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ESTABLISHMENT OF REAL-TIME PCR FOR ANALYZING mRNA ABUNDANCE IN *CHLORELLA VULGARIS* EXPOSED TO XENOBIOTICS

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Abstract: Among the aquatic organisms, algae are one of the most susceptible organisms to pollution in water. Transcriptional abundance has been gradually used as a surrogate for gene expression in environmental biology. In the present study, real-time PCR assay was established to quantify the transcript abundances of three photosystem genes (*psa*B, *psb*C and *rbc*L) in *Chlorella vulgaris*, and the acute toxicity of three herbicides (glufosinate, atrazine and diclofop-methyl) was assessed using this method. The results demonstrated that real-time PCR is an efficient technique to assess the toxicity of xenobiotic compounds in aquatic system.

Key words: Real-time PCR; *Chlorella vulgaris*; Photosynthesis; Transcript abundance
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The water bodies of densely populated and agricultural and industrialized areas usually receive high and diverse loading of contaminants. Among tested organisms, *Chlorella vulgaris*, a green alga has several attributes that make it suitable for pollution bioassays. Algae are one of the first organisms to react to changes in nutrient loading[1], besides they are sensitive to toxicants[2−4]. To determine the environmental impact of complex contaminants, toxicity-based approaches, rather than chemical or physiological approaches, are better adopted in risk assessment. More relevant biological information from physiological and biochemical studies at the molecular level needs to be incorporated into the toxicity and risk assessment procedures. There is a significant lag of such approaches due mainly to difficulties in gene transcription. One of the challenges in environmental and molecular toxicology is to establish the assay of estimating gene transcript abundance responding to a variety of toxicants in specific species.

It is crucial to develop simple analysis methods that detect gene expression sensitively. Tremendous progress has been made since quantitative methods for analysis of nucleic acids became available[5]. A PCR-based quantitative assay, first described by Holland, *et al.* [6] and referred to as a real-time PCR, recently emerged and applies to a wide range of research such as Wang, *et al.*[7] Real-time PCR combines the sensitivity of PCR with real-time measurement of amplification and thus allows quantification of the original target concentration. PCR product formation is monitored by determining the increase in fluorescence either due to binding of the amplicon to a fluorescent DNA stain, such as SYBR green, or due to the release of a fluorescent moiety from an oligonucleotide probe specific for the amplicon. The research here was to establish quantificational method, which directed at detection of mRNA as a surrogate for gene expression of algae in the water environment. We selected photosynthesis-related genes in a freshwater alga *Chlorella vulgaris* as target genes. These genes are *psa*B, which is part of the *psa*A/B operon of the chloroplast genome and codes for the photosystem I (PSI) reaction center protein; *psb*C, which codes for chlorophyll-protein complexes (CP43), an integral membrane protein component of photosystem II (PSII) and one of the interior transducers of excitation energy from the light-harvesting pigment proteins to the photochemi-

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cal reaction center; and *rbc*L, coding for the large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) in algae, which is the key enzyme of the Calvin-Benson-Basham cycle, catalyzing the first step in which CO_2 is reductively assimilated into organic carbon. The objective of this study was to establish a real-time PCR assay to monitor the effects of pollutes on aquatic plant by assessing transcripts of these photosynthesis related genes.

1 Materials and Methods

1.1 The cultivation of Alage

Chlorella vulgaris (No.24) was purchased from the Institute of Hydrobiology, Chinese Academy of Sciences, and maintained in Shuisheng-4 medium[8]. Algal cells were cultured at (25±0.5)℃ in flasks (250 mL) containing 50 mL of medium and illuminated with fluorescent lights (2500 lx) for daily cycles of 16h light and 8h dark. The algal cell culture density was monitored spectrophotometrically at 685 nm (A_{685}, optical density at 685 nm). The regression equation between the density of algal cells ($y\times10^5$ /mL) and A_{685} (x) was established as y=162.1x+1.3463 (r^2=99.34%).

1.2 The treatment of the acute toxicity

Two hundred μg/L atrazine (Sigma), 20 μg/mL glufosinate (AgrEvo) and 20 μg/mL diclofop-methyl (Sigma) solutions in the culture medium containing algal cells at log phase were prepared (A_{685}=0.08). Selection of these concentrations was based on the 96-h EC50 value of atrazine (900 μg/L), glufosinate (20 μg/mL) and diclofop-methyl (20 μg/mL), which obtained in our preliminary experiments from the concentration-response curves of cell density for *C. vulgaris*. Three replicate cultures were prepared for each treatment concentration. Samples were withdrawn after 16h for RNA extraction.

1.3 RNA extraction

To extract RNA from algal cells, 50 mL of cultures were transferred to centrifuge tubes and centrifuged at 14000 r/min for 10 min. Isolated algae cells were disrupted in liquid nitrogen in a ceramic mortar, and then 500 μL of TRIzol reagent (Invitrogen, Carlsbad California, USA) was added. Total RNA was isolated according to the manufacturer's instructions for the reagent including DNase treatment. Nucleic acid concentrations were measured spectrophotometrically at 260 nm. The 260/280 nm ratios were determined and referred to as the purity of the total RNA extracted. The integrity was tested by electrophoresis in 1% agarose formaldehyde gel[9].

1.4 The method of Real-time PCR

To facilitate the real-time PCR analysis of the selected genes under the same reaction conditions, primers were first designed by TaKaRa Biochemicals (Dalian, China). 18S rRNA gene was selected as housekeeping gene, and *psa*B, *psb*C and *rbc*L were target genes. Gene sequences have been described in the GenBank database, which accession numbers were X13688, 809130, 809108 and AF499684, respectively. For 18S rDNA amplification, two primers were 5'-TTGACGGAAGGGCACCA-3' and 5'-CACCACC CATAGAATCAAGAAAGAG-3' to amplify 127 bp region, *psa*B primers were 5'-GCTGG-TCAATCTTTGGCTTC-3' and 5'-AAAGTCTCCGG-TCCGATGGT-3' to amplify 90bp region, *psb*C primers were 5'-GAACATCACCACCACCAGGA-3' and 5'-CGGTGCTTGGCTTTTAGTTTG-3' to amplify 80bp region, and *rbc*L primers were 5'-CTTGGACGACT-GTATGGACTG-3' and 5'-ATACCGTGAGGAGGACC-TTG-3' to amplify 271bp fragment.

Reverse transcription (RT) was carried out using a M-MLV reverse transcriptase kit (TaKaRa Biochemicals). The real-time quantitative PCR was performed using a PCR instrument (Applied Biosystems7300, Foster, California, USA). A reaction mixture for each PCR run was prepared with the SYBR Green PCR Core Reagents (TaKaRa Biochemicals). The cycle parameters consisted of one cycle of 10s at 95°C and then 40 cycles of 5s at 95°C followed by 31s at 60°C. Data were collected at the end of each extension step. The relative quantification of gene expressions among the treatment groups was analyzed by the $2^{-\Delta\Delta Ct}$ method[10], where Ct was the cycle number at which the fluorescent signal rises statistically above the background. The 18S rRNA transcript was used to standardize the results by eliminating variations in the quantity and quality of mRNA and cDNA. Each mRNA level was expressed as the mRNA ratio of itself to 18S rRNA.

Data were presented as mean ± standard error of the mean (SEM) and tested for statistical significance by analysis of variance (ANOVA) followed by the Fisher's post hoc test using StatView 5.0 program (Statistical Analysis System, Institute, Cary, NC, USA). Values were considered significantly different when P was less than 0.05 or 0.01.

2 Results

The RNA quality was easily verified by visualization on an ethidium bromide agarose gel (Fig. 1), which showed no degradation. The intactness of the RNA was indicated by the integrity of the ribosomal bands. Real-time PCR were performed for the expression of all 4 genes and agarose gel (3.0%) electrophoresis for the fragments of 18S rRNA, *psa*B, *psb*C and *rbc*L showed single expected amplicons of 127, 90 80 and 271bp, respectively (Fig. 2). It meant that real-time PCR amplification was specific.

In order to prove whether the real-time PCR was fea-

sible for monitoring pollution bioassays, the expression change of these photosynthesis related genes in *Chlorella* cells exposed individually to three sorts of herbicides glufosinate, atrazine and diclofop-methyl for 16h was detected. As shown in Fig. 3, the mRNA level of *psa*B exhibited some decrease in the presence of glufosinate, atrazine and diclofop-methyl for 16h, but it had no significant changes.

*psb*C exhibited different responses to three sorts of herbicides (Fig. 4). Herbicides resulted in a decrease of the *psb*C transcript abundance by 49%—74% as compared to the control sample. The maximum decrease in the *psb*C transcript abundance was observed after 16h exposure to 20 μg/mL of glufosinate. All these decreases were significant at $P<0.01$ level.

The influence of three herbicides on the transcript level of *rbc*L was similar to that of *psb*C. The transcript abundance of *rbc*L significantly decreased after 16h exposure (Fig. 5). The minimum abundance of *rbc*L observed at 200μg/L of atrazine was only 6% of that of the

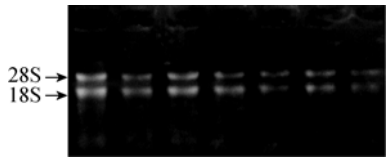

Fig. 1 1% agrose gel electrophoresis detection of total RNA extracted from *Chlorella vulgaris* with Trizol reagent

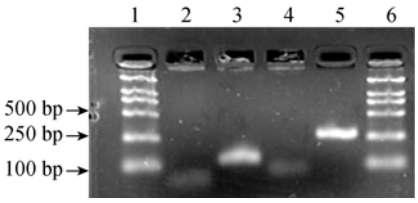

Fig. 2 Real time-PCR assay of extracted RNA from *Chlorella vulgaris* with Trizol reagent. Line 1 and 6: molecular weight marker, line 2-5: PCR amplification of *psb*C, 18S rDNA, *psa*B and *rbc*L, respectively

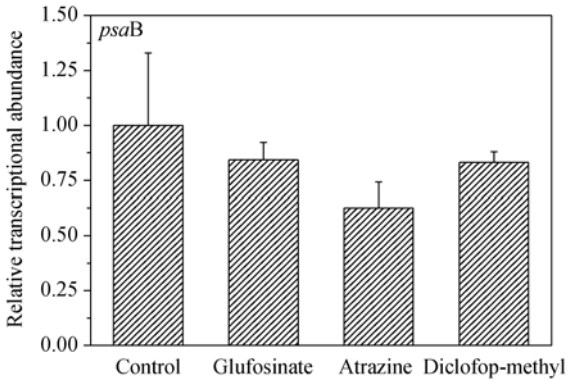

Fig. 3 Expressions of *psa*B in *Chlorella vulgaris* exposed to glufosinate, atrazine and diclofop-methyl

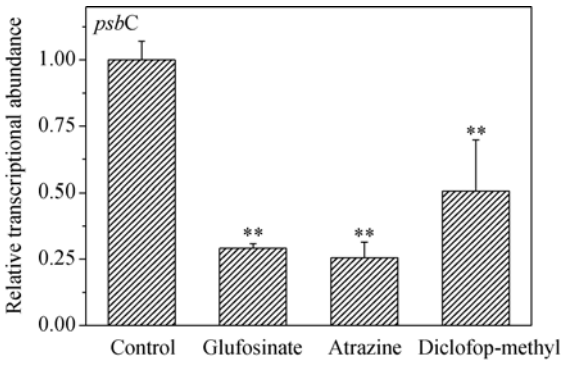

Fig. 4 Expressions of *psb*C in *Chlorella vulgaris* exposed to glufosinate, atrazine and diclofop-methyl
Values were normalized against 18S rRNA as housekeeping gene and represent the relative mRNA expression (mean±standard error) of three replicate cultures. Asterisks represent statistically significant differences compared with the control (*$P<$ 0.05, **P <0.01): the same as follows

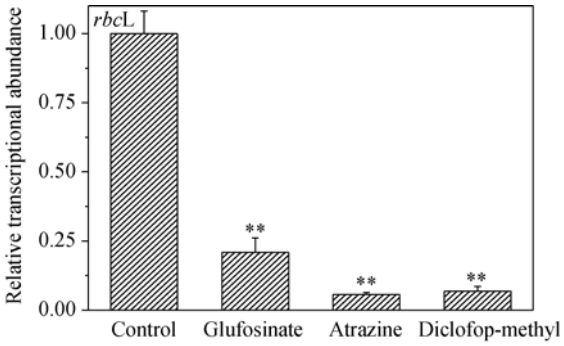

Fig. 5 Expressions of *rbc*L in *Chlorella vulgaris* exposed to glufosinate, atrazine and diclofop-methyl

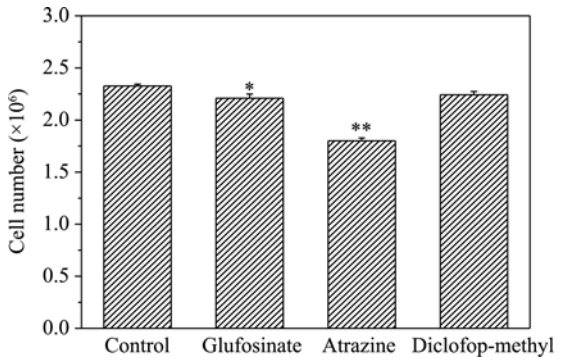

Fig. 6 The alga cell numbers of *Chlorella vulgaris* exposed to glufosinate, atrazine and diclofop-methyl

control sample, and the abundances of *rbc*L at diclofop-methyl and glufosinat were only 7% and 21% of that of the control sample. And as shown in Fig. 6, algae cell numbers decreased after 16-h herbicides exposure.

3 Discussion and Conclusion

In this study, we mainly choosed three herbicides atrazine, diclofop-methyl and Glufosinate. Atrazine is used to control annual broadleaf weeds in agricultural

fields, sods, turfs and residential lawns[11]. It is one of the most heavily employed herbicieds in the world with the usage of 2800 and 35828 tons/year in China and the United States, respectively[12,13]. Diclofop-methyl is commonly applied on the same field for consecutive years to control annual weed grasses in many Italy areas, and make the soils and water pollution[14]. Glyphosate and glufosinate are broad spectrum, nonselective, post-emergence herbicides extensively used in various applications for weed control in aquatic systems and vegetation control in non-crop areas[15]. And these toxic herbicides are frequently detected in surface water.

In this research, we established real-time PCR method to assess the effects of herbicides on gene expressions of unicellular green alga *Chlorella vulgaris*. Significant advances have been made since quantitative methods for analysis of nucleic acids via various hybridization techniques became available. But these hybridization techniques often involve the use of radiolabeled probes for detection of a particular mRNA, and are time-consuming, costly, and more important, release radioactive wastes. The real-time PCR assay is one of the most appropriate methods that sensitively detect gene expression without the above-mentioned shortcomings. Even though real-time PCR was originally developed for clinical applications, it has recently been applied to microbial ecology. For example, it has been used for detection of small-subunit rRNA and for determination of *narG* gene copy abundance[16] in the environment.

Our results clearly showed the strong influence of three herbicides on the transcription of photosystem genes in this study. And these results were also in confirmatory to the change of cell number. As shown in Fig. 6, algae cell numbers decreased after 16-h herbicides exposure. Glufosinate, atrazine and diclofop-methyl targeted different gene in plant or algae. Glufosinate, isolated from microorganisms, is an active ingredient of a non-selective herbicide. Glufosinate irreversibly inhibits glutamine synthetase (GS)[17], which results in an accumulation of ammonium derived from nitrate reductase activity and photorespiratory pathway. Atrazine inhibits photosynthesis by blocking electron transport during the Hill reaction of photosystem II in plant species[18]. Diclofop-methyl belonging to the aryloxyphenoxypropionate (APP) group is potent inhibitors of the enzyme acetyl-CoA carboxylase (ACCase), an important enzyme in acyl-lipid biosynthesis. As other environmental stresses, herbicides inhibited the expression abundance of target gene and then caused oxidative damage either directly or indirectly by triggering an increased level of reactive oxygen species (ROS), and resulted in plant death or slower growth ultimately. Photosynthesis is the most basic physiological metabolism in plants and some algae, which converts light energy to chemical energy and stores it in the bonds of sugar. In herbicide metabolic process, photosynthesis would be disrupted directly or indirectly, and the expression of photosynthesis-related genes decreased.

In conclusion, photosynthesis-related genes in *Chlorella vulgaris* were employed by the real-time PCR assay in the present study, and the expression of these genes was all inhibited by three different sorts of herbicides. The results showed that the pollution of herbicides in aquatic system could be monitored by this method. The technique has the advantages of high sensitivity, reliability, and accuracy, and it would gain a great attention for application in algal bioassays and monitoring.

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生物异源物质对小球藻基因转录研究体系的建立

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摘要: 藻类是水生生态系统中的敏感物种。在环境生物学研究中, 基因转录水平通常作为衡量基因表达水平变化的指标。研究建立了定量 PCR 技术(Real-time PCR)检测小球藻基因转录变化的研究体系。以 3 个主要光合作用基因(*psa*B、*psb*C、*rbc*L)为靶标基因, 研究了除草剂草丁膦(Glufosinate)、阿特拉津(Atrazine)和禾草灵(Diclofop-methyl)对基因转录的影响, 结果表明 3 种供试药物对小球藻光合作用基因 *psb*C 和 *rbc*L 相对表达量有显著地抑制作用。这表明 Real-time PCR 可以作为评定水生环境中异源化合物毒性的有效方法。

关键词: Real-time PCR; 普通小球藻; 光合作用; 基因转录