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

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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 analysed 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 plant¹⁻². Regeneration was widely used for vegetative propagation of excellent variety, detoxification and the obtain of transgenic crops³⁻⁴. 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 content⁵, and distinguished tolerance for a variety of toxic substances⁶⁻⁷, has been applied as a monocotylous model plant for gene-expression systems. In duckweed, stable transformation mediated by *Agrobacterium* depends on efficient callus regeneration protocols.

Here, we use transcriptome sequencing technology to explore the molecular mechanism of plant hormones regulating callus regeneration⁸. 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 hormones⁵. The balance of auxin and cytokinin is the basis for vitro tissue culture⁹. 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 allelopathy¹⁰⁻¹¹. For example, the VOCs of *Artemisia frigida* Willd play an allelopathic role on the seed germination of pasture grasses¹². 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

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 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 repair", "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 log² 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 HOMEODOMAIN 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 log² 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 receptors¹³⁻¹⁴, have been up-regulated in

regenerating callus. Histidine phosphate transfer protein (AHP), interacting with CRE1 and CKI1, has been up-regulated by 2.9662 log² 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 cycle¹⁵⁻¹⁶. A-ARR has been down-regulated by 4.5266 log² 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*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 cyclohexane peak area of the callus (0.85*10⁷), the cyclohexane peak area of the regenerating callus 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 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 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

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 analysed to investigate the potential functions of

KEGGs and DEGs in the callus treat hydrolyzing O-glycosyl compounds by cyclohexane. As shown in Fig. 7a, “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. 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 “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 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. 7c).

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

Comparison 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 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 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	Cyclohexa ne vs Callus_Re ad_count	Callus_Read_ count	Regenerating callus vs Callus_log2Fol d Change	Cyclohexan e 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	/	126.89808 6	427.8033881	/	-1.7564	1.96E- 13	1.49E- 12
auxin-responsive protein IAA	Cluster-7966. 10326	/	2912.3188 25	6803.456966	/	-1.2242	1.54E- 20	1.99E- 19
auxin-responsive protein IAA	Cluster-7966. 9984	/	757.42813 55	2282.819782	/	-1.5911	5.19E- 39	2.10E- 37
auxin-responsive protein IAA	Cluster-7966. 7990	/	882.14582 83	7136.36841	/	-3.0168	1.35E- 109	9.48E- 107
auxin-responsive protein IAA	Cluster-7966. 3823	/	24.871927 46	135.7070458	/	-2.4536	3.10E- 16	2.89E- 15
auxin-responsive protein IAA	Cluster-7966. 9412	/	68.990295 52	688.2924139	/	-3.3241	1.37E- 77	3.22E- 75
auxin-responsive protein IAA	Cluster-7966. 8499	/	2134.9153 79	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	/	821.41272 57	2005.683263	/	-1.2889	1.79E- 22	2.67E- 21
auxin response factor	Cluster-7966. 4925	/	2164.1001 71	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	/	2164.1001 71	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	/	0.0052 791	0.0175 25
SAUR family	Cluster-2913.	88.1496484	/	25.01594296	1.8182	/	1.24E-	7.06E-

protein	0						05	05
SAUR family	Cluster-3967.	0.343464407	/	9.713352671	-4.7418	/	0.0020	0.0075
protein	0						559	26
SAUR family	Cluster-6172.	95.9333365	/	263.0061674	-1.4501	/	0.0004	0.0019
protein	19466						6131	407
SAUR family	Cluster-6172.	33.87690781	/	139.8713152	-2.0608	/	1.44E-	1.61E-
protein	1791						09	08
SAUR family	Cluster-6172.	200.5704202	/	541.4632883	-1.4326	/	3.18E-	2.84E-
protein	18366						08	07
SAUR family	Cluster-6172.	61.01981071	/	123.8718257	-1.034	/	0.0078	0.0248
protein	17182						308	21
SAUR family	Cluster-6172.	11.05396801	/	235.4106465	-4.4395	/	6.48E-	7.95E-
protein	17013						30	28
SAUR family	Cluster-5374.	11.47905177	/	33.5277143	-1.5175	/	0.0161	0.0466
protein	0						76	31
SAUR family	Cluster-6172.	51.66034612	/	993.507599	-4.2614	/	8.32E-	1.46E-
protein	13654						34	31
SAUR family	Cluster-1875.	/	191.21578	56.27788869	/	1.7696	1.48E-	1.04E-
protein	0		98				12	11
SAUR family	Cluster-7966.	/	9.7587392	60.95804546	/	-2.6418	4.36E-	1.89E-
protein	1555		58				07	06
SAUR family	Cluster-3489.	/	26.374019	109.0773706	/	-2.0508	1.81E-	1.17E-
protein	0		35				11	10
SAUR family	Cluster-7372.	/	50.624257	235.2504774	/	-2.2123	5.44E-	4.64E-
protein	0		17				15	14
SAUR family	Cluster-7966.	/	163.41277	768.464197	/	-2.2365	2.29E-	6.09E-
protein	7594		87				31	30
SAUR family	Cluster-7966.	/	217.55911	523.5357639	/	-1.2668	5.94E-	6.34E-
protein	11015		06				18	17
SAUR family	Cluster-7966.	/	222.97542	490.5957546	/	-1.1396	7.22E-	3.04E-
protein	4605		24				07	06
SAUR family	Cluster-7966.	/	23.922131	206.5310611	/	-3.1169	4.05E-	8.18E-
protein	15997		49				27	26
SAUR family	Cluster-7966.	/	88.770555	876.2847193	/	-3.3056	1.04E-	9.68E-
protein	11607		71				55	54
Ethylene-responsive	Cluster-6172.	96.47590512	/	1004.215639	-3.3831	/	1.88E-	2.15E-
transcription factor 3	9509						29	27
Ethylene-responsive	Cluster-6172.	97.2605432	/	1695.501734	-4.1228	/	1.27E-	2.68E-
transcription factor 3	14530						35	33
cysteine-rich	Cluster-6172.	11.82009373	/	80.9107182	-2.7672	/	1.41E-	9.59E-
receptor	505						06	06
Zinc finger	Cluster-6172.	66.60847947	/	133.4054107	-1.0012	/	0.0117	0.0352
	2152						46	46
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	/	0.0001	0.0006
							3142	126
Zinc finger	Cluster-2857.0	1.304040245	/	11.91316299	-3.1698	/	0.0031	0.0111
							694	29

Table 3 Gene expression in plant regeneration of Cytokine

Description	Gene-id	Regenerating callus vs Callus_Read_c ount	Cyclohexa ne vs Callus_Re ad_count	Callus_Re ad_count	Regenerating callus vs Callus_log2Fo ld Change	Cyclohexa ne vs Callus_log 2Fold 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-13
histidine-containing phosphotransfer peotein	Cluster-6172.20325	165.1914481	/	21.04093208	2.9662	/	3.08E-13	5.97E-13
histidine-containing phosphotransfer protein	Cluster-7966.4523	/	264.877881	801.4125562	/	-1.5983	3.14E-23	4.90E-23
histidine-containing phosphotransfer protein	Cluster-2808.0	/	3.444274279	19.24749268	/	-2.4633	0.0049514	0.01214
two-component response regulator ARR-A family	Cluster-6172.12818	118.8137608	/	737.8963989	-2.6308	/	4.93E-15	1.22E-15
two-component response regulator ARR-A family	Cluster-4229.0	14.43168456	/	54.47624248	-1.8958	/	0.0091425	0.028425
Histidine kinase CKII	Cluster-6172.4116	765.058398	/	305.7574303	1.3238	/	5.13E-10	6.17E-10

213

Table 4 Gene expression in plant regeneration of Brassinosteroid

Description	Gene-id	Regenerating callus vs Callus_Read_c ount	Cyclohexa ne vs Callus_Re ad_count	Callus_Re ad_count	Regenerating callus vs Callus_log2F old Change	Cyclohexa ne vs Callus_log 2 Fold Change	pval	padj
BRI1 kinase inhibitor 1	Cluster-6172.8113	291.510962	/	769.9837	-1.4001	/	3.62E-07	2.73E-07
brassinosteroid resistant 1/2	Cluster-6172.9208	243.3127962	/	545.7915	-1.1678	/	3.52E-07	2.66E-07
brassinosteroid resistant 1/2	Cluster-6401.0	/	43.03949153	146.1382	/	-1.7659	9.00E-11	5.52E-11

brassinosteroid resistant 1/2	Cluster-6172.2 0298	33.13147023	/	156.7584	-2.2381	/	5.50E-0 7	4.01E
cyclin D3	Cluster-6172.6 746	932.3401808	/	2811.633	-1.5916	/	3.80E-2 1	1.91E

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215 **Discussion**

216 In line with previous studies, we established an effective way to *in vitro* callus
217 regeneration in duckweed. Interestingly, we found that one regenerating callus
218 promoted another callus to regenerate. Genomes and transcriptome sequencing
219 (especially plant hormones) and volatile substances were studied to reveal the
220 molecule framework of plant regeneration in duckweed .

221 Plant hormones played a crucial role during callus regeneration¹. Through our
222 study, we hope to have a deeper understanding of the regulatory mechanism of callus
223 regeneration. Callus were induced by auxin, similar as lateral root primordium¹⁷⁻¹⁹. In
224 Arabidopsis, the callus tissue formed root stem cell niche, by regulation the
225 expression of root stem cell regulators, including WOX²⁰⁻²³. According to our results,
226 ARF, AUX/IAA, GH3, ARF1, SAUG and other response factor have been
227 down-regulated significantly during the callus redifferentiation (Fig. 3). In the auxin
228 signaling pathway, the interaction between ARF and AUX /IAA could regulate the
229 genes expression of auxin early response. Moreover, ERF3, WOX11 and ZFP were
230 found to be related to the ARs, LRs and RHs of initiation in *Spirodela*⁸, which might
231 lead associated to the regeneration in duckweed.

232 Cytokinins and auxin have synergistic or antagonistic interactions with each
233 other²⁴. As a phytohormone, cytokinin could not only control key aspects of

environmental responses, such as biotic and abiotic stress responses, but also regulate various developmental processes including cell proliferation, leaf formation, and root formation and growth²⁵⁻²⁶. Cytokinins promoted plant regeneration by regulating the generation of somatic embryogenesis in *Fumariaceae* and Rice²⁷⁻²⁸. 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 expression 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 hormones²⁹. 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 allelopathy³⁰. Plants in different growing environments, such as biological stress or abiotic stress, might release different VOCs to improve their resistance to external interference³¹⁻³³. In previous studies, VOCs have been shown to mediate cell to cell communication, thereby leading to stress responses in plants³⁴. 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/IAA⁸. 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 indiciated 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 al³⁵⁻³⁶. 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 culture in 1968³⁷. 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 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 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 Fluorometer (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 reads⁶. 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 default³⁹. 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.

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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

Figure2

Figure3

Figure4

a

<u>Name:</u> 1, 3-dimethyl benzene	<u>Formula:</u> C8H10	<u>MW:</u> 106	<u>CAS#:</u> 108-38-3	<u>NIST#:</u> 228063						
<u>ID#:</u> 11515	<u>DB:</u> replib	<u>Other DBs:</u> Fine, TSCA, RTECS, HODOC, NIH, EINECS, IRDB								
<u>Contributor:</u> Japan AIST/NIMC Database- Spectrum MS-NW- 45										
<u>10 largest peaks:</u>	91 999	106 709	105 287	77 112	87 120	39 76	92 75	51 74	79 70	65 59

Figure5a

b

<u>Name:</u> 4-methyl-2-pentanol		<u>Formula:</u> C ₆ H ₁₄ O		<u>MW:</u> 102		<u>CAS#:</u> 108-11-2		<u>NIST#:</u> 333936			
<u>ID#:</u> 3612		<u>DB:</u> replib		<u>Other DBs:</u> Fine, TSCA, RTECS, HODOC, NIH, EINECS, IRDB							
<u>Contributor:</u> NIST Mass Spectrometry Data Center											
<u>10 largest peaks:</u>		45 999	43 320	69 308	41 235	87 120	39 117	57 100	44 97	84 88	56 86

Figure5b

c

<u>Name:</u> Cyclohexane		<u>Formula:</u> C6H14O		<u>MW:</u> 84	<u>CAS#:</u> 110-82-7	<u>NIST#:</u> 228008				
<u>ID#:</u> 5014	<u>DB:</u> replib		<u>Other DBs:</u> Fine, TSCA, RTECS, EPA, HODOC, NIH, EINECS, IRDB							
<u>Contributor:</u> Japan AIST/NIMC Database- Spectrum MS-NW- 14										
<u>10 largest peaks:</u>	56 999	84 713	41 504	55 309	69 258	42 250	43 230	39 149	57 140	27 116

Figure 5c

Cyclohexane

4-methyl-2-pentanol

1, 3-dimethyl benzene

Figure6

Figure7

a

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b

c

Figure8abc

d

Cyclohexane vs Callus

Regenerating callus vs Callus

e

Cyclohexane vs Callus

Regenerating callus vs Callus

Figure8de

Figure9

Genomes and transcriptome sequencing

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VOCs analysis

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	$C_{22}H_{34}O_4$

Why?

Cyclohexane

Cyclohexane treatment

Transcriptome analysis of callus treated by Cyclohexane

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.

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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 $\log_{10}(\text{FPKM}+1)$ 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-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.

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).

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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.