AHP), interacting with CRE1 and CKI1, has been up-regulated by 2.9662 log ^{2} Fold Change. Type-A ARABIDOPSIS RESPONSE REGULATORS (A-ARR) plays a role as a negative feedback regulator, which inhibit the activity of type-B ARABIDOPSIS RESPONSE REGULATORS （B-ARR） and form a negative feedback cycle[15-16]. A-ARR has been down-regulated by 4.5266 log ^{2} Fold Change. It might be lead to overall up-regulated in cytokinins during the callus regenerating.

Fig. 4 Comparing regenerating callus and callus was related to cytokinins metabolism response and cytokinins signal transduction pathway.

Changes of VOCs during callus regeneration

The VOCs of regenerating callus have 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.5. 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 $0.84*10^7$, 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 cyclohexane peak area of the callus ($0.85*10^7$), the cyclohexane peak area of the regenerating callus was $1.28*10^7$, $4.3*10^6$ higher than that of callus. And the peak area of 4-methyl-2-pentanol was $2.1*10^7$, 2.33 times than that of callus.

Fig. 5 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.

Table 1 The main components of VOCs from regenerating callus and callus

Designation	Chemical formula	RG Peak area （$*10^7$）	CL Peak area （$*10^7$）	Acquisition time (min)
Cyclohexane	C_6H_{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	$C_6H_{14}O$	2.1	0.9	3.81
1, 3-dimethyl benzene	C_8H_{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

Callus regeneration was 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. 6 showed, cyclohexane promoted the regeneration of callus significantly. After 16 days cyclohexane treatment, roots formed from the callus. The newborn roots could be distinctly observed shown as red arrow. However, 1, 3-dimethyl benzene and 4-methyl-2-pentanol groups have no obvious phenomenon of regeneration in 16 days.

Fig. 6 Effects of 16 days' treatment of callus by three VOCs (cyclohexane, 4-methyl-2-pentanol and 1, 3-dimethyl benzene).

Transcriptome analysis identifies KEGGs and DEGs in callus treated by cyclohexane

Transcriptome analysis has been analyed to investigate the potential functions of

KEGGs and DEGs in the callus treat hydrolyzing O-glycosyl compoundsed by cyclohexane. As shown in Fig. 7a, “RNA transport” and “glycolysis/gluconeoge, and galaclose metabolism ” were in the biological process with the most down-regulated KEGGs. “Ribosome” was the the top-enriched pathway (Richfactor>0.55). It was followed by “photosynthesis”, and “oxidative phosphorylation” (Fig. 7b).

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. 7c, “DNA integration”, “ribonucleoprotein complex” and “structrural 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 biological process with the most up-regulated DEGs. “ribonucleoprotein complex” and “structrural molecule activity” were were in biological process with the most down-regulated DEGs. (Fig. 7c).

Fig. 7 In the context of "Cyclohexane vs CL", the top 20 KEGG pathways of up-regulated DEGs.

Comparsion of the expression of genes related to 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 auxin signal pathway (Table 2). AUX/IAA and GH3 has been down regulated in both callus treated with cyclohexane and in the regenerating callus. A majority of SAUR have been down-regulated during regeneration and treated with cyclohexane (Fig. 8a). 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. 8b). The gene regulation in regeneration and treated with cyclohexane is different. The CRE1 has been up-regulated in the regenerating callus, and that has been down-regulated in callus treated with cyclohexane.

Thirdly, the expression of genes related to brassionosteroid signal has been investigated. In the brassionosteroid 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 brassionosteroid signal pathway, the expression of BZR1/2 has been down-regulated in in callus treated with cyclohexane and the regenerating callus.

Moreover, the expression of genes related to ethylene signal has been investigated (Fig. 8c). The expression of ETR and EBF1/2 has been up-regualted in callus treated with cyclohexane and the regenerating callus. transcription factor MYC2（MYC2）, plays a role in jasmonic acid signal pathway, has been up-regulated in both cyclohexane treatment and regenerating callus (Fig. 8d). There is no significant difference in gibberellin signal pathway during cyclohexane treatment (Fig. 8e).

Fig. 8 The pathway of biosynthesis of five types of plant hormone. Red meant high expression, and blue meant low expression.

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_log2 Fold Change	pval	padj
auxin-responsive protein IAA	Cluster-6172.2761	25.79057461	/	291.8684987	-3.499	/	1.53E-20	7.20E-19
auxin-responsive protein IAA	Cluster-6172.9506	1350.903101	/	7436.536506	-2.4616	/	8.50E-33	1.36E-30
auxin-responsive protein IAA	Cluster-6172.9484	3097.048347	/	8093.560243	-1.3863	/	9.12E-09	8.99E-08
auxin-responsive protein IAA	Cluster-6172.6741	115.191085	/	752.9653036	-2.7163	/	4.06E-30	5.09E-28
auxin-responsive protein IAA	Cluster-6172.4574	329.7352314	/	2581.00597	-2.9677	/	5.94E-28	5.78E-26
auxin-responsive protein IAA	Cluster-7966.13997	/	126.898086	427.8033881	/	-1.7564	1.96E-13	1.49E-12
auxin-responsive protein IAA	Cluster-7966.10326	/	2912.318825	6803.456966	/	-1.2242	1.54E-20	1.99E-19
auxin-responsive protein IAA	Cluster-7966.9984	/	757.4281355	2282.819782	/	-1.5911	5.19E-39	2.10E-37
auxin-responsive protein IAA	Cluster-7966.7990	/	882.1458283	7136.36841	/	-3.0168	1.35E-109	9.48E-107
auxin-responsive protein IAA	Cluster-7966.3823	/	24.87192746	135.7070458	/	-2.4536	3.10E-16	2.89E-15
auxin-responsive protein IAA	Cluster-7966.9412	/	68.99029552	688.2924139	/	-3.3241	1.37E-77	3.22E-75
auxin-responsive protein IAA	Cluster-7966.8499	/	2134.915379	8945.722998	/	-2.067	4.70E-93	1.83E-90
auxin response factor	Cluster-6172.11643	642.3349812	/	5159.885188	-3.0056	/	1.02E-27	9.71E-26
auxin response factor	Cluster-7966.6357	/	821.4127257	2005.683263	/	-1.2889	1.79E-22	2.67E-21
auxin response factor	Cluster-7966.4925	/	2164.100171	4677.229108	/	-1.1117	8.03E-30	1.93E-28
auxin-responsive GH3 gene family	Cluster-6172.10088	766.109379	/	5210.198946	-2.7661	/	7.72E-22	4.27E-20
auxin-responsive GH3 gene family	Cluster-7966.4925	/	2164.100171	4677.229108	/	-1.1117	8.03E-30	1.93E-28
SAUR family protein	Cluster-6172.1833	1482.626306	/	191.0797756	2.9556	/	1.56E-18	5.86E-17
SAUR family protein	Cluster-6172.15713	151.8512412	/	76.03947766	1.0014	/	0.0052791	0.017525
SAUR family	Cluster-2913.	88.1496484	/	25.01594296	1.8182	/	1.24E-	7.06E-

protein	0						05	05
SAUR family protein	Cluster-3967.0	0.343464407	/	9.713352671	-4.7418	/	0.0020559	0.007526
SAUR family protein	Cluster-6172.19466	95.9333365	/	263.0061674	-1.4501	/	0.00046131	0.0019407
SAUR family protein	Cluster-6172.1791	33.87690781	/	139.8713152	-2.0608	/	1.44E-09	1.61E-08
SAUR family protein	Cluster-6172.18366	200.5704202	/	541.4632883	-1.4326	/	3.18E-08	2.84E-07
SAUR family protein	Cluster-6172.17182	61.01981071	/	123.8718257	-1.034	/	0.0078308	0.024821
SAUR family protein	Cluster-6172.17013	11.05396801	/	235.4106465	-4.4395	/	6.48E-30	7.95E-28
SAUR family protein	Cluster-5374.0	11.47905177	/	33.5277143	-1.5175	/	0.016176	0.046631
SAUR family protein	Cluster-6172.13654	51.66034612	/	993.507599	-4.2614	/	8.32E-34	1.46E-31
SAUR family protein	Cluster-1875.0	/	191.2157898	56.27788869	/	1.7696	1.48E-12	1.04E-11
SAUR family protein	Cluster-7966.1555	/	9.758739258	60.95804546	/	-2.6418	4.36E-07	1.89E-06
SAUR family protein	Cluster-3489.0	/	26.37401935	109.0773706	/	-2.0508	1.81E-11	1.17E-10
SAUR family protein	Cluster-7372.0	/	50.62425717	235.2504774	/	-2.2123	5.44E-15	4.64E-14
SAUR family protein	Cluster-7966.7594	/	163.4127787	768.464197	/	-2.2365	2.29E-31	6.09E-30
SAUR family protein	Cluster-7966.11015	/	217.5591106	523.5357639	/	-1.2668	5.94E-18	6.34E-17
SAUR family protein	Cluster-7966.4605	/	222.9754224	490.5957546	/	-1.1396	7.22E-07	3.04E-06
SAUR family protein	Cluster-7966.15997	/	23.92213149	206.5310611	/	-3.1169	4.05E-27	8.18E-26
SAUR family protein	Cluster-7966.11607	/	88.77055571	876.2847193	/	-3.3056	1.04E-55	9.68E-54
Ethylene-responsive transcription factor 3	Cluster-6172.9509	96.47590512	/	1004.215639	-3.3831	/	1.88E-29	2.15E-27
Ethylene-responsive transcription factor 3	Cluster-6172.14530	97.2605432	/	1695.501734	-4.1228	/	1.27E-35	2.68E-33
cysteine-rich receptor	Cluster-6172.505	11.82009373	/	80.9107182	-2.7672	/	1.41E-06	9.59E-06
Zinc finger	Cluster-6172.2152	66.60847947	/	133.4054107	-1.0012	/	0.011746	0.035246
Zinc finger	Cluster-6172.	48.98729315	/	12.34615377	1.9959	/	0.0009	0.0036

	19271						3637	929
Zinc finger	Cluster-2307.0	24.64384028	/	90.12586254	-1.8617	/	0.00013142	0.0006126
Zinc finger	Cluster-2857.0	1.304040245	/	11.91316299	-3.1698	/	0.0031694	0.011129

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	padj
cytokinin receptor（arabidopsis histidine kinase 2/3/4）	Cluster-6172.6079	7743.071642	/	2946.788739	1.3939	/	4.11E-13	7.79E
histidine-containing phosphotransfer peotein	Cluster-6172.20325	165.1914481	/	21.04093208	2.9662	/	3.08E-13	5.97E
histidine-containing phosphotransfer protein	Cluster-7966.4523	/	264.877881	801.4125562	/	-1.5983	3.14E-23	4.90E
histidine-containing phosphotransfer protein	Cluster-2808.0	/	3.444274279	19.24749268	/	-2.4633	0.0049514	0.012
two-component response regulator ARR-A family	Cluster-6172.12818	118.8137608	/	737.8963989	-2.6308	/	4.93E-15	1.22E
two-component response regulator ARR-A family	Cluster-4229.0	14.43168456	/	54.47624248	-1.8958	/	0.0091425	0.028
Histidine kinase CKI1	Cluster-6172.4116	765.058398	/	305.7574303	1.3238	/	5.13E-10	6.17E

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
brassinosteroid resistant 1/2	Cluster-6172.9208	243.3127962	/	545.7915	-1.1678	/	3.52E-07	2.66E
brassinosteroid resistant 1/2	Cluster-6401.0	/	43.03949153	146.1382	/	-1.7659	9.00E-11	5.52E

brassinosteroid resistant 1/2	Cluster-6172.20298	33.13147023	/	156.7584	-2.2381	/	5.50E-07	4.01E
cyclin D3	Cluster-6172.6746	932.3401808	/	2811.633	-1.5916	/	3.80E-21	1.91E

Discussion

In line with previous studies, we established an effective way to *in vitro* 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 the molecule framework of plant regeneration in duckweed .

Plant hormones played a crucial role during callus regeneration[1]. Through our study, we hope to have a deeper understanding of the regulatory mechanism of callus regeneration. Callus were induced by auxin, similar as lateral root primordium[17-19]. In Arabidopsis, the callus tissue formed root stem cell niche, by regulation the expression of root stem cell regulators, including WOX[20-23]. According to our results, ARF, AUX/IAA, GH3, ARF1, SAUG and other response factor have been down-regulated significantly during the callus redifferentiation (Fig. 3). In the auxin signaling pathway, the interaction between ARF and AUX /IAA could regulate the genes expression of auxin early response. Moreover, ERF3, WOX11 and ZFP were found to be related to the ARs, LRs and RHs of initiation in *Spirodela*[8], which might lead associated to the regeneration in duckweed.

Cytokinins and auxin have synergistic or antagonistic interactions with each other[24]. As a phytohormone, cytokinin could not only control key aspects of environmental responses, such as biotic and abiotic stress resopnses, but also regulate various developmental processes including cell proliferation, leaf formation, and root formation and growth[25-26]. Cytokinins promoted plant regeneration by regulating the generation of somatic embryogenesis in *Fumariaceae* and Rice[27-28]. 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. 4). And the expreission of cytokinins synthesis was up-regulated, thereby promoting the differentiation of shoots. The transcriptome analysis suggested the similar result with Arabidopsis, giving evidence that the regulation of auxin and cytokinins leads to regeneration. Besides, plant regeneration has been regulated by other hormones[29]. In our results, we found that gibberellin, jasmonic acid and increased significantly, while genes related to gibberellin and brassinolide were down-regulated during callus regenerating (Fig. 8).

Plants release VOCs to the environment to affect their own or other biological life processes in the process of plants growth and development. This phenomenon was called allelopathy[30]. Plants in different growing environments, such as biological stress or abiotic stress, might release different VOCs to improve their resistance to external interference[31-33]. In previous studies, VOCs have been shown to mediate cell to cell communication, there by leading to stress responses in plants[34]. 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. 6).

Here, the regulation of gene expression related to hormone in callus treated with cyclohexane, which promoted regeneration, suggested the role of auxin during regeneration. AUX/IAA and GH3 has been down regulated in both callus treated with cyclohexane, which is similar with that in the regenerating callus (Fig. 8). And adventitious root initiation and enlongation has been promoted by AUX/IAA[8]. Interestingly, the root formation has been enhanced significantly by cyclohexane treatment (Fig. 6).

Altogether, we propose a hypothesis how callus regenerate 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 indiciatied that VOCs might played an crucial role in the process of plant regeneration. It also makes clear that allelopathy does affect plant growth and development.

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 descripted as Wang et al. and Yang et al[35-36]. 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 was designed by Gamborg for soybeans tissue culturein 1968[37]. The induction medium contained plant hormones 15 mg/l dicamba, 3.5 mg/l 2, 4-D, 6-BA 2mg/l and 1.5% sucrose. The pH of medium was adjusted to 6.2-6.4 and then it was sterilizated at 121℃ 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 formated. The calli were transferred to the subculture medium. Subculture medium contains B5 medium, 10 mg/L 4-chlorophenoxyacetic acid (CPA) and 2 mg/L 2ip. 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 3 days culture in B5 subculture medium, the calli were cultured in B5 medium with 20 ml cyclohexane in a large 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.

Fig. 9 System of co-culture and dynamic headspace air-circulation.

The Co-culture of regenerating callus and callus

The callus was cultured on 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 placed together in a closed environment for co-culture described as Fig. 9a.

VOCs Collection and analysis

Shown as Fig. 9b, the VOCs released from callus and regenerating duckweed were collected using the dynamic headspace air-circulation method described by Zuo et al. (2018)[38]. There were 3 conical flasks of callus or regenerating callus for each group. The chemical composition analysis of VOCs was performed by thermal-desorption system/ gas chromatography/ mass spectrum (TDS/GC/MS). And the GC/MS data was 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 Flurometer (Life Technologies, CA, USA). And then, RNA integrity was 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. The filtering contents were followed as our previous study: Removed adapters; Removed reads whose proportion of N is greater than 10%; Remove low-quality reads[6]. 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[39]. Overall, a reference sequence, with an average length of 1928 bp and a total length of 282527137 bp, was obtained for subsequent analysis.

Data analysis

The experiment were 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 error bar. The graphs in this 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.

References

1. Ikeuchi M, Ogawa Y, Iwase A, Sugimoto K. Plant regeneration: cellular origins and molecular mechanisms. Development. 2016 May 1;143(9):1442–51.

2. Fehér A. Callus, Dedifferentiation, Totipotency, Somatic Embryogenesis: What These Terms Mean in the Era of Molecular Plant Biology? Front Plant Sci. 2019 Apr 26;10:536.

3. Lardon R, Wijnker E, Keurentjes J, Geelen D. The genetic framework of shoot regeneration in Arabidopsis comprises master regulators and conditional fine-tuning factors. Commun Biol. 2020 Dec;3(1):549.

4. Motte H, Vereecke D, Geelen D, Werbrouck S. The molecular path to in vitro shoot regeneration. Biotechnology Advances. 2014 Jan;32(1):107–21.

5. Li J, Jain M, Vunsh R, Vishnevetsky J, Hanania U, Flaishman M, et al. Callus induction and regeneration in Spirodela and Lemna. Plant Cell Reports. 2004 Feb 1;22(7):457–64.

6. Yao J, Sun J, Chen Y, Shi L, Yang L, Wang Y. The molecular mechanism underlying cadmium resistance in NHX1 transgenic Lemna turonifera was studied by comparative transcriptome analysis. Plant Cell Tiss Organ Cult. 2020 Oct;143(1):189–200.

7. Yang L, Chen Y, Shi L, Yu J, Yao J, Sun J, et al. Enhanced Cd accumulation by Graphene oxide (GO) under Cd stress in duckweed. Aquatic Toxicology. 2020 Dec;229:105579.

8. An D, Zhou Y, Li C, Xiao Q, Wang T, Zhang Y, et al. Plant evolution and environmental adaptation unveiled by long-read whole-genome sequencing of *Spirodela*. Proc Natl Acad Sci USA. 2019 Sep 17;116(38):18893–9.

9. Shim S, Kim HK, Bae SH, Lee H, Lee HJ, Jung YJ, et al. Transcriptome comparison between pluripotent and non-pluripotent calli derived from mature rice seeds. Sci Rep. 2020 Dec;10(1):21257.

10. Zhang G, Zhao F, Chen L, Pan Y, Sun L, Bao N, et al. Jasmonate-mediated wound signalling promotes plant regeneration. Nat Plants. 2019 May;5(5):491–7.

11. Turlings TCJ, Tumlinson JH, Lewis WJ. Exploitation of Herbivore-Induced Plant Odors by Host-Seeking Parasitic Wasps. Science. 1990 Nov 30;250(4985):1251–3.

12. Zhang RM, Zuo ZJ, Gao PJ, Hou P, Wen GS, Gao Y. Allelopathic effects of VOCs of Artemisia frigida Willd. on the regeneration of pasture grasses in Inner Mongolia. Journal of Arid Environments. 2012 Dec;87:212–8.

13. Hwang I, Sheen J. Two-component circuitry in Arabidopsis cytokinin signal transduction. Nature. 2001 Sep;413(6854):383–9.

14. Binglian Z, Jiaqiang S, Suzhi Z, Yan D, Jianru Z. Cytokinin signal transduction: Known simplicity and unknown complexity. 2003;48(13):8.

15. Guo Hao. Research Progress in Signal Transduction Mechanism of Cytokinin. OJNS. 2020;08(03):85–91.

16. Hwang I, Sheen J, Müller B. Cytokinin Signaling Networks. Annu Rev Plant Biol. 2012 Jun 2;63(1):353–80.

17. Atta R, Laurens L, Boucheron-Dubuisson E, Guivarc'h A, Carnero E, Giraudat-Pautot V, et al. Pluripotency of Arabidopsis xylem pericycle underlies shoot regeneration from root and hypocotyl explants grown *in vitro*. The Plant Journal. 2009 Feb;57(4):626–44.

18. Hirota A, Kato T, Fukaki H, Aida M, Tasaka M. The Auxin-Regulated AP2/EREBP Gene *PUCHI* Is Required for Morphogenesis in the Early Lateral Root Primordium of *Arabidopsis*. The Plant Cell. 2007 Aug 27;19(7):2156–68.

19. Sugimoto K, Jiao Y, Meyerowitz EM. Arabidopsis Regeneration from Multiple Tissues Occurs via a Root Development Pathway. Developmental Cell. 2010 Mar;18(3):463–71.

20. Liu J, Sheng L, Xu Y, Li J, Yang Z, Huang H, et al. *WOX11* and *12* Are Involved in the First-Step Cell Fate Transition during de Novo Root Organogenesis in *Arabidopsis*. Plant Cell. 2014 Mar;26(3):1081–93.

21. Shimotohno A, Heidstra R, Blilou I, Scheres B. Root stem cell niche organizer specification by molecular convergence of PLETHORA and SCARECROW transcription factor modules. Genes Dev. 2018 Aug 1;32(15–16):1085–100.

22. Haecker A, Groß-Hardt R, Geiges B, Sarkar A, Breuninger H, Herrmann M, et al. Expression dynamics of *WOX* genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. Development. 2004 Feb 1;131(3):657–68.

23. Pi L, Aichinger E, van der Graaff E, Llavata-Peris CI, Weijers D, Hennig L, et al. Organizer-Derived WOX5 Signal Maintains Root Columella Stem Cells through Chromatin-Mediated Repression of CDF4 Expression. Developmental Cell. 2015 Jun;33(5):576–88.

24. Azizi P, Rafii MY, Maziah M, Abdullah SNA, Hanafi MM, Latif MA, et al. Understanding the shoot apical meristem regulation: A study of the phytohormones, auxin and cytokinin, in rice. Mechanisms of Development. 2015 Feb;135:1–15.

25. Karunadasa SS, Kurepa J, Shull TE, Smalle JA. Cytokinin-induced protein synthesis suppresses growth and osmotic stress tolerance. New Phytol. 2020 Jul;227(1):50–64.

26. Romanov GA, Lomin SN, Schmülling T. Cytokinin signaling: from the ER or from the PM? That is the question! New Phytol. 2018 Apr;218(1):41–53.

27. Sagare AP, Lee YL, Lin TC, Chen CC, Tsay HS. Cytokinin-induced somatic embryogenesis and plant regeneration in Corydalis yanhusuo (Fumariaceae) — a medicinal plant. Plant Science. 2000 Dec;160(1):139–47.

28. Raghava Ram NV, Nabors MW. Cytokinin Mediated Long-term, High-frequency Plant Regeneration in Rice Tissue Cultures. Zeitschrift für Pflanzenphysiologie. 1984 Feb;113(4):315–23.

29. Ikeuchi M, Iwase A, Rymen B, Lambolez A, Kojima M, Takebayashi Y, et al. Wounding Triggers Callus Formation via Dynamic Hormonal and Transcriptional Changes. Plant Physiol. 2017 Nov;175(3):1158–74.

30. Cheng F, Cheng Z. Research Progress on the use of Plant Allelopathy in Agriculture and the Physiological and Ecological Mechanisms of Allelopathy. Front Plant Sci [Internet]. 2015 Nov 17 [cited 2021 May 26];6. Available from: http://journal.frontiersin.org/Article/10.3389/fpls.2015.01020/abstract

31. Raghava T, Ravikumar P, Hegde R, Karunakara AC, Kush A. Effect of insect herbivory on the volatile profile of tomato cultivars. 2009;7.

32. Loreto F, Barta C, Brilli F, Nogues I. On the induction of volatile organic compound emissions by plants as consequence of wounding or fluctuations of light and temperature. Plant Cell Environ. 2006 Sep;29(9):1820–8.

33. Jud W, Vanzo E, Li Z, Ghirardo A, Zimmer I, Sharkey TD, et al. Effects of heat and drought stress on post-illumination bursts of volatile organic compounds in isoprene-emitting and non-emitting poplar: Post-illumination bursts in poplar. Plant, Cell & Environment. 2016 Jun;39(6):1204–15.

34. Zuo Z-J, Zhu Y-R, Bai Y-L, Wang Y. Volatile communication between Chlamydomonas reinhardtii cells under salt stress. Biochemical Systematics and Ecology. 2012 Feb;40:19–24.

35. Wang Y, Kandeler R. Promotion of Flowering by a Tumor Promoter. Journal of Plant Physiology. 1994 Nov;144(6):710–3.

36. Yang L, Han H, Liu M, Zuo Z, Zhou K, Lü J, et al. Overexpression of the Arabidopsis photorespiratory pathway gene, serine: glyoxylate aminotransferase (AtAGT1), leads to salt stress tolerance in transgenic duckweed (Lemna minor). Plant Cell Tiss Organ Cult. 2013 Jun;113(3):407–16.

37. Gamborg OL, Miller RA, Ojima K. Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research. 1968 Apr;50(1):151–8.

38. Zuo Z, Yang L, Chen S, Ye C, Han Y, Wang S, et al. Effects of nitrogen nutrients on the volatile organic compound emissions from Microcystis aeruginosa. Ecotoxicology and Environmental Safety. 2018 Oct;161:214–20.

39. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 2011 Jul;29(7):644–52.

Author contributions

Sun, J.S. provided resource support and orchestrated the arrangement. Yang, L. designed the experiment,, analyzed the data and wrote the manuscrip. Sun, J.G. finished the experiment and counted the experimental results. Yao, J. supervised the experimental project and the subsequent editing of the manuscript. Wang, Y. Y. completed the data statistics and forms. Yan, C.Y. completed the chart drawing and part of the experiment. Wu, J.Y. adjusted the overall format of the manuscript and completed basic experiments. Ren, Q.T. cultured the experimental plants. Zhao, L. provided technical assistance.

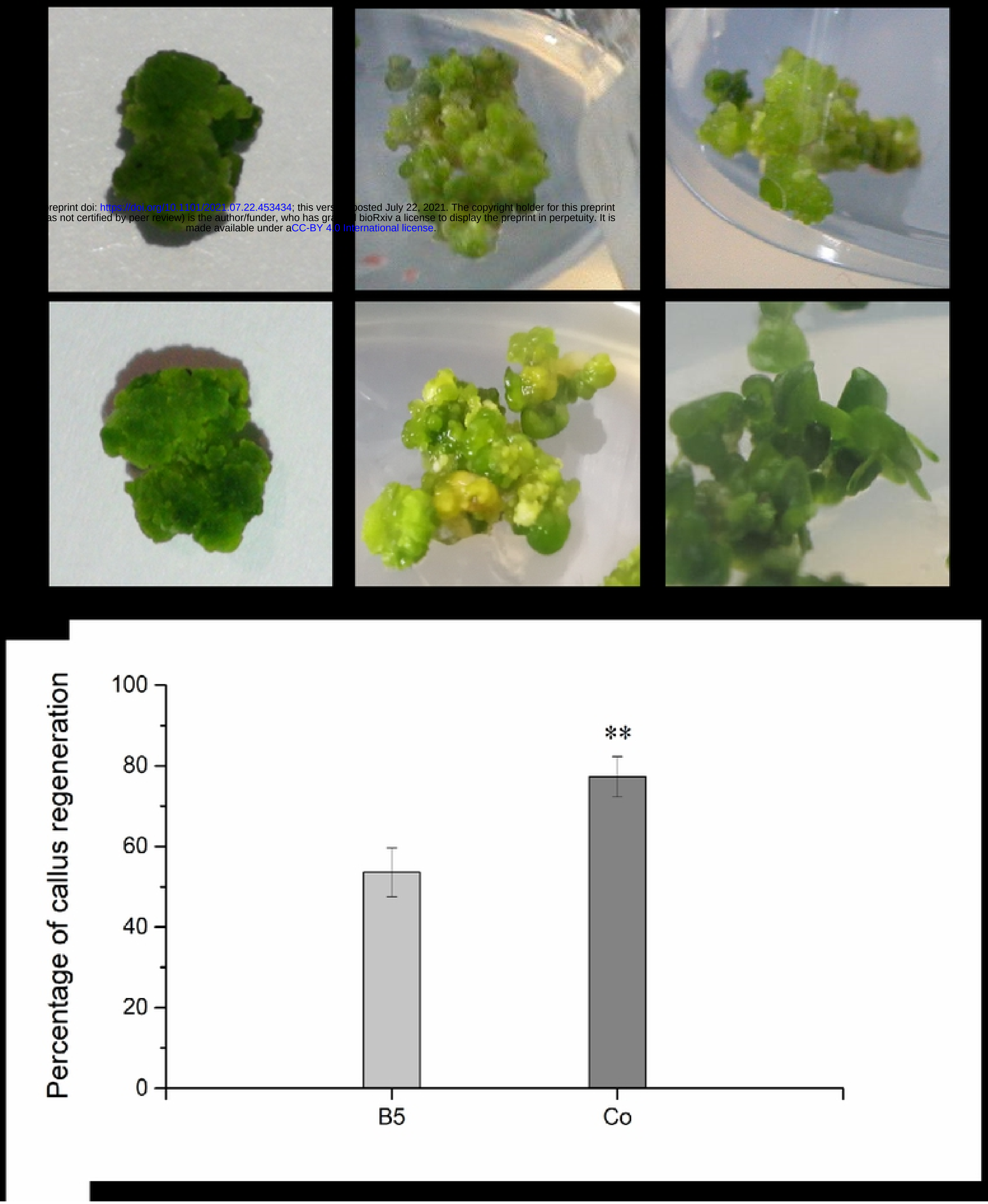


Figure1

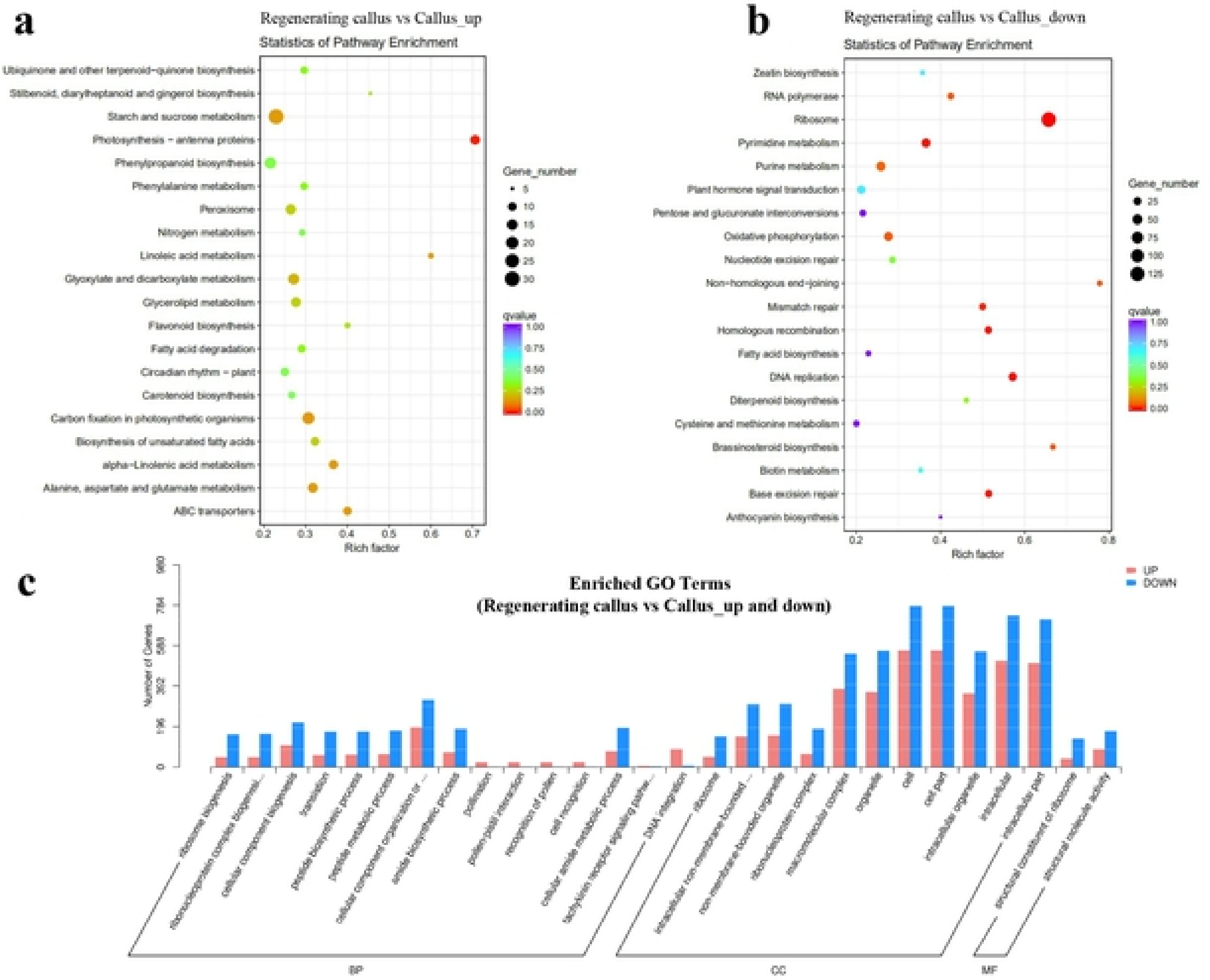
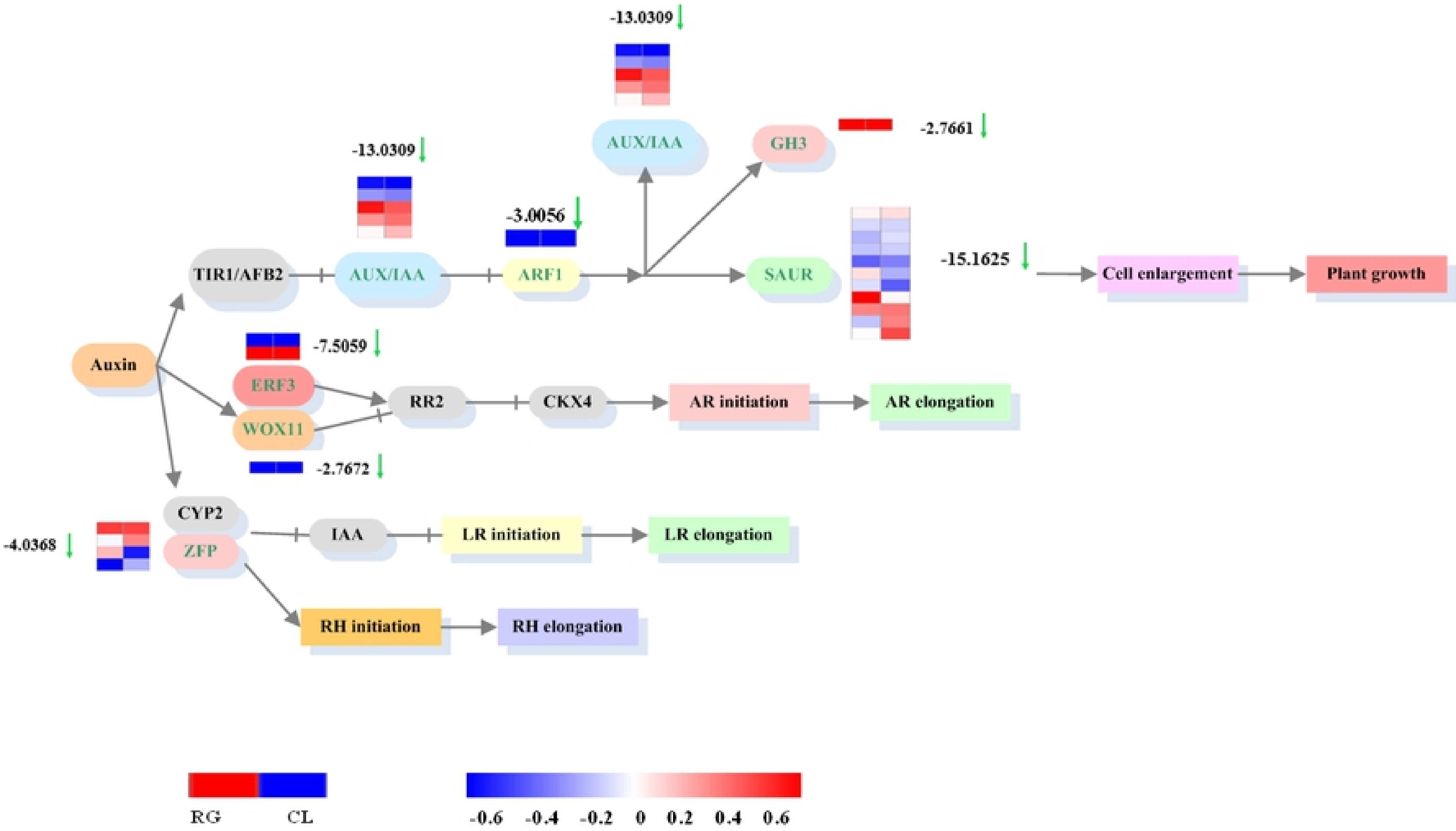

a
Regenerating callus vs Callus_up
Statistics of Pathway Enrichment
Ubiquinone and other terpenoid−quinone biosynthesis
Stilbenoid, diarylheptanoid and gingerol biosynthesis
Starch and sucrose metabolism
Photosynthesis − antenna proteins
Phenylpropanoid biosynthesis
Phenylalanine metabolism
Peroxisome
Nitrogen metabolism
Linoleic acid metabolism
Glyoxylate and dicarboxylate metabolism
Glycerolipid metabolism
Flavonoid biosynthesis
Fatty acid degradation
Circadian rhythm − plant
Carotenoid biosynthesis
Carbon fixation in photosynthetic organisms
Biosynthesis of unsaturated fatty acids
alpha−Linolenic acid metabolism
Alanine, aspartate and glutamate metabolism
ABC transporters
0.2
0.3
0.4
0.5
0.6
0.7
Rich factor
Gene_number
5
10
15
20
25
30
qvalue
1.00
0.75
0.50
0.25
0.00
b
Regenerating callus vs Callus_down
Statistics of Pathway Enrichment
Zeatin biosynthesis
RNA polymerase
Ribosome
Pyrimidine metabolism
Purine metabolism
Plant hormone signal transduction
Pentose and glucuronate interconversions
Oxidative phosphorylation
Nucleotide excision repair
Non−homologous end−joining
Mismatch repair
Homologous recombination
Fatty acid biosynthesis
DNA replication
Diterpenoid biosynthesis
Cysteine and methionine metabolism
Brassinosteroid biosynthesis
Biotin metabolism
Base excision repair
Anthocyanin biosynthesis
0.2
0.4
0.6
0.8
Rich factor
Gene_number
25
50
75
100
125
qvalue
1.00
0.75
0.50
0.25
0.00
c
Enriched GO Terms
(Regenerating callus vs Callus_up and down)
UP
DOWN
Number of Genes
0
196
392
588
784
980
ribosome biogenesis
ribonucleoprotein complex biogenesis...
cellular component biogenesis
translation
peptide biosynthetic process
peptide metabolic process
cellular component organization or ...
amide biosynthetic process
pollination
pollen-pistil interaction
recognition of pollen
cell recognition
cellular amide metabolic process
tachykinin receptor signaling pathw...
DNA integration
ribosome
intracellular non-membrane-bounded ...
non-membrane-bounded organelle
ribonucleoprotein complex
macromolecular complex
organelle
cell
cell part
intracellular organelle
intracellular
intracellular part
structural constituent of ribosome
structural molecule activity
BP
CC
MF

Figure2



-13.0309
AUX/IAA
GH3
-2.7661
-13.0309
-3.0056
TIR1/AFB2
AUX/IAA
ARF1
SAUR
-15.1625
Cell enlargement
Plant growth
-7.5059
Auxin
ERF3
RR2
CKX4
AR initiation
AR elongation
WOX11
-2.7672
CYP2
-4.0368
ZFP
IAA
LR initiation
LR elongation
RH initiation
RH elongation
RG
CL
-0.6
-0.4
-0.2
0
0.2
0.4
0.6

Figure3

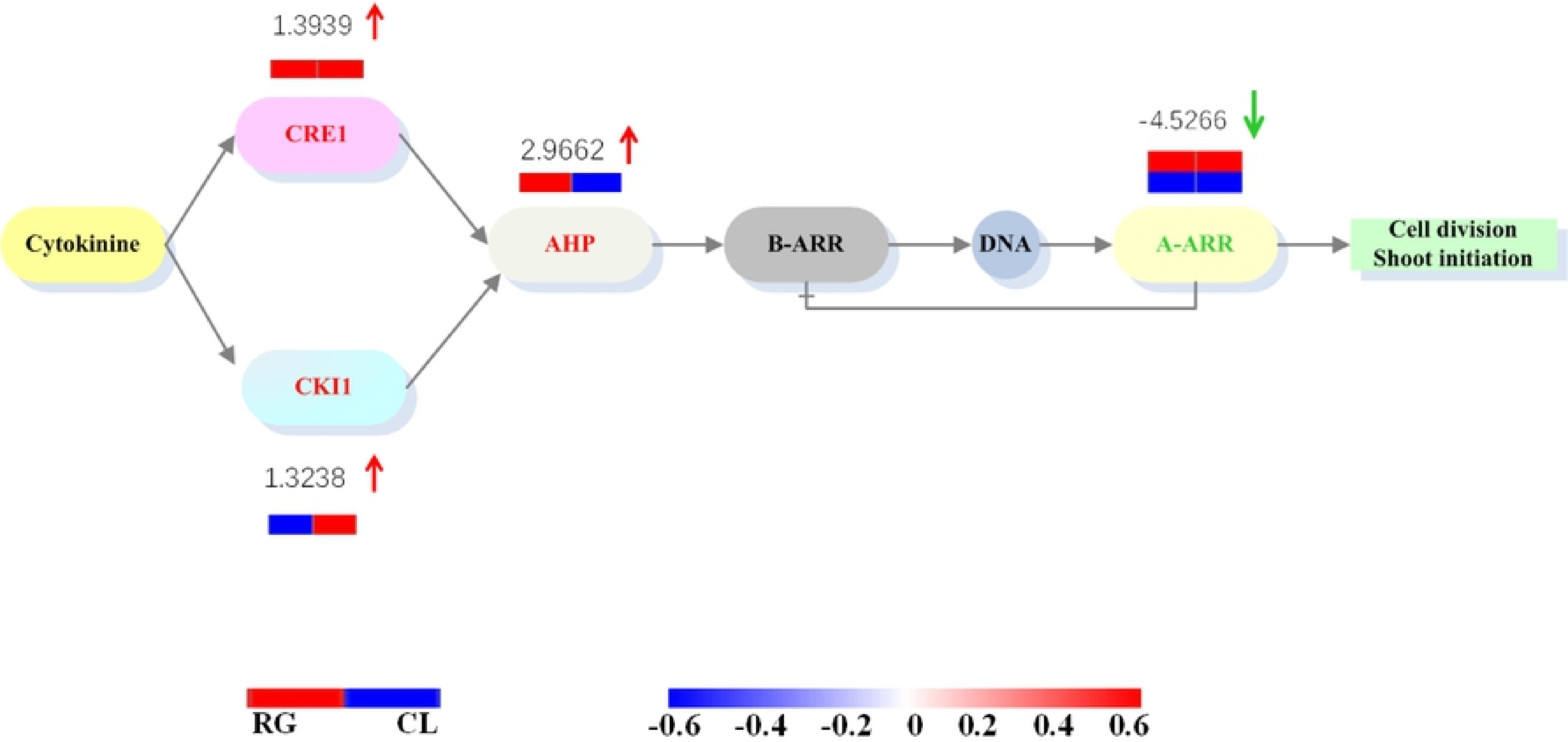
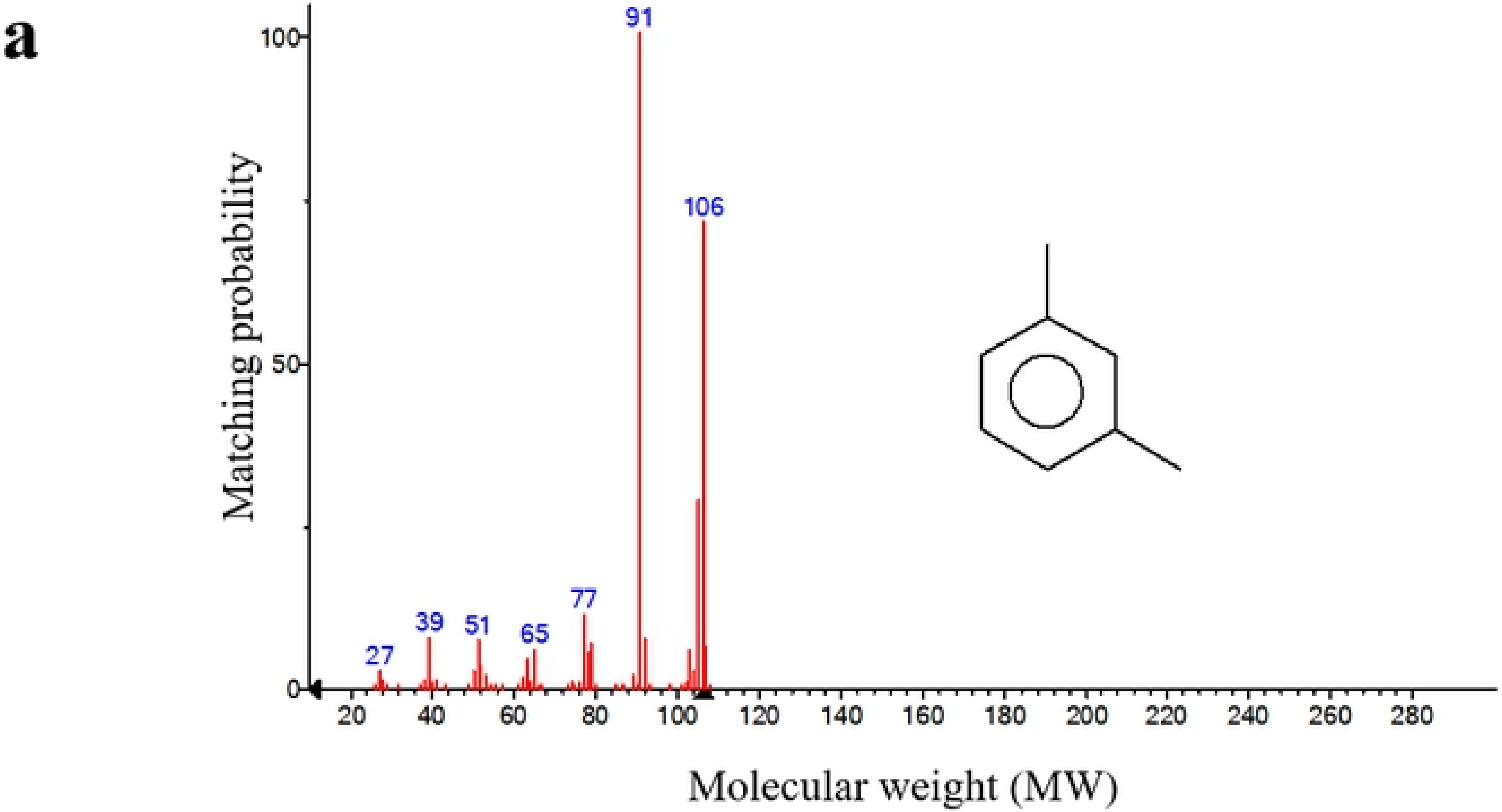
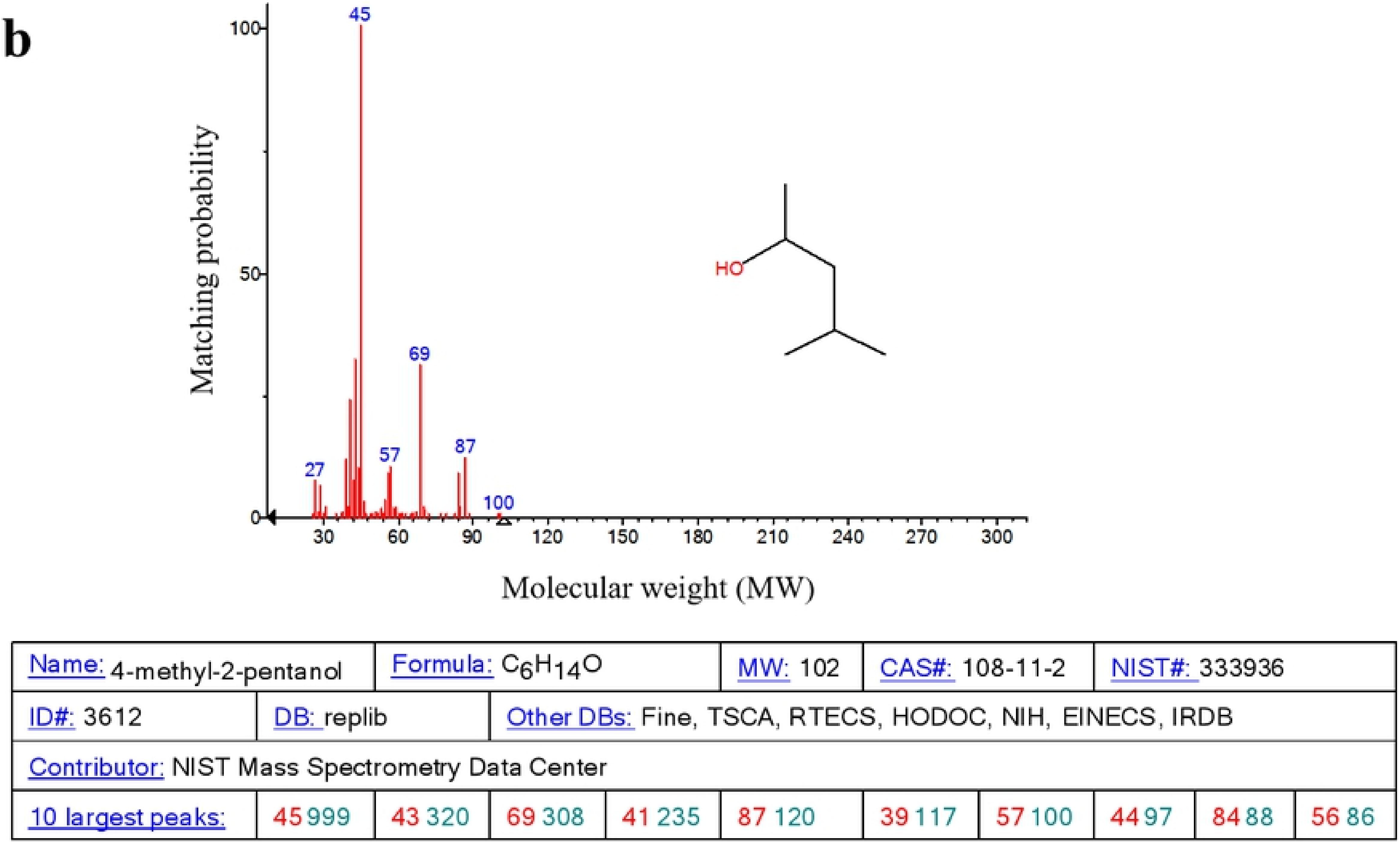

1.3939
CRE1
2.9662
-4.5266
Cytokinine
AHP
B-ARR
DNA
A-ARR
Cell division
Shoot initiation
CKI1
1.3238
RG
CL
-0.6
-0.4
-0.2
0
0.2
0.4
0.6

Figure4



Name: 1, 3-dimethyl benzene			Formula: C_8H_{10}		MW: 106	CAS#: 108-38-3		NIST#: 228063		
ID#: 11515	DB: replib		Other DBs: Fine, TSCA, RTECS, HODOC, NIH, EINECS, IRDB							
Contributor: Japan AIST/NIMC Database- Spectrum MS-NW- 45										
10 largest peaks:	91 999	106 709	105 287	77 112	87 120	39 76	92 75	51 74	79 70	65 59

Figure5a



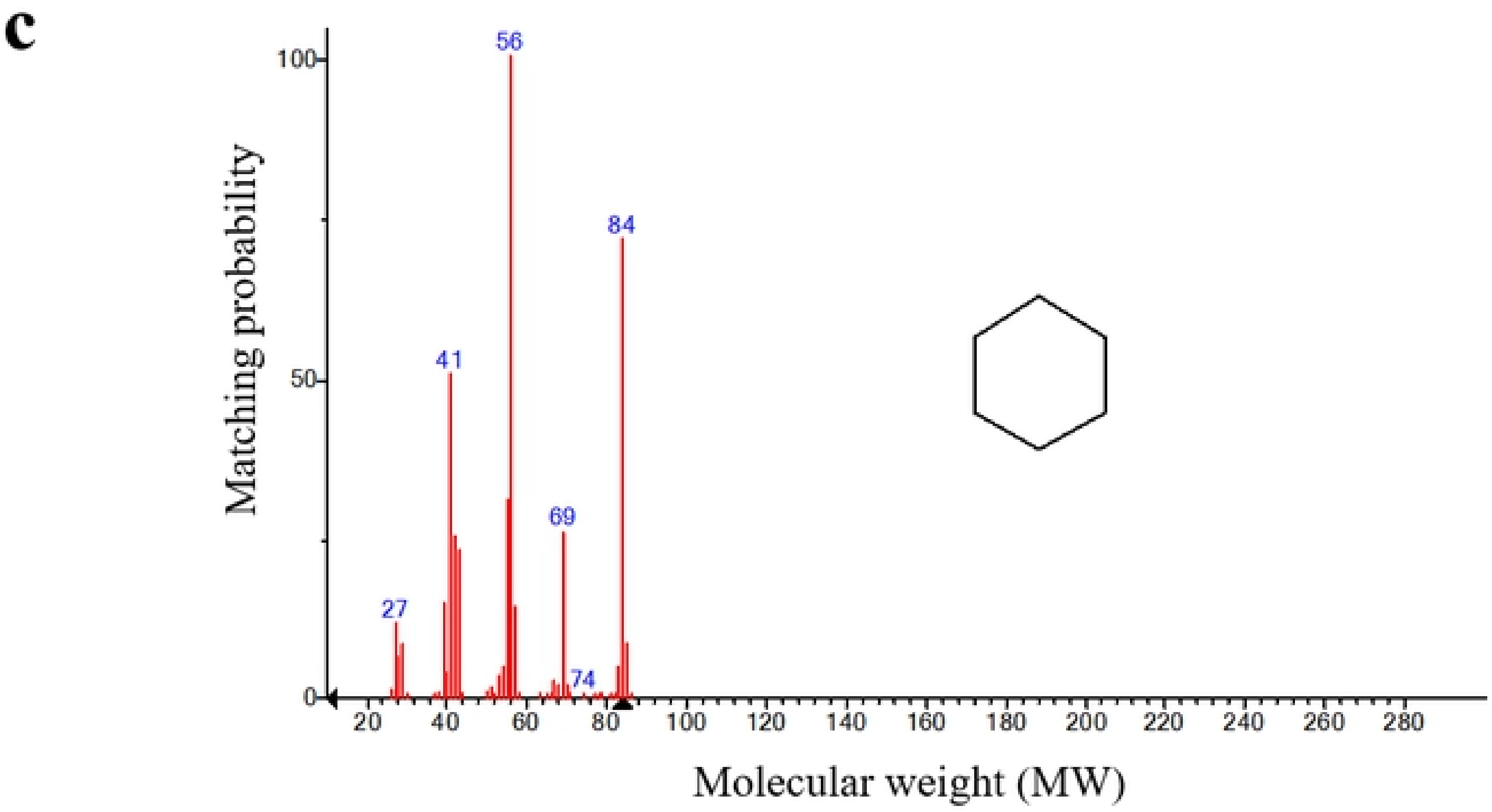
Name: 4-methyl-2-pentanol | Formula: $C_6H_{14}O$ | MW: 102 | CAS#: 108-11-2 | NIST#: 333936

ID#: 3612 | DB: replib | Other DBs: Fine, TSCA, RTECS, HODOC, NIH, EINECS, IRDB

Contributor: NIST Mass Spectrometry Data Center

10 largest peaks:	45 999	43 320	69 308	41 235	87 120	39 117	57 100	44 97	84 88	56 86

Figure5b



Name: Cyclohexane		Formula: $C_6H_{14}O$			MW: 84	CAS#: 110-82-7		NIST#: 228008		
ID#: 5014	DB: replib	Other DBs: Fine, TSCA, RTECS, EPA, HODOC, NIH, EINECS, IRDB								
Contributor: Japan AIST/NIMC Database- Spectrum MS-NW- 14										
10 largest peaks:	56 999	84 713	41 504	55 309	69 258	42 250	43 230	39 149	57 140	27 116

Figure5c

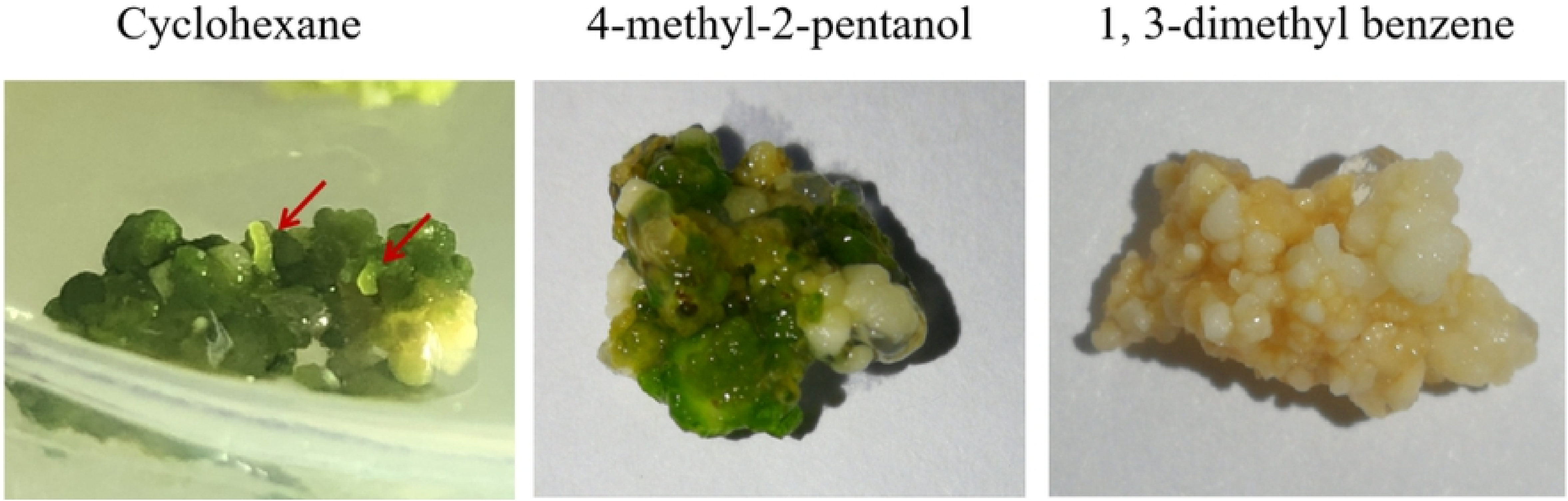


Figure6

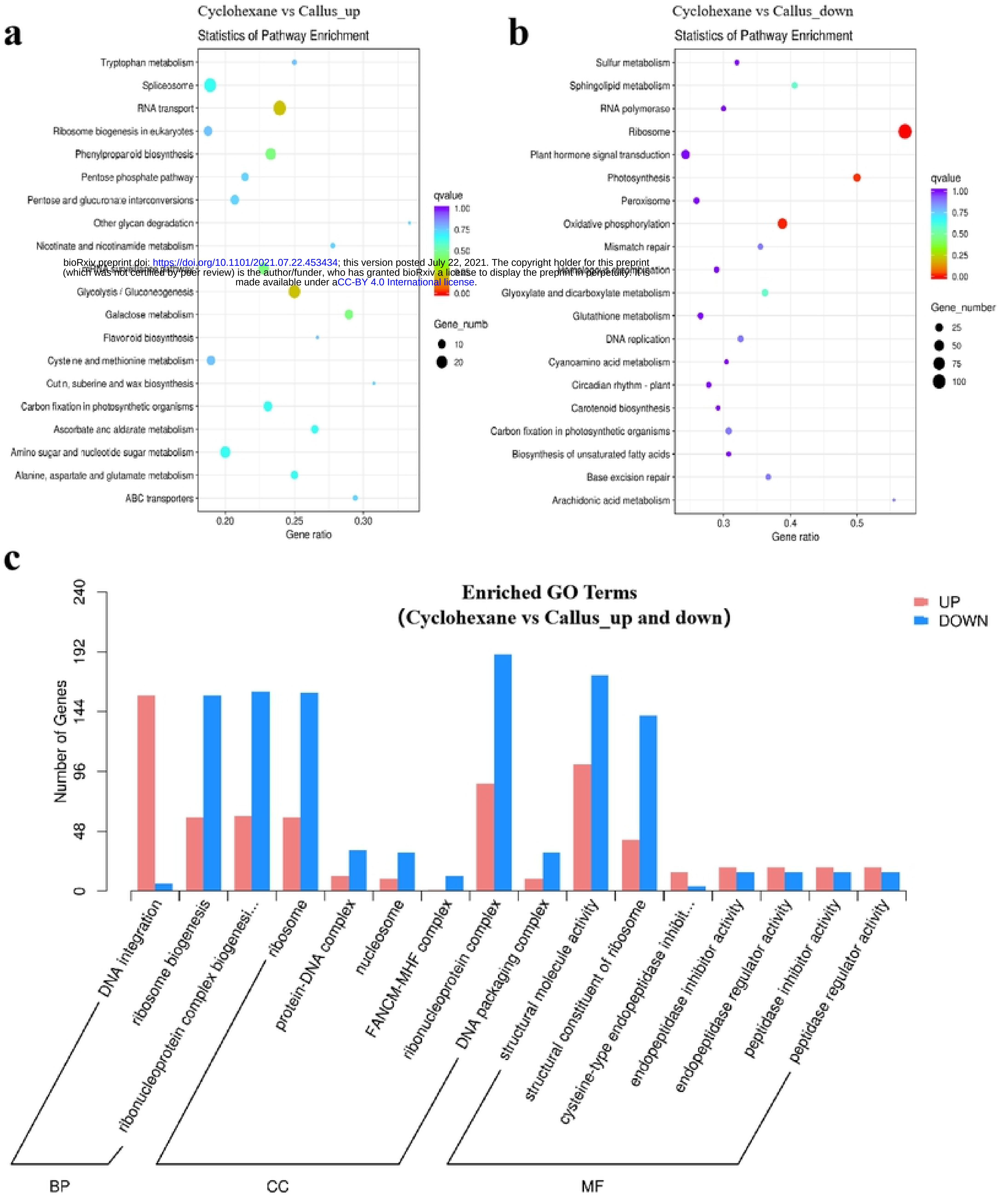

a
Cyclohexane vs Callus_up
Statistics of Pathway Enrichment
Tryptophan metabolism
Spliceosome
RNA transport
Ribosome biogenesis in eukaryotes
Phenylpropanoid biosynthesis
Pentose phosphate pathway
Pentose and glucuronate interconversions
Other glycan degradation
Nicotinate and nicotinamide metabolism
mRNA surveillance pathway
Glycolysis / Gluconeogenesis
Galactose metabolism
Flavonoid biosynthesis
Cysteine and methionine metabolism
Cutin, suberine and wax biosynthesis
Carbon fixation in photosynthetic organisms
Ascorbate and aldarate metabolism
Amino sugar and nucleotide sugar metabolism
Alanine, aspartate and glutamate metabolism
ABC transporters
0.20
0.25
0.30
Gene ratio
qvalue
1.00
0.75
0.50
0.25
0.00
Gene_numb
10
20
b
Cyclohexane vs Callus_down
Statistics of Pathway Enrichment
Sulfur metabolism
Sphingolipid metabolism
RNA polymerase
Ribosome
Plant hormone signal transduction
Photosynthesis
Peroxisome
Oxidative phosphorylation
Mismatch repair
Homologous recombination
Glyoxylate and dicarboxylate metabolism
Glutathione metabolism
DNA replication
Cyanoamino acid metabolism
Circadian rhythm - plant
Carotenoid biosynthesis
Carbon fixation in photosynthetic organisms
Biosynthesis of unsaturated fatty acids
Base excision repair
Arachidonic acid metabolism
0.3
0.4
0.5
Gene ratio
qvalue
1.00
0.75
0.50
0.25
0.00
Gene_number
25
50
75
100
c
Enriched GO Terms
(Cyclohexane vs Callus_up and down)
UP
DOWN
Number of Genes
0
48
96
144
192
240
DNA integration
ribosome biogenesis
ribonucleoprotein complex biogenesi...
ribosome
protein-DNA complex
nucleosome
FANCM-MHF complex
ribonucleoprotein complex
DNA packaging complex
structural molecule activity
structural constituent of ribosome
cysteine-type endopeptidase inhibit...
endopeptidase inhibitor activity
endopeptidase regulator activity
peptidase inhibitor activity
peptidase regulator activity
BP
CC
MF

Figure7

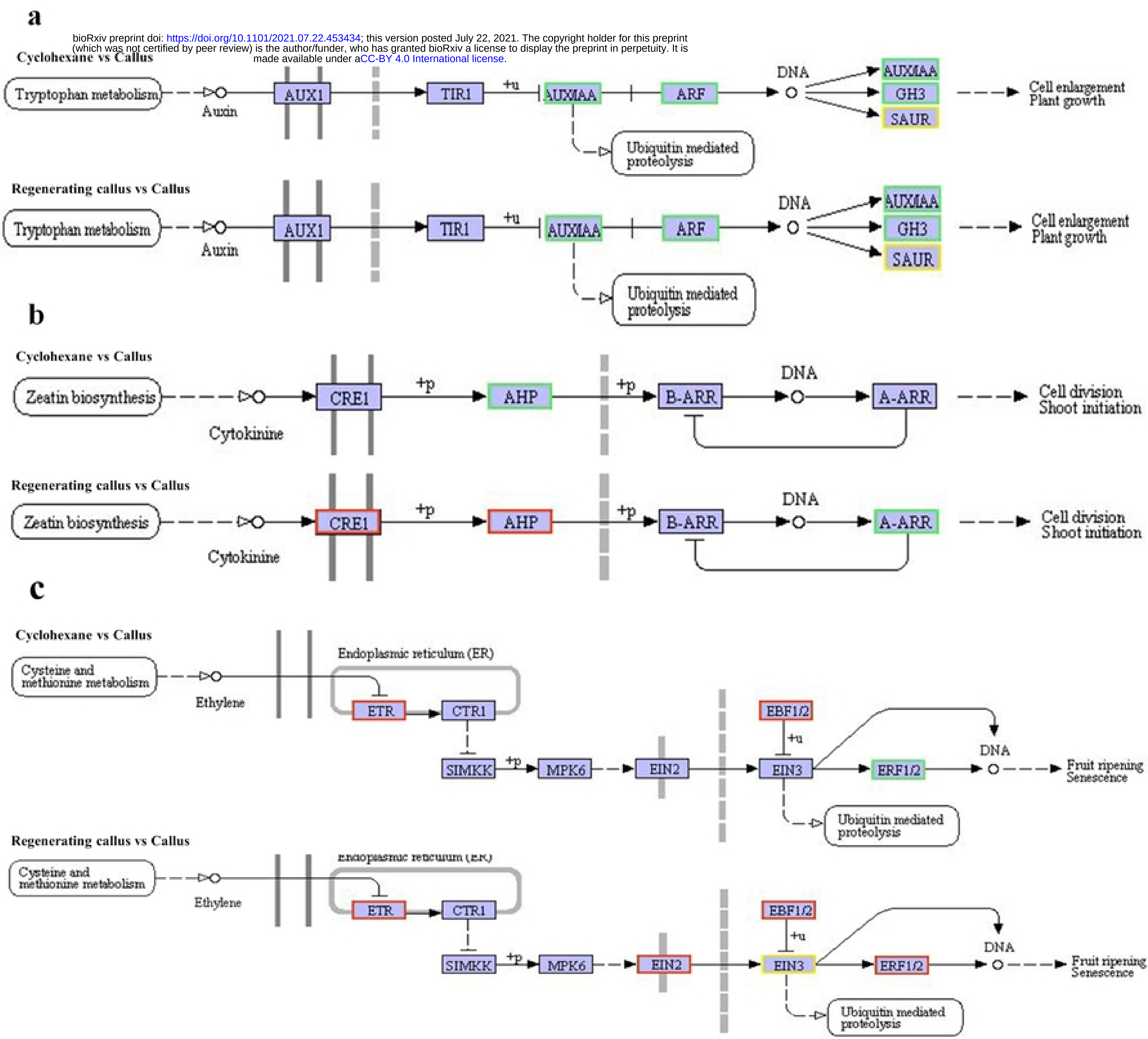

a
Cyclohexane vs Callus
Tryptophan metabolism
Auxin
AUX1
TIR1
+u
AUX/IAA
ARF
DNA
AUX/IAA
GH3
SAUR
Cell enlargement
Plant growth
Ubiquitin mediated proteolysis
Regenerating callus vs Callus
Tryptophan metabolism
Auxin
AUX1
TIR1
+u
AUX/IAA
ARF
DNA
AUX/IAA
GH3
SAUR
Cell enlargement
Plant growth
Ubiquitin mediated proteolysis
b
Cyclohexane vs Callus
Zeatin biosynthesis
Cytokinine
CRE1
+p
AHP
+p
B-ARR
DNA
A-ARR
Cell division
Shoot initiation
Regenerating callus vs Callus
Zeatin biosynthesis
Cytokinine
CRE1
+p
AHP
+p
B-ARR
DNA
A-ARR
Cell division
Shoot initiation
c
Cyclohexane vs Callus
Cysteine and methionine metabolism
Ethylene
Endoplasmic reticulum (ER)
ETR
CTR1
SIMKK
+p
MPK6
EIN2
EBF1/2
+u
EIN3
ERF1/2
DNA
Fruit ripening
Senescence
Ubiquitin mediated proteolysis
Regenerating callus vs Callus
Cysteine and methionine metabolism
Ethylene
Endoplasmic reticulum (ER)
ETR
CTR1
SIMKK
+p
MPK6
EIN2
EBF1/2
+u
EIN3
ERF1/2
DNA
Fruit ripening
Senescence
Ubiquitin mediated proteolysis

Figure8abc

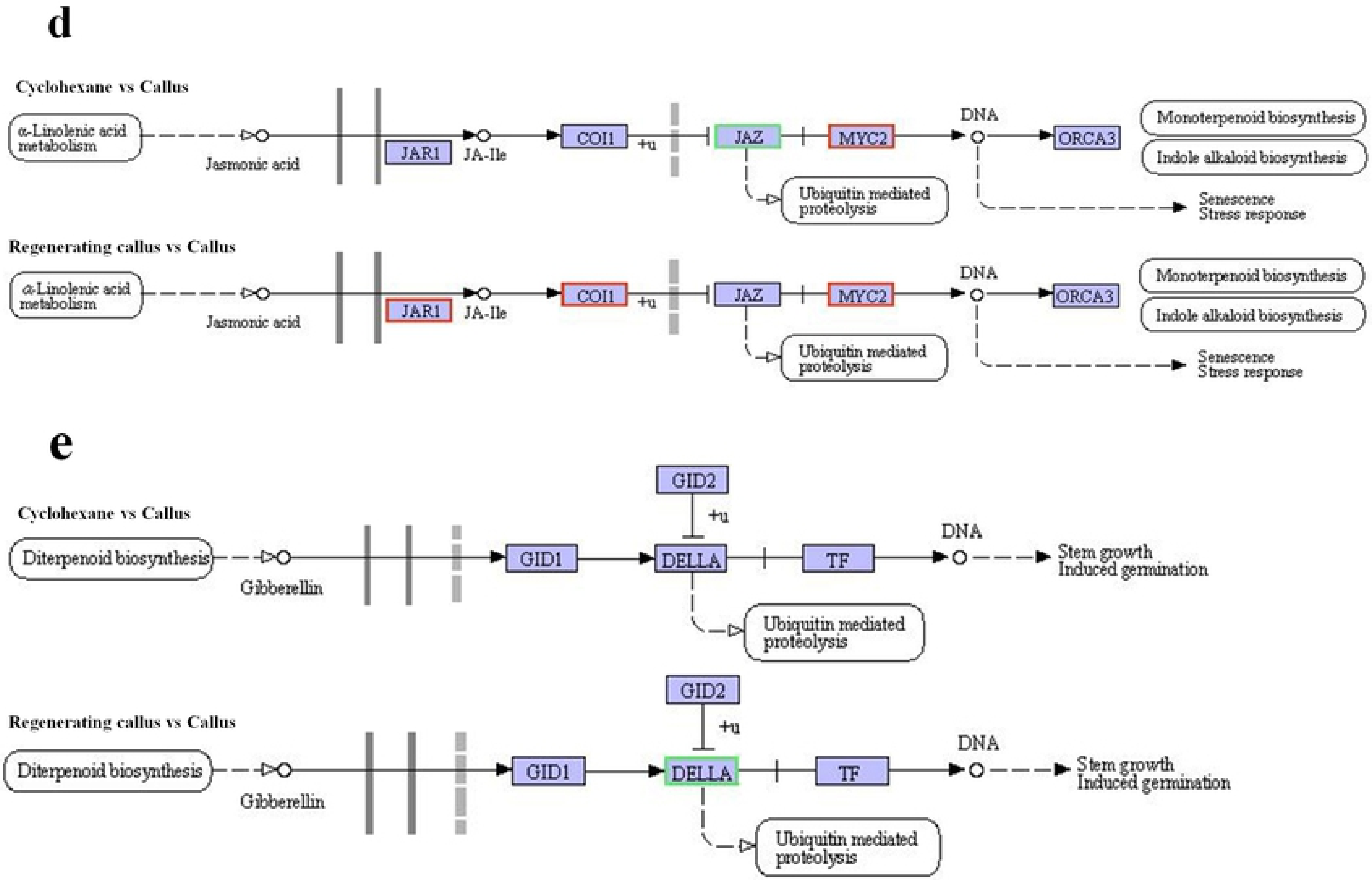

d
Cyclohexane vs Callus
α-Linolenic acid metabolism
Jasmonic acid
JAR1
JA-Ile
COI1
+u
JAZ
MYC2
DNA
ORCA3
Monoterpenoid biosynthesis
Indole alkaloid biosynthesis
Ubiquitin mediated proteolysis
Senescence
Stress response
Regenerating callus vs Callus
α-Linolenic acid metabolism
Jasmonic acid
JAR1
JA-Ile
COI1
+u
JAZ
MYC2
DNA
ORCA3
Monoterpenoid biosynthesis
Indole alkaloid biosynthesis
Ubiquitin mediated proteolysis
Senescence
Stress response
e
Cyclohexane vs Callus
GID2
+u
Diterpenoid biosynthesis
Gibberellin
GID1
DELLA
TF
DNA
Stem growth
Induced germination
Ubiquitin mediated proteolysis
Regenerating callus vs Callus
GID2
+u
Diterpenoid biosynthesis
Gibberellin
GID1
DELLA
TF
DNA
Stem growth
Induced germination
Ubiquitin mediated proteolysis

Figure8de

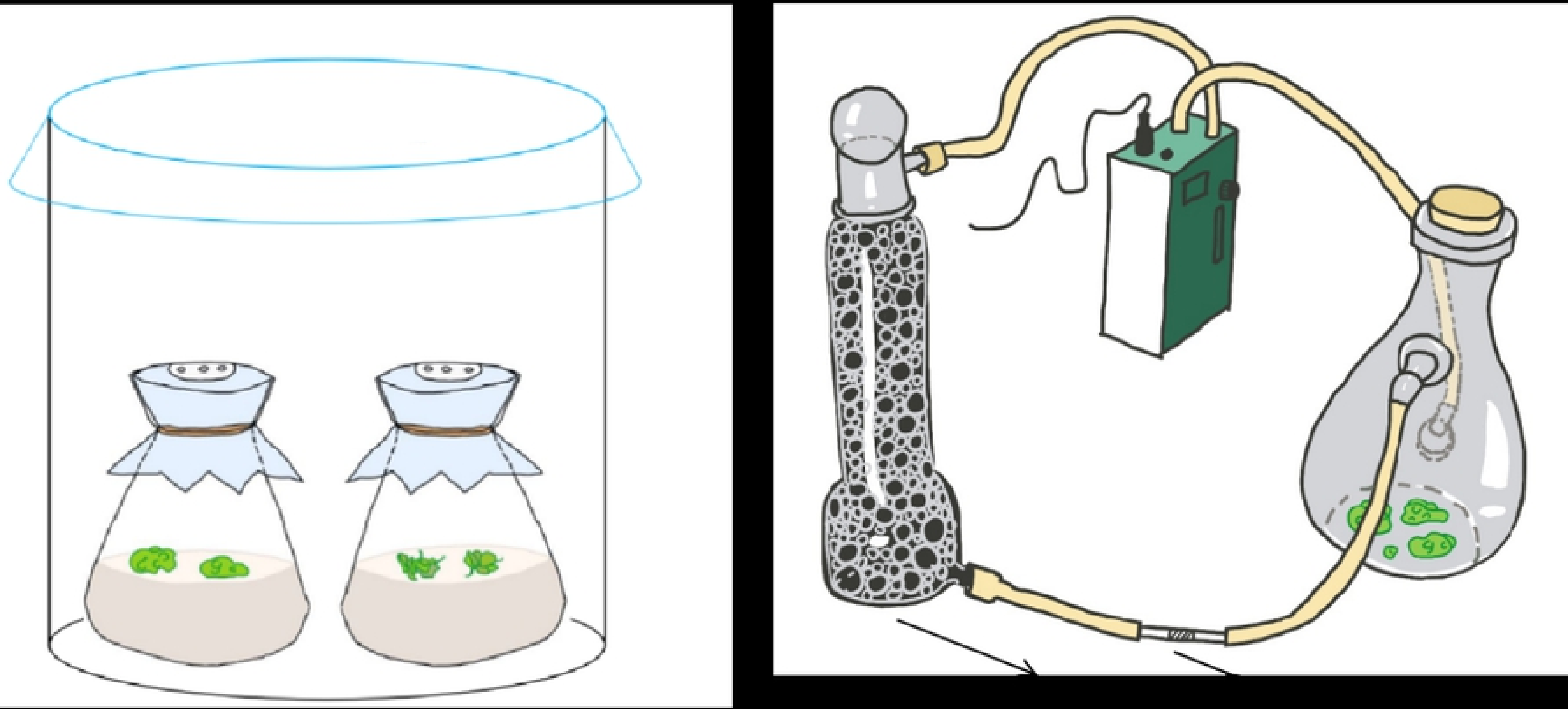
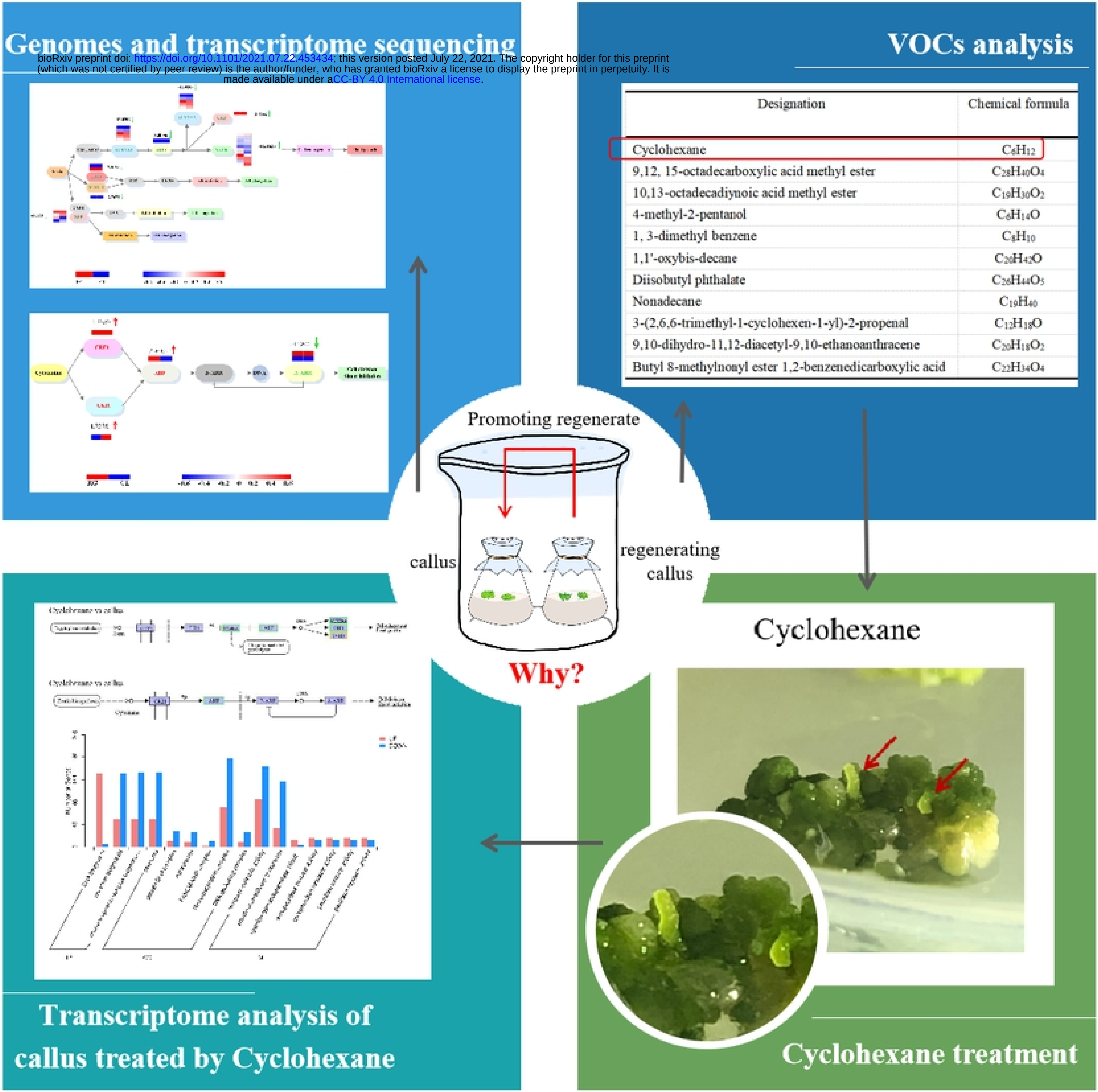


Figure9



Designation	Chemical formula
Cyclohexane	C_6H_{12}
9,12, 15-octadecarboxylic acid methyl ester	$C_{28}H_{40}O_4$
10,13-octadecadiynoic acid methyl ester	$C_{19}H_{30}O_2$
4-methyl-2-pentanol	$C_6H_{14}O$
1, 3-dimethyl benzene	C_8H_{10}
1,1'-oxybis-decane	$C_{20}H_{42}O$
Diisobutyl phthalate	$C_{26}H_{44}O_5$
Nonadecane	$C_{19}H_{40}$
3-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-propenal	$C_{12}H_{18}O$
9,10-dihydro-11,12-diacetyl-9,10-ethanoanthracene	$C_{20}H_{18}O_2$
Butyl 8-methylnonyl ester 1,2-benzenedicarboxylic acid	$C_{22}H_{34}O_4$

graphical abstract

Fig. 1 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.

Fig. 2 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.

Fig. 3 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 log10 (FPKM+1) from high to low. Red meant high expression, blue meant low expression.

Fig. 4 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. CKI1, CRE1 and AHP were up-regulated, but negative feedback regulator A-ARR was down-reg+ulated. The colour in this figure legend from red to blue, which meant log10 (FPKM+1) from high to low. Red meant high expression, blue meant low expression.

Fig. 5 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.

Fig. 6 Effects of 16 days' treatment of callus by three VOCs (cyclohexane, 4-methyl-2-pentanol and 1, 3-dimethyl benzene).

Fig. 7 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.

Fig. 8 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.

Fig. 9 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.