Expression of Penaeidins 3a in Transgenic Duckweed Confers Antibacterial Ability

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Research

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Abstract

Background

With the development of aquaculture, fish and shrimp diseases have been paid more and more attention in the world. How to improve the immunity of aquatic animals was an urgent problem to be solved. Duckweed (*Lemnaceae*), as a eukaryote, could be an ideal feedstock for the production of antimicrobial peptides.

Result

Penaeidins 3a (Pen 3a) from *Litopenaeus vannamei* was expressed under the control of CaMV-35S promoter in duckweed, *Lemna turionifera* 5511. Bacteriostatic test by Pen3a duckweed extract showed the antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Transcriptome analysis of WT and Pen3a duckweed showed different results, and the protein metabolic process was the most up-regulated DEGs. In Pen 3a transgenic duckweed, the expression of sphingolipid metabolism and phagocytosis process-related genes have been significantly up-regulated. Quantitative proteomics suggested a remarkable difference in protein enrichment in metabolic pathways.

Conclusion

Our study provide a novel solution on aquaculture and water purification. The Pen 3a transgenic duckweed extraction inhibit the growth of gram-negative bacteria, gram-positive bacteria, which could be applied to control the bacteria in lake. The results could lay the foundation for the subsequent production of antibiotics.

Background

Duckweed (*Lemnaceae*) could be applied as an alternative feedstock for biofuels and bioreactors with several advantages. Firstly, the biomass of duckweed can double in 2 or 3 days with a high production than other plants [1] and maybe harvest for various purposes including bioreactors. Secondly, duckweed is a widely distributed aquatic floating plant with high adaptability[2], leading to salt stress tolerance in transgenic duckweed (*Lemna minor*). And duckweed grows year-round in some water systems with a warm climate. Thirdly, the protein content of duckweed is very high, which is suitable for expressing foreign protein effectively[3]. Moreover, duckweed, because of its high nutritional value, could be employed as human food and animal feed[4]. Therefore, duckweed could be applied as an ideal feedstock for biofuels, animal feed, and bioreactors.

Antimicrobial peptides (AMPs) play a role in resisting bacteria, viruses, and other microorganisms[5], including resistance disease which infected aquatic organisms. Penaeid shrimp aquaculture, with a value

of about US\$32 billion per year, has been consistently affected worldwide by devastating diseases that cause a severe loss in production[6]. Antibiotics have been suggested for controlling vibriosis in aquaculture, but with the problem of human health and the environment[7–9]

Penaeidins, as a natural immune substance extracted from *Litopenaeus vannamei*[10] have an inhibitory effect on gram-negative bacteria and gram-positive bacteria due to the sequence divergence in the N-terminal proline-rich domain (PRD) and subsequent conformational differences[11–13]. Penaeidins from shrimp hemocytes were difficult to isolate from natural samples[14].

Duckweeds are an excellent candidate for penaeidins expression in the aquatic system[15]. Firstly, duckweeds are effective and economical bioreactors of various types[16]. Especially, duckweeds reproduce by vegetative propagation ways during long-day conditions. And the daughter fronds are produced by budding within a single pouch of the mother frond, leading to the stable genetic bioreactor. Secondly, the target product could be secreted into the liquid medium—leading to a simpler product purification[17]. Thirdly, duckweeds could be applied as feed. And the expression of penaeidins improves the nutritional value of feed. Also, compared with prokaryotes such as *Escherichia coli*, duckweed, as a eukaryote, has the advantage of expressing penaeidins with higher activity. Using duckweed as a bioreactor to produce penaeidins would be a good solution to the major public health challenge of microbial antibiotic resistance.

Here, we report a successful penaeidins 3a transformation of duckweed and the bacteriostatic test on the extract of penaeidins 3a transgenic duckweed, as well as the direct bacteriostatic activity in the liquid medium.

Results

GUS activity and molecular analysis of transgenic duckweed

Pen3a transgenic duckweeds (designated as Pen3a) were obtained mediated by *Agrobacterium*, and five independent transgenic lines were cultured in a liquid medium. GUS activity has been detected in roots and fronds of Pen3a duckweed (Fig. 1a). These 5 independent transgenic lines have been detected by PCR identification (Fig. 1b). RNA sequence result showed that the mRNA transcripts were expressed in 5 transgenic lines, but not in wild type (WT) duckweed (Table 1). These results indicated that Peaeidin 3a. gene had been successfully integrated into the duckweed genome. And Table 1 shows that the read count of *Litopenaeus vannamei* Penaeidin-3. The wild-type duckweed's read count was 0. The expression of Pen3a_K has the highest read count among the five transgenic lines.

Table 1
Gene expression of Penaeidin in duckweed

Lines	Gene-id	Description	readcount
Pen3a_D	Cluster-6169.0	Penaeidin-3 OS=Litopenaeus vannamei	1.84520081740231
Pen3a_E	Cluster-6169.0	Penaeidin-3 OS=Litopenaeus vannamei	5.418213813
Pen3a_H	Cluster-6169.0	Penaeidin-3 OS=Litopenaeus vannamei	5.67877520684982
Pen3a_J	Cluster-6169.0	Penaeidin-3 OS=Litopenaeus vannamei	4.75452543036578
Pen3a_K	Cluster-6169.0	Penaeidin-3 OS=Litopenaeus vannamei	18.3589398900158
WT	Cluster-6169.0	Penaeidin-3 OS=Litopenaeus vannamei	0

Transcriptome analysis of WT and Pen3a duckweed

The gene expression of five transgenic and wild-type duckweed samples has been analyzed. Overview of the expression profile of the DEGs has been shown in Fig. 2a. The cluster analysis between Pen3a duckweed and WT duckweed was significantly different. To understand the potential functions of DEGs in the Pen 2a duckweed, gene ontology (GO) enrichment analysis has been conducted (Fig. 2b). Three different classifications represent three basic classifications of GO term, biological processes (BP), cellular components (CC), and molecular functions (MF). “biosynthetic process” in BP category “cell and cell part” in CC and “structural molecule activity” in MF category were most down-regulated DEGs. And the most up-regulated DEGs in the BP section were “protein metabolic process” and in the MF category was “ion binding”.

Molecular mechanism changes in growth-related KEGG pathway

Sphinganine act as signaling molecules during biotic and abiotic stresses. The expression of key enzymes in sphingolipid metabolism has been shown in Fig. 3. The expression of 3-dehydrosphinganine reductase (KDSR) was up-regulated in the pathway of sphinganine. The gene expression of sphinganine C4-monooxygenase (SUR2), which catalyzes the synthesis of phytosphingosine and phytoceramide, has been enhanced. Acid ceramidase (ASA1) that catalyzes phytoceramide to form phytosphingosine has been up-regulated. Very-long-chain ceramide synthase (LAG1, 2.3.1.297), promoting the mutual conversion between sphinganine and dihydroceramide, was increased. The glucosylceramidase (GBA, 3.2.1.45) gene expression of synthetic N-Acylsphingosine was been promoted. Alkaline ceramidase (ASA3, 3.5.1.23) and sphingoid base N-stearoyltransferase (CERS1, 2.3.1.299), which promoted the conversion between ceramide and sphingosine, was up-regulated[18].

The expression of key proteins in autophagy has been up-regulated in the KEGG pathway

Phagocytosis was the basic biological defense mechanism. Phagosomes play an important role in sequestering cytoplasmic components, which are delivered to the vacuole for breakdown. The difference of gene expression between transgenic duckweed Pen3a and WT in the phagosome metabolic pathway was studied (Fig. 4). Phagolysosomes could degrade antigens to form immunogenic peptides. V-type H⁺-transporting ATPase subunit A (V-ATPase) gene expression was up-regulated in all three stages of phagolysosome formation. Tubulin alpha (TUBA), as an important protein in microtubules, was up-regulated after the expression of Penaeidins 3a gene. The expression of protein transport protein Sec61 subunit alpha (Sec61A) has been up-regulated. The Sec61 complex promotes the ubiquitination of misfolded peptide chains or unassembled protein subunits. These results effectively confirmed the enhanced antibacterial ability of transgenic duckweed Pen3a.

Label-free Quantitative Proteomics

In this analysis, a Label-free algorithm was used to label the proteomic data quantitatively. As Fig. 5a, in the process of protein cluster analysis, Pen 3a and WT samples and quantitative information of proteins were classified. The protein clustering results of the Pen 3a vs WT comparison group showed a significant difference. In the significant difference analysis of quantitative results, the proteins that meet the screening criteria of expression difference multiple greater than 1.5 times and P-value < 0.05 in the three repeated experimental data in the sample group are selected as significant differentially expressed proteins. We used the protein expression difference multiple (Fold change) between the two groups of samples and the P-value obtained by the T-test to draw the Volcano plot. The abscissa of the volcano map is the fold change and the ordinate is the significance (P-value). The volcano plot of pen3a vs WT comparison group shows the relative quantitative information of significant difference proteins in the two groups of samples, in which the red point is the significantly up-regulated protein, the green point is the significant down-regulated protein, and the gray point is the non-significant difference protein (Fig. 5b). To further reveal the function of proteins, we analyzed the bacterial secretion system and protein export of the protein interaction network (Fig. 5c). The dotted and solid lines indicate a confidence score. The default lowest value is 400. The solid line indicates a higher value and the dotted line indicates a lower value. Round nodes represent proteins/genes and red represents up-regulation and green represents down-regulation. Rectangular nodes represent KEGG Pathway Biological process, the significance P-value was represented by yellow-blue gradient, and yellow color means p-value was low, blue means P-value was high. Taking the KEGG pathway as the unit and all qualitative proteins as the background, the significance level of protein enrichment of each pathway was analyzed and calculated by Fisher's exact test, to determine the significantly affected metabolic and signal transduction pathways. As Fig. 5d shows that, different colors represent different classifications of metabolic pathways. Among them, the difference in protein enrichment in the metabolic pathway was the most significant.

Effects of lake water on WT duckweed and Pen3a duckweed

Plants possess innate immune systems responding to bacteria and fungi. In this study, we investigated the growth of WT and Pen3a duckweed cultured in lake water for 14 days. And the WT duckweed and Pen

3a duckweed showed different phenotypes. The roots of wild duckweed were longer than Pen3a duckweed, and the frond of Pen3a duckweed still keep three frond group, the state of the WT duckweed's frond group have been broken up (Fig. 6a). According to statistics, after 14 days of cultivation in the lake, the broken-up rate of roots of WT duckweed was 31.37%, and that of Pen3a duckweed was 20.16% (Fig. 6b). The broken-up roots of Pen3a duckweed were shorter than WT duckweed. These phenomena indicated some self-protection mechanism of transgenic duckweed in response to adverse environments.

Antibactericidal tests of Pen3a duckweed extracting solution

A bacteriostatic test by Pen3a duckweed extract has been performed. The antibacterial activity against the Gram-negative bacteria *Escherichia coli* and the Gram-positive *Staphylococcus aureus* was obvious. The inhibition zone of the different concentrations of the extract on *E. coli* and *S. aureus* were shown in Figure 7 (the concentrations were 50%, 75%, and 100%). The inhibition zone of *E. coli* was about 19.2 ± 0.6 mm when the extraction of concentration was 100%(Fig. 8a) and the inhibition zone of around 15.5 ± 0.5 mm was observed for *S. aureus* (Fig. 8b). The results showed that Pen3a duckweed had an inhibitory effect on bacteria.

Microbiomes with statistical difference

Linear discriminant analysis Effect Size (LEfSe) was used to analyze the difference in the abundance of bacteria in Qiushui Lake. LEfSe, developed by Regata et al. in 2011, was suitable for statistical analysis of metagenomic data from multiple microbiomes[19]. From Fig. 8a, we could infer the evolutionary relationship of microorganisms with significant differences between groups. Among the three treatments, LEfSe detected 88 bacterial clades, of which LDA values were higher than 2.5 (Fig. 8b). These microorganisms mainly belong to *Nitrospirae*, *Sphingobacteriia*, *Synechococcophycideae*, and *Opiritae*. In control, the genus *Nitrospira* which belongs to the phylum *Nitrospirae* was substantially higher. Nevertheless, both WT and Pen3a duckweed significantly decreased the bacterial abundance and effectively inhibited the growth of *Nitrospirae*.

Composition and difference analysis of bacterial community structure in different treatment of lake water

The analysis of taxonomic indicated that the dominant four phyla were Proteobacteria, Cyanobacteria, Actinobacteria, and Bacteroidetes after 2 different treatments (Fig. 9a). This result was no significant difference. Compared with the control group, the relative abundance of Chlorobi has increased in WT and Pen3a duckweed treatment groups. Pen3a duckweed could inhibit the growth of Firmicutes. After 14 days in the lake water, the abundance of Firmicutes after Pen3a duckweed treatment was 0.382%, while that after WT duckweed treatment was 7.104%. To explore the similarity between different groups, we constructed a cluster tree of samples to investigate the similarities and differences (Fig. 9b). The results showed that WT duckweed and Pen3a duckweed completely inhibited the growth of *Nitrospirae* (sequence number percent was 0).

Discussion

Antimicrobial peptides, including penaeidins, are constituted by divergent classes of peptide isoforms in shrimp (Molecular Characterization and Antibacterial Activity Analysis of Two Novel Penaeidin Isoforms from Pacific White Shrimp, *Litopenaeus vannamei*), which are the terminal immunity effectors in immunity response (Discovery of Synthetic Penaeidin Activity against Antibiotic-resistant Fungi). Some strategies could be built to control certain diseases in shrimp aquaculture. For example, penaeidins have been found to play a role in restricting white spot syndrome virus infection by antagonizing the envelope proteins to block viral entry (Penaeidins restrict white spot syndrome virus infection by antagonizing the envelope proteins to block viral entry). Two penaeidin isoforms from *Litopenaeus vannamei*, named Lva-PEN 2 and Lva-PEN 3, could strongly bind to bacteria and possess antiprotease activity (Molecular Characterization and Antibacterial Activity Analysis of Two Novel Penaeidin Isoforms from Pacific White Shrimp, *Litopenaeus vannamei*). In this study, the bacteriostatic test showed that Pen3a_K duckweed extract inhibited *E. coli* and *S. aureus*.

Duckweed provides an advantage to express a foreign protein. For example, the M2e peptide of Avian Influenza Virus H5N1 had a high-yield expression in transgenic duckweed, promising for the development of a duckweed-based expression system to produce an edible vaccine against avian influenza (High-Yield Expression of M2e Peptide of Avian Influenza Virus H5N1 in Transgenic Duckweed Plants). Also, duckweed was considered a promising source of protein for food products due to its high protein content and environmentally friendly production (Duckweed as human food. The influence of meal context and information on duckweed acceptability of Dutch consumers). Here, we report a workable way to express penaeidins in duckweed. Described as Fig. 10, the Pen 3a duckweed could be both cultured in industrial and pond conditions, making use of the shrimp-culturing wastewater. Duckweed, co-cultured with shrimp wastewater in an outdoor water recirculation system, could be applied to obtain penaeidins extraction, feed production, and play a role in bacteria inhibition.

It has been reported that duckweed hosts a similar bacterial assemblage as the terrestrial leaf microbiome in taxonomic (Duckweed hosts a taxonomically similar bacterial assemblage as the terrestrial leaf microbiome). Also, Duckweed is a kind of traditional Chinese medicine. Here, the microbiome study showed the ability of duckweed as well as Pen 3a transgenic duckweed to play a role in controlling the bacterial abundance and effectively inhibited the growth of *Nitrospirae*. Scientists showed that duckweed could be cultured in swine wastewater for nutrient recovery and biomass production (Growing duckweed in swine wastewater for nutrient recovery and biomass production). And we investigated the tolerance and the growth of duckweed in lake water. Shown as Fig. 6, the Pen3a duckweed showed a decreased root abscission and an enhanced frond condition, suggesting a better growth in the lake. Hence, the design of Pen 3a transgenic duckweed could be cultured in the lab, industrial condition, and native lake with important technological applications.

Conclusions

In summary, Pen 3a transgenic duckweed has been obtained by the agrobacterium-mediated transformation. After Pen 3a gene was transferred, the expression of sphingolipid metabolism and phagocytosis process-related genes have been up-regulated. Furthermore, the results of quantitative proteomics in phagosome metabolic pathway has changed significantly. And both WT and Pen3a duckweed decreased the bacterial abundance and effectively inhibited the growth of *Nitrospirae*. And Pen3a duckweed displayed better growth in the lake. In the future, the design of Pen 3a transgenic duckweed could be cultured in the lab, industrial condition, and native lake with important technological applications.

Materials And Methods

Construction of antimicrobial peptide VPS vector

Penaeidins 3a (Pen3a) cDNA was obtained from RNA of *Litopenaeus vannensis* by reverse transcription as a template. The product was connected to CaMV-35S promoter and NOS terminator of Tobacco Mosaic virus by PCR and enzyme digestion. After double enzyme (*Bgl*I and *Pst*I) digestion, the plant expression vector p1301 -Pen 3a was constructed (Fig. 11).

Duckweed culture condition

The experimental material *Lemna turionifera* 5511 was collected from Xiqing District, Tianjin. The culture method refers to the liquid medium described by Wang and Kandeler[20]. Briefly, the liquid culture medium consisted of 0.4 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.4 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 1.0 mM KNO_3 , 0.4 mM KH_2PO_4 , 0.4 mM $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 50 μM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 50 μM KCl , 6.1 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 69 μM H_2BO_3 , 30 μM $\text{K}_2\text{H}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, 56.7 μM $\text{FeNH}_4\text{-EDTA}$, 13.8 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.8 μM $\text{ZnNa}_2\text{EDTA} \cdot 4\text{H}_2\text{O}$, 4.8 μM $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 18.6 μM $\text{Na}_2\text{-EDTA} \cdot 2\text{H}_2\text{O}$ and the pH was adjusted to 5.8. Duckweed was cultured at $23 \pm 2^\circ\text{C}$ with a light intensity of $95 \mu\text{mol m}^{-2} \text{s}^{-1}$ and the photoperiod was 16 h of light and 8 h of darkness.

Plant transformation and GUS activity detection

The methods of callus induction and regeneration of duckweed were referred to Yang *et al.* (2012)[2]. Briefly, the p1301-pen 3a vector has been transformed to *Agrobacterium tumefaciens* EHA105. The duckweed callus was co-cultured with *Agrobacterium* ($\text{OD}_{600} = 0.6$) and vacuumed for 30 s three times, then cultured with slow shaking at 28°C for 30 minutes under dark conditions. The callus was transferred to B5 medium at 28°C and cultivated under dark conditions overnight. The callus was cultivated on a selection medium containing 1 mg L^{-1} Hygromycin and 160 mg L^{-1} Cefalexin for 3 days. Then transfer to regeneration medium containing 1 mg L^{-1} Hygromycin, 160 mg L^{-1} Cefalexin, and 0.1 M Serine Cultivate for 30 days until the callus regenerates. Histochemical stable b-glucuronidase (GUS) stain has been performed to determine the transformation of duckweed by Biosharp GUS Staining Kit (Biosharp; according to instruction).

RNA isolation and quantification

RNA samples from duckweed callus were extracted by the Tiangen kit (Tiangen RNAsimple total RNA kit). mRNA was purified from total RNA using poly-T oligo-attached magnetic beads and detected on 1% agarose gel to ensure the sample quality. The purity of RNA was measured by the Nanophotometer (IMPLEN, CA, USA). RNA concentration was analyzed using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 assay kit of the Agilent BioAnalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 1.5 µg RNA was used for library preparation for transcriptome sequencing. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA).

Sequencing data filtering and transcript assembly

Data images of sequencing fragments measured by high-throughput sequencers are transformed into sequence data (readings) by CASAVA base recognition. The raw data obtained from sequencing included a small number of reads with sequencing adaptors or low sequencing quality. Filtered content: removed adaptors; removed reads whose proportion of N is greater than 10%; remove low-quality reads. 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[21]. Overall, a reference sequence with an average length of 1928 bp and a total length of 282527137 bp was obtained for subsequent analysis. The combination comparison of differential clustering gene analysis can obtain the differential gene sets, and the FPKM values of the union of all comparative combinations of differential gene sets in the six samples are used for hierarchical clustering analysis.

DNA extraction and polymerase chain reaction(PCR)

Total DNA was isolated from duckweed using TAKARA MiniBEST Plant Genomic DNA Extraction Kit (TAKARA; according to the manufacture's instruction). DNA concentration was quantified by NanoDrop spectrophotometer. Experimental setup and execution were conducted using a Veriti® 96-Well Thermal cycler, according to the protocol provided by the manufacturer (ABI, USA). PCR products were analyzed by agar gel electrophoresis. The forward primer and reverse primers are TACGCGGAGCACCAGACGGA and TCAACCGGAATATCCCTTT.

Bacteriomic and bioinformatics analysis

Firstly, raw data FASTQ files were imported into the format which could be operated by the QIIME2 system using qiime tools import program. Then, the QIIME2 DADA2 plug-in was used for quality control, pruning, denoising, splicing, and removal of chimera to obtain the final feature sequence table[22]. The QIIME2 feature-classifier plugin was then used to align ASV sequences to a pre-trained GREEN GENES 13_8 99% database (trimmed to the V3V4 region bound by the 338F/806R primer pair) to generate the taxonomy table[23]. Any contaminating mitochondrial and chloroplast sequences were filtered using the QIIME2 feature-table plugin. Secondly, ANCOM, ANOVA, Kruskal Wallis, LEfSe, and DEseq2 were used to identify bacteria that differed in abundance between groups and samples[24–26]. Thirdly, diversity metrics were calculated using the core-diversity plugin within QIIME2. Feature level alpha diversity indices, such as observed OTUs, Chao1 richness estimator, Shannon diversity index, and Faith's

phylogenetic diversity index were calculated to estimate the microbial diversity within an individual sample. Beta diversity index was used to assess the differences in microbial community structure among samples and was subsequently demonstrated by PCoA and NMDS maps[27]. Finally, redundancy analysis (RDA) was performed to reveal the association of microbial communities to environmental factors based on relative abundances of microbial species at different taxa levels using the R package “vegan”.

Antibacterial test of transgenic duckweed extract

Pen 3a duckweed cultured for 14 days was frozen in liquid nitrogen and thoroughly ground. And then, it has been diluted by 2 g to 5 ml phosphate buffer saline (PBS, PH 7.2-7.4, 10 mM). The inoculation rings were used to pick the strains on the inclined surface of the test tube in sterile water with glass beads, and the spores were dispersed by shaking with the hand for several minutes. The mixed spore suspension was prepared after filtration. Mix the bacteria liquid with 15-20 ml melted agar medium, then self-cooling it. The filter paper was soaked in antimicrobial peptides and placed in the center of the plate with bacteria. After culture for 2-3 days, the presence and size of the bacteriostatic circle around the filter paper were observed.

Label-free Quantification Proteomics

Protein was extracted from tissue samples using SDT lysis buffer (4% SDS, 100 mM DTT, 100 mM Tris-HCl pH 8.0), which were boiled for 5 min and further ultrasonicated and boiled again for another 5 min. Undissolved cellular debris was removed by centrifugation at 16000g for 15 min. The supernatant was collected and quantified with a BCA Protein Assay Kit (Bio-Rad, USA). Protein digestion (200 µg for each sample) was performed with the FASP method described[28]. Then LC-MS/MS analysis was performed. LC-MS/MS was performed on a Q Exactive Plus mass spectrometer coupled with Easy 1200 nLC (Thermo Fisher Scientific). Sequence database searching and data analysis: The MS data were analyzed using MaxQuant software version 1.6.0.16. MS data were searched against the UniProtKB *Rattus norvegicus* database (36080 total entries, downloaded 08/14/2018). The quantitative protein ratios were weighted and normalized by the median ratio in Maxquant software. Only proteins with fold change ≥ 1.5 -fold and a p-value < 0.05 were considered for significantly differential expressions. Bioinformatics analysis of bioinformatics data was carried out with Perseus software[29], Microsoft Excel, and R statistical computing software.

Declarations

Ethics approval and consent to participate

The plants used in the experiment comply with relevant institutional, national, and international guidelines and legislation. This material has not been published in whole or in part elsewhere and the manuscripts are not currently being considered for publication in another journal.

Consent for publication

All authors have been personally and actively involved in substantive work leading to the manuscript and will hold themselves jointly and individually responsible for its content.

Competing interests

There is no interest conflict in submitting this paper, and all authors have approved this manuscript for publication. This work is original research that has not been published previously and is not considered for other publications elsewhere. All of us listed have approved this manuscript.

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Authors' contribution

Sun, J.S. provided resource support and orchestrated the arrangement. Yang, L. designed the experiment, analyzed the data, and wrote the manuscript. Sun, J.G. finished the experiment and counted the experimental results. Ren, Q.T. completed the data statistics and forms. Ma, X. completed the chart drawing and part of the experiment. Wang, Y.Y. supervised the experimental project and the subsequent editing of the manuscript. Wang, W.Q. adjusted the overall format of the manuscript and completed basic experiments. He, Y.M. cultured the experimental plants.

Availability of data and material

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

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Figures

a **b**

Figure 1

Identification of duckweed by GUS staining and RT-PCR. a GUS activity in transgenic lines of Pen3a duckweed. b RT-PCR identification of the Pen3a duckweed. MK represented Marker, WT was wild-type duckweed, D, E, H, J, K was Pen3a_D, Pen3a_E, Pen3a_H, Pen3a_J, Pen3a_K duckweed.

Figure 2

a Heat map of cluster analysis of DGEs in six genotype duckweed. b Go enrichment histogram of down-regulated differential genes. Enriched GO Terms of Pen3a_K versus WT. c Go enrichment histogram of up-regulated differential genes.

SPHINGOLIPID METABOLISM

Figure 3

Gene changes in sphingolipid metabolism KEGG pathway. Changes in gene expression of sphingolipid metabolism pathway in KEGG pathway of Pen3a duckweed compared with WT duckweed.

Figure 4

Changes of duckweed phagosome KEGG pathway between Pen 3a and WT. The block's color meant unigenes coding corresponding proteins, which was determined by the expression pattern. The red blocks indicated the up-regulated genes.

Figure 5

Proteomic analysis. a Results of hierarchical cluster analysis of expressed proteins to Pen3a and WT comparison group. The protein abundance was shown as color code, blue means lower abundance, and red means higher abundance. Abundance difference factor in protein hierarchy clustering $p < 0.05$. b Volcano Plot between Pen3a and WT comparable group. The red dots were significantly up-regulated proteins, the green dots were significantly down-regulated proteins, and the gray dots were non-

significantly different proteins. c Interaction network results of differentially expressed proteins in Pen3a vs WT comparison group. d KEGG enrichment pathway statistics of Pen3a vs WT comparison group.

Figure 6

The phenotype of duckweed cultured in the lake for 14 days. a Difference of root length between WT and Pen3a duckweed after 14 days of culture. scale bar =10 mm; b Root-removed rate of WT and Pen3a duckweed.

a

E. coli

50%

75%

100%

b

S. aureus

Figure 7

Zone of inhibition of various bacteria by Pen 3a duckweed extracting solution. a Inhibition zone of different concentrations Pen 3a duckweed extracting solution on Escherichia coli. b Inhibition zone of different concentrations Pen 3a duckweed extracting solution on staphylococcus aureus.

Figure 8

a LefSe results on lake microbial communities. Cladogram corresponds to different taxonomic levels of families and genera in the phylum, and the lines between the levels represent the relationships. Each circle node represents a species, the yellow node represents no significant difference between groups, and the non-yellow node represents that the species is the characteristic microorganism of the corresponding color group and the abundance is significantly higher in this group. The color fan-shaped area is marked with the subordinate classification range of the characteristic microorganism. Blue indicates the control group, green indicates wild-type treatment, yellow indicates transgenic antimicrobial peptide duckweed treatment. b Histogram of the LDA scores computed for abundance between three treatments. Each horizontal bar represents a species, and the length of the bar corresponds to the LDA scores. The higher the LDA value, the greater the difference. The histogram could well explain the greatest differences between microbial communities. The LDA scores of indicator microbiota of the three types of sediments were greater than 2.5 logs 10.

Figure 9

Abundance of the bacterial community in lake water after 14 days treatments. a Phylum taxonomic distribution. The abscissa represents three processing methods, and the ordinate represents the ratio of the number of series at the level of the gate to the total annotation data. It represents the top 20 microbial phyla with relative abundance. b Heatmap of microbial community structures at the phylum level.

Figure 10

The design of Pen 3a transgenic duckweed technology applications.

Figure 11

T-DNA schematic representation of the final plasmid pCAMBIA-1301-Pen3a. Poly A: the terminator of the HPT gene and the GUS terminator; HTP: hygromycin resistance gene; P35S: the promoter of CaMV-35S; Pen3a: penaeidin 3a.1 gene; GUS: β -glucuronidase gene.