Cloning and Differential Expression Analysis of a new *rbcS* Gene from *Lemna gibba*

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ABSTRACT: A novel Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit gene (named ssu4d) was cloned from Lemna gibba by a novel chromosome walking technology, genomic DNA (gDNA) region of ssu4d with the size of 1, 346 bp, was isolated. The full-length of ssu4d cDNA (named ssu4dc), contained a 522 bp open reading frame encoding a protein of 174 amino acids, was also isolated. Sequence analysis of ssu4dc and ssu4d showed that ssu4d contained an intron which is located from +355 nt to +1125 nt downstream transcriptional iniative site. ssu4dc contained 54 bp of 5' untranslated region (UTR), and an open reading frame of 174 amino acids consisting of a chloroplast transit peptide with 57 amino acids and a mature protein of 117 amino acids. The deduced amino acid sequence of ssu4dc shared 95-96% identity with L. gibba RbcS protein. Real time-PCR analysis showed differential expression of individual rbcS genes in light-grown Lemna gibba. And the levels of SSU4dc mRNA was regulated by the action of phytochrome, there was variability in the amount of expression of SSU4dc RNA comprared to the SSU1 and SSU5B from Lemna gibba.

KEYWORD: cloning; new rbcS gene; lemna gibba; transcriptional analysis

1 INTRODUCTION

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) which is the most abundant protein in nature catalyzes both the carboxylation of ribulose bisphosphate during CO_2 fixation and the oxygenation of the same substrate during photorespiration (Carmo-Silva and Salvucci, 2011). Rubisco is currently the target enzyme for improving the efficiency of photosynthesis in the hope increasing the yield and growth of crops (Spreitzer, 2003). Meanwhile, the small subunits of Rubisco were produced by rbcS gene family in the nuclear genome, which are synthesized as 20-kDa precursors in the cytoplasm and then processed to 15-kDa during transport into the chloroplast (Thanh et al., 2011). In plants, the 55-kDa large subunits are encoded by a single rbcL gene in the chloroplast genome(Spreitzer and Salvucci, 2002).The nucleotide and amino acid sequences of the RbcS vary among different organisms, while the primary structure of the large subunits is largely conserved (Whitney and Andrews, 2001; Mueller-Cajar and Whitney, 2008).

The duckweed *Lemna gibba* is an aquatic monocot in which the *rbcS* is encoded by a 12-to 14-member gene family (Buzby et al., 1990). The transcription of this gene family and the mRNA level of at least six of the genes have been shown to be regulated by phytochrome, genomic clones for six members of the gene family and a cDNA clone for a seventh have been isolated and characterized (Silverthorne et al., 1990; Stiekema et al., 1983). Each genomic sequence isolated contains a single intron at the position of the second intron of dicots (Silverthorne et al., 1990). The introns vary in length but have a high degree of homology with one another, whereas the 3' untranslated regions are sufficiently divergent over their full length to allow their use as gene-specific probes. Using gene-specific probes, the expression of individual rbcS sequences in total steady state RNA was shown to be under the control of phytochrome (Silverthorne et al., 1990). However, there is considerable variation in the levels of expression of individual rbcS genes in two different organs: the roots and fronds. This variation could be explained by transcriptional differences, in some post-transcriptional event(s) (Silverthorne and Tobin, 1990). The most extreme differences between transcription rates versus steady state mRNA levels were measured for SSU1 and SSU5B (Acevedo-Hernandez et al., 2005).

In an effort to increase our knowledge of lemna gibba photosynthesis and elucidate relationship

among *rbcS* genes from *lemna gibba*, a new *rbcS* gene (ssu4d) was isolated from Lemna gibba. The isolation of cloned *ssu4d* gene of the Lemna rbcS family has allowed us to examine the expression of these closely related genes and to determine whether there are differences in the effect of phytochrome action on individual family member.

2 MATERIALS AND METHODS

2.1 *Plant materials and growth conditions*

Axenic cultures of duckweed (*Lemna gibba*) were maintained in 150 ml flasks containing 50 ml SH basal medium supplemented with 1% sucrose and cultured in a growth chamber at 28 ℃ either under constant fluorescent lighting (90–150 μmol photons m^{-2} s^{-1}) with a photoperiod of 16 h of light and 8 h of dark. Dark-grown duckweeds were harvested using green safe-light conditions at 0.01 μmol m^2 s^{-1}. For light-pulses, Duckweeds were treated with FR and RL pulses pulse of 2 min and 10 min reapectively. FR and RL pulses were at 120, 140 μmol m^2 s^{-1} respectively. Duckweeds were harvested for total RNA extraction 24 h after the onset of the last irradiation under a dim green safelight.

2.2 *Cloning and Sequence analysis of L. gibba rbcS gene*

Genomic DNA was isolated from fronds of *L. gibba* using the modified CTAB extraction method (Luo et al., 2006). The PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Germany). Based on the known *L. gibba rbcS* gene sequence, the generacy primers (PF1 and PR1) were designed (table 1) to obtain about 400 bp DNA fragment by PCR, This DNA fragment was cloned into the pMD18-T vector (TakaRa) for sequencing, as the 400 bp DNA fragment contained the 5' sequence of the new *rbcS* gene, and the 3' region of the new rbcS gene was obtained by self-formed PCR (Wang et al., 2007) and the primers (sp1, sp2, sp3) were listed (table1. By bioinformatics analysis, series of 5' forwars primers (PF2a, PF2b and PF2c) were designed to confirmed the transcriptional iniation site of *ssu4dc* gene (table 1). The primers (table 1) was designed according to the cloned DNA sequence and used for cloning of cDNA of ssu4d (Table 1). The PCR fragments were cloned into the pMD18-T vector (TaKaRa, Dalian, China), the positive clones were sequenced. The DNA sequences were analyzed using DNAMAN5 software. Similarity searches were done online at the nucleotide level with BLASTn and at amino acid level with BLASTp.

Table 1. Primers used for cloning of *rbcS* gene from *L.gibba*

Primers	**Primers sequence (5' to 3')**	**Purpose**
PF1	MGATAAGRTGTAATCCW	cloning of *rbcS* gene
PR1	TGGAAGCCATCATCGACGAAGCCAT	from *L.gibba*
PF2a PF2b PF2c	AGAGACCCCAGAGCTTCCGAG GAGACCCCAGAGCTTCCGAG CAGAGACCCCAGAGCTTCCGAG	series of forward primers used for cloning of cDNA of *ssu4dc*
PR2	TTAGGTGGGCTTGTAGGCGATGAAG	reverse primer for cloning of *ssu4dc* cDNA
SP1	GCTTCGTCGATGATGGCTTCCACCG	3'-walking
SP2	CGGCGACGAGGAAGGCTAACGATCTGT	3'-walking
SP3	GGGGCTGAAGAAGTTCGNNNNNNNNNNCTACCT	3'-walking

2.3 *Real-time PCR analysis of rbcS genes*

Total Lemna gibba RNA was isolated from L.gibba using a Trizol reagent (Invitrogen) following the manufacturer's instructions. Single-strand cDNA was synthesized from 2 μg total RNA from each sample by reverse transcription (RT)-PCR using an RT Kit (TaKaRa, Dalian, China) according to the manufacture's instruction. Each real-time PCR reaction (15 μl total volume) contained 2 μl of template (cDNA), 7.5 μl of 2× Sensi Mix (Quantace, London, UK), 0.3 μl of 50× SYBR Green I Solution (Quantace), 0.33 μM of each of the forward and reverse primers and ultrapure H_2O. The product of ssu4dc cDNA was amplified with two specific primers (ssu4d-TF: 5'- AGAGACCCCAGAGCTTC CGAGAAGA-3' as forward primer and ssu4d-TR: 5'-AGCCGTTGCGGAGGAGGTAGTCG-3' as reverse primer), the product of ssu5B cDNA with 5B-F(5'-TGGCTTCGTCGATGATGGCTTCCAC3) and 5B-R (5′- GTTGTGGAAAGACCACGGGGAT ACG -3'); the product of ssu1cDNA with 1-F (5′-TTTCCACCGCCGCCGTGGCCCGCGT-3′) and 1-R (5′-GAATGACAGATACTTTTTGATTTAT -3′); using specific primers actin-F (5'-ATCCAATTCTC CTCACTGAAGCCCC-3') and actin-R (5'-GGGGCTTCAGTGAGGAGAATTGGAT-3') as control. Reactions were performed in a Step-one plus real-time PCR system (applied biosystem). Initial denaturation was performed at 95 ℃ for 10 minutes, followed by 45 cycles of: denaturation at 95 ℃ for 30seconds; annealing at 55 ℃ for 30seconds; and extension at 72 ℃ for 60 seconds. Each sample was run in triplicate in two separate reactions generating six readings per gene.

3 RESULTS

3.1 *Isolation of the ssu4d gene from Lemna gibba*

There is a conserved region found in rbcS genes of Lemna gibba (GenBank accession No. X17231.1, X17230.1, X17232.1, X17235.1, X17234.1 X17233.1, and X00137.1). A pair of generacy primers were designed based on this region. A putative novel rbcS gene about 400 bp fragment was obtained and sequenced, and eventually, a new rbcS gene with a length of 1346 bp (designated as ssu4d) was cloned from L. gibba genomic DNA by SEFA-PCR. Sequence blast analysis revealed that ssu4d shared 95-96 % identity with the known L. gibba rbcS genes. According to the sequence of this putative novel *rbcS* gene, specific primers were designed to amplify the *rbcS* gene cDNA, transcriptional site was confirmed by designing a series of 5'forward primers based on the putative transcriptional site which was inferred by the analysis of known *rbcS* genes from L gibba. A 579 bp *rbcS* gene cDNA fragment was first generated by RT-PCR. Sequence analysis of this cDNA fragment showed ssu4dc shared 95-96 % identity with Lemna gibba rbcS genes. The integrity of the genomic DNA (1346 bp in length) and cDNA (579 bp in length) sequences of ssu4d was confirmed by nest PCR.

3.2 *Sequence Analysis of L. gibba ssu4d gene*

Comparison of gDNA and cDNA sequences of ssu4d indicated that the position of the single intron in ssu4d gene was located from +355 nt to +1125 nt downstream the transcriptional iniation site. The intron, with the size of 770 nt, was quite different from the other intron of L. gibba rbcS gene in length; the 5' splice site of the intron is TT and the 3' splice site is AC, which indicated that ssu4d contained different splicing mechnasim. cDNA fragment of 579 bp was analysed and searched against NCBI database to find the conserved domain and putative function. It was found that the Lemna gibba cDNA (ssu4dc) possessed a 522 bp open reading frame (ORF) from 55 bp to 579 bp of the sequence apart from a 54 bp 5'untranslated region (5'-UTR) which was underlined (figure. 1), and this cDNA encoded for a protein of which those domains were found similar to other L. gibba RbcS (figure. 1). The deduced amino acid sequences of ssu4d gene are shown (figure. 1) .The transit peptide is less conserved than the mature SSU polypeptide when the deduced amino acid sequence of ssu4dc from L.gibba was compared with those sequences from other organisms in GenBank database using BLASTX search. The result indicated that ssu4d conserved segments have broad homology to the cloned L. gibba RbcS with the similarity and identity.

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X17235.1    ----MMVSTAAVARVRPAQTNMVGAFNGCRSSVAFPATRKANNDLSTLPSSGGRVSCMQV
X00137      ----------------------------------------------------------MQV
SSU4dc      MAASMMSSTAAVASV-AKTSMVAPFNGLRSAVAFPATRKAN-DLSTLPSNGGRVSCMQV
                                                                    ***

X17235.1    WPPEGLKKFETLSYLPPLSVEDLAKEVDYLLRNDWVPCIEFSKEGFVYRENNASPGYYDG
X00137      WPPEGLKKFETLSYFPLSSVEDLAKEVDYLLRNDWVPCIEFSKEGFVYRENNASPGYYDG
SSU4dc      WPPEGLKKFETLSYLPPLSVEALSKEVDYLLRNGWIPCVEFSKEGFVYRQYHASPGYYDG
            ************.* ****.*********.*:**.*********::*********

X17235.1    RYWTMWKLPMFGCTDASQVIAEVEEAKKAYPEYFVRIIGFDNKRQVQCISFIAYKPT
X00137      RYWTMWKLPMFGCTDASQVIAEVEEAKKAYPEYFVRIIGFDNKRQVQCISFIAYKPT
SSU4dc      RYWTMWKLPMFGCTDASQVIAEVEEAKKAYPEYFVRIIGFDNKRQVQCISFIAYKPT
            **************************************************
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Fig.1 Comparison of *Lemna gibba rbcS* sequences encoding the mature SSU protein. The deduced amino acid sequence of X17235.1, X00137 from *L.gibba* are shown.The deduced transient peptide of Ssu4dc was underlined with boldfaced letters. An asterisk(*) denotes the position of residues identical with X17235.1.

3.3 *Differential expression analysis of rbcS gene*

The 5 '-UTR (untranslated region) and 3 '- UTR of rbcS genes from Lemna gibba represent distinct sequences according to the sequence comparison analysis. This made it possible to use such sequence to analyze the expression of specific genes. It was demonstrated that the ssu1 sequence is the most highly represented, followed by ssu5B in Lemna gibba (Silverthorne et al., 1990). In an attempt to examine the expression of ssu4dc, Real-time PCR analysis of the cDNA clone were performed using primers designed based on the 5'- UTR and 3 '- UTR. Our results indicated that the SSU1 was the most highly represented, followed by ssu4dc and SSU5B, the expression level of ssu4dc gene was higher than that of SSU5B in light condition (Fig. 2).

Since the rbcS genes were expressed in white light-grown plants, we determined whether the different expression of rbcS gene under phytochrome regulation. The cDNA clone was used to template to evaluate the different expression of rbcS gene. The results of real-time PCR analysis demonstrated that the expression of each rbcS gene could be increased by red light pulses, although the magnitude of this response varies widely between the genes (Fig. 3). The responses to red light can be reversed by far-red light(Fig. 3). Although the expressed levels of the SSU40A, SSU40B and ssu4dc mRNAs are very different, they could also be demonstrated to be phytochrome-regulated.

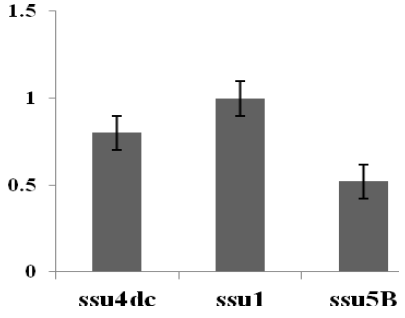
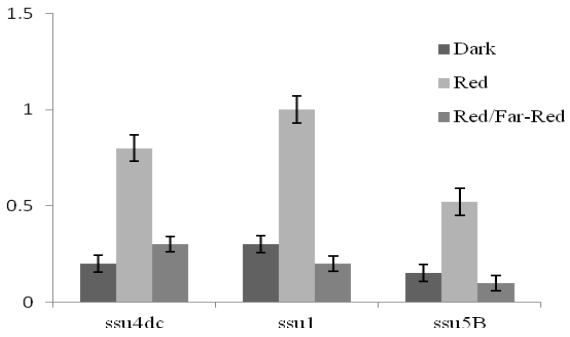


Fig. 2. Differential expression of individual *rbcS* genes in light-grown plants. The amount of expression of each gene is normalized to the expression of SSUI. The mean of triplicate determinations is shown.



RT-PCR

Fig. 3 phytochrome-regulated expression of individual *rbcS* genes. The relative expression levels of SSU4dc, SSU1 and SSU5B are shown in arbitrary units. Dark, Red, and Red/Far-red refer to the growth conditions described in Materials and methods. The mean of triplicate determinations is shown.

4 DISCUSSION

In this paper, a novel L. gibba rbcS gene (ssu4d), which was 1,346 bp long, was cloned from L. gibba, and the isolated 579-bp cDNA of ssu4d (ssu4dc) contained a 522-bp open reading frame encoding a protein of 174 amino acids, BLAST revealed that the cloned cDNA sequence of ssu4dc was highly similar to the rbcS genes from other species and the deduced ssu4dc protein also showed high identity to the RBCS proteins from other plant species via multialignments. Bioinformatics analysis showed that SSU4dc protein might belong to the rbcS supergene family. There was only one intron located between +355 and +1225, which indicated that ssu4dc gene was quite different from the cloned rbcS genes from duckweed. ssu4dc contained 54 bp of 5' untranslated region (UTR), and an open reading frame of 174 amino acids consisting of a chloroplast transit peptide with 57 amino acids and a mature protein of 117 amino acids. The deduced amino acid sequence of ssu4dc shared 95-96 % identity with Lemna gibba RBCS.

Quantitative analysis indicated that SSU4dc mRNA accumulated to much lower levels than SSU1 mRNA in steady state nuclear mRNA isolated from fronds, but higher levels than SSU5B mRNA. The expression levels of the SSU1, SSU5B and ssu4dc mRNAs varied very different, we could not rule out the possibility that the differential expression of individual rbcS genes is the result of more than one regulatory event. The analysis of these regulatory steps may provide a clue as to the mechanisms of phytochrome regulation of rbcS gene expression.

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