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Some molecular studies related to leaf rolling in *Ctenanthe Setosa*

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Abstract

The changes in total RNA content under drought stress condition that eventually causes leaf rolling was investigated in *Ctenanthe setosa*, a member of Marantaceae family in Zingiberales. Degree of leaf rolling (%) and total RNA content were measured under drought stress condition. It was determined that total RNA content decreased while degree of leaf rolling was increasing. In addition, partial sequence of small subunit ribosomal DNA (18S rDNA) of *Ctenanthe setosa* was determined to use as a control marker in polymerase chain reactions (PCR) during molecular studies. When compared with presumably functional sequences, 18S rDNA partial sequence of *Ctenanthe setosa* shows greater complete sequence similarity of 18S small subunits of *Marantochloa atropurpurea, Maranta bicolor* and *Calathea loeseneri* which are the members of same family. Obtained sequence also resembled *Strelitzia nicolai* and *Phenakospermum guyannense* belonging another family, Strelitziaceae from Zingiberales order. In addition, it seemed like to *Ravenala madagascariensis, Musa acuminate, Heliconia indica, Orchidantha fimbriata* and *Orchidantha siamensis* from same order. These results have been pointed out that obtained 18S rDNA partial sequence is true for *Ctenanthe setosa.*

Keywords: *Ctenanthe setosa*, leaf rolling, 18S rDNA, partial sequence, Total RNA.

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Introduction

Ctenanthe setosa (Marantaceae) is a tropical herbaceous perennial plant, and is cultivated as a greenhouse ornamental and houseplant for its attractive foliage. *C. setosa* respond to water deficit stress through mechanism such as leaf rolling, osmotic adjustment, proline accumulation and inducing of antioxidant system (Terzi and Kadioglu, 2006; Kadioglu and Terzi, 2007). Leaf rolling is an adaptive trait reducing water loss via transpiration thus, controlling plant water metabolism by relieving water stress (Omarova et al., 1995). The rolling also increases drought resistance in cereal crops (Townley-Smith and Hurd, 1979). In recent years, some biochemical studies have been performed on drought stress during leaf rolling in *C. setosa* (Kadioglu and Turgut, 1999; Ayaz *et al*., 2000; Kadioglu *et al*., 2002; Terzi and Kadioglu, 2006). However, there is no a report on total RNA changes during leaf rolling in plants.

Plants respond to various environmental stresses at molecular and cellular levels as well as physiological level. Genes induced during drought-stress conditions are thought to function not only in protecting cells from water deficit stress but also in the regulation of genes for signal transduction in the drought-stress response (Shinozaki *et al*., 2003). To understand responses to stresses involving a water deficit component, many genes induced by periods of water deficit have been identified and characterized (Bray, 1997). However there is no any study on inducible genes by water deficit stress in *C. setosa*. Polymerase chain reactions (PCR) have been generally used in these molecular studies. Moreover, sequences of 18S rDNA of species are generally used as a control marker in PCR. New sequence markers are also required to answer several issues (Nickerson and Drouin, 2004). In other words, it was firstly required a control marker such as 18S rDNA in these molecular studies. However, sequence of 18S rDNA of *C. setosa* has not been recorded by now.

The study investigates the relationship between total RNA content and leaf rolling in *Ctenanthe setosa*. In addition, our studies focus on determination of 18S rDNA sequence by using degenerate primers designed as based on 18S rDNA gene of *Pinus taeda* L in *C. setosa*.

Materials and Methods

Growth of the plants and stress applications

Ctenanthe setosa (Rosc.) Eichler (Marantaceae) was vegetatively propagated from their rhizomes and grown for twelve months in plastic pots containing peat and sand (5:1) in a growth chamber with the following parameters: 16 h light and 8 h darkness at 25 °C, relative humidity 70 % and 300 µmol m-2 s-1 of photon flux density. Some plants were regularly watered (control) and the others were withheld water through 56 days. The leaf rolling started on 32nd days after drought treatment. Degree of leaf rolling (%) and total RNA content were determined during drought stress.

Degree of leaf rolling

Degrees of leaf rolling (%) were measured according to Premachandra *et al.* (1993). The width of mid-portion of leaves was measured and leaf rolling degree was calculated as percentage reduction in leaf width by rolling. Samples were taken from the plants at three different stages from stage I up to stage III (control (0 % degree of leaf rolling): stage I, 50-60 %: stage II and 70 %-more: stage III).

Determination of total RNA content

Total RNA was isolated by using EZ-RNA Total RNA Isolation Kit (Biol. Indust.) according to instructions to producer firm. Leaf samples (0.1 g) were grounded in liquid nitrogen by using mortar and pestle. Samples were transferred to centrifuge tubes and added 0.5 ml denaturing solution (Solution A). Homogenates were stored for 5 min at room temperature and added 0.5 ml extraction solution (solution B) per 0.5 ml denaturing solution. Tubes were vigorously shaken for 15 sec, stored at room temperature for 10 min and then centrifuged at 12.000 g for 15 min at 4°C. The aqueous colorless (upper) phases were transferred to a fresh tube. RNA from the aqueous phase was precipitated by mixing with 0.5 ml isopropanol per 0.5 ml denaturing solution. It was stored at room temperature for 10 min and then centrifuged at 12.000 g for 8 min at 4 °C. The RNA pellet was washed with 1 ml 75 % ethanol after supernatant was removed. Then it was centrifuged at 7.500 g for 5 min at 4°C. The RNA pellet was air-dried for 5 min after the ethanol was removed and dissolved in nuclease free water. Total RNAs were displayed by 1.4 % agarose gel containing 0.5 µg/ml etidium bromur in 1 X Tris-Acetic acid-Ethylenediaminetetraasetic acid (TAE) buffer prepared by using deionise water containing diethyl pyrocarbonate (DEPC). Then gel was examined in BioDoc Analyse System (Biometra). Total RNA contents were determined by reading at 260 O.D via a spectrophotometer (Agilent 8453 E). RNA content was expressed as µg RNA per mg fresh weight.

DNA isolation

To determine 18S rDNA partial sequence of *C. setosa*, DNA isolation was firstly performed by using Genomic DNA isolation kit (Fermentas). Fresh leaf sample (100 mg) was grounded in liquid nitrogen with a mortar and pestle. Lysis solution (400 µl) was added and incubated at 65 °C for 10 min. It was gently mixed for several times after chloroform (600 µl) was added and centrifuged at 10.000 rpm for 2 min. The upper phase was transferred to a new tube, added 800 µl precipitation solution and mixed gently. It was centrifuged at 10.000 rpm for 2 min and discarded the supernatant. DNA pellet was dissolved in 1.2 M NaCl, precipitated with 96 % ethanol and supernatant was discarded after centrifuged 4 min. DNA pellet was washed with 70 % cold ethanol and dissolved in nuclease free water.

Amplification, cloning and sequencing of 18S rDNA

Total DNA used as a template was isolated from *C. setosa*. Amplification of the small subunit complete 18S rDNA was performed according to previously described methods (Padmanabhan *et al.* 1997). Reaction mixture was prepared as 50 ng of template DNA with 1 X reaction buffer, 200 µM (each) deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 5 pmole (each) primer, and 1 U of Taq DNA polymerase. Amplification was carried out with 40-cycle program (each cycle consisting of denaturation at 94°C for 90 s, annealing at 50 °C for 90 s, and extension at 72 °C for 120 s), followed by a final extension step at 72 °C for 5 min in a DNA thermal cycler (Biometra). The experiment was associated with negative (without DNA template) control. PCR products were analyzed by 1.4 % agarose gel electrophoresis. Then gel was examined in BioDoc Analyse System (Biometra).

The PCR product was cloned into pGEM-T easy vector (Promega A1360, Madison, USA) as per the instructions given by the supplier. Ligation products were transformed into DH5α cells. Transformed were checked by digesting the isolated plasmid with ECORI. The true clones containing the insert were analyzed by automated sequencing (Macrogen, Core). The sequence obtained was compared with those from GenBank by using the BLAST program (Altschul *et al*., 1990).

Oligonucleotide primers

PCR amplification of 18S rDNA gene from total DNA was done by using UNI Primers, forward, 5'- ACC AGA CAA ATC GCT CCA CC-3'; reverse, 5'- GGT GAC GGA GAA TTA GGG TTC-3' which were designed according to the nucleotide sequence of 18S rDNA gene of *Pinus taeda* L (Padmanabhan *et al*., 1997).

Statistical analysis

ANOVA of the means of six replicates with the Duncan Multiple Comparison test, and significance was determined at $P< 0.05$.

Results and Discussion

Leaf rolling increased in *Ctenanthe setosa* while drought stress period was increasing. Total RNA content also significantly declined compared to the stage I (control, unrolled) while stages of leaf rolling enhanced (Table 1). Displaying of agarose gel electrophoresis supposed to these results. Furthermore, in agarose gel electrophoresis, it was observed that densities of some chloroplast and mitochondrial RNA bands rose at stage II and III compared to stage I (Figure 1). Thus we suggest that some transcript levels increases during leaf rolling although total RNA content decreases. Indeed, it was reported that changes on some genes in transcriptional level occurred during stress in some plant species (Mittler and Zilinkas, 1994; Ashok *et al*., 2001). In the present study, decreasing of total RNA content while some RNA levels are increasing, results from declining of transcript levels which belong to some enzyme and protein. Nevertheless, Kadioglu and Turgut (1999) recorded that protein level declined during leaf rolling in *C. setosa.*

On the other hand, in present study, we firstly reported 18S rDNA small subunit partial sequence of *C. setosa*. DNA fragment multiplied by PCR from genomic DNA of *C. setosa* by using degenerate primer combination was shown in Figure 2. A sequence was obtained by using degenerate primers designed as based on 18S rDNA gene of *Pinus taeda* L in *C. setosa*. The sequence obtained was compared with those from GenBank by using the BLAST program and the 18S rDNA small subunit partial sequence was demonstrated in Figure 3. Several drought-responsive genes have been cloned and characterized in various plant species (Gosti *et al*., 1995; Yordanov *et al*., 2000; Reddy *et al*., 2004). In these molecular studies, PCR has been used. Some physiological studies have been done under drought stress condition during leaf rolling in *Ctenanthe setosa* (Kadioglu and Turgut, 1999; Ayaz *et al*., 2000; Saruhan *et al*., 2006; Terzi and Kadioglu, 2006; Saglam *et al*., 2008) but there is no any study at molecular level. We suggest that in the near future, primers based on 18S rDNA small subunit partial sequence are designed to use as a control marker on the molecular studies in *C. setosa*.

18S rDNA partial sequence of *C. setosa* was compared with those from GenBank, percentages of similarities were determined and results were shown in Table 2. In the present study, 18S rDNA sequence shows greater sequence similarity of 18S small subunits of *Marantochloa atropurpurea* (99 %), *Maranta bicolor* (99 %) and *Calathea loeseneri* (98 %) which are the members of same family. The sequence

Table 1. Total RNA content during leaf rolling in *C. setosa*. Means ± SD of six replicates.

Drought Period (day)	Stages of Leaf Rolling	Total RNA Content (ºg/mg fw)
0	I	2,1 ± 0,2* b
48	II	1,8 ± 0,2 a
56	III	1,3 ± 0,1 a

*Different letters indicate significant differences between means of six replicates.

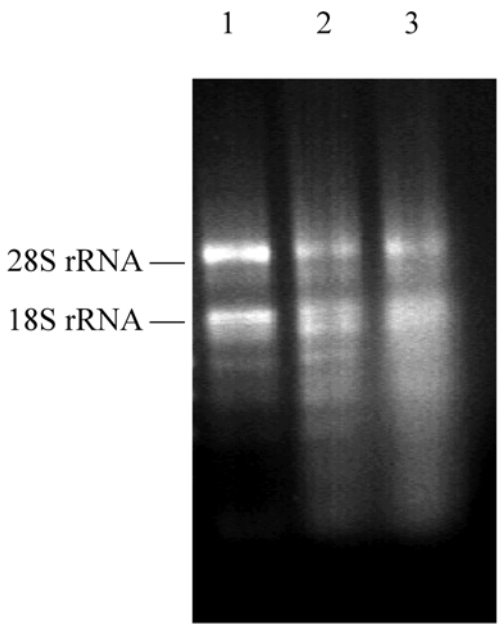

Figure 1. Displaying of total RNAs in agarose gel. Line 1. stage I (control), line 2: stage II, line 3: stage III.

Figure 2. DNA fragment by PCR from genomic DNA of *Ctenanthe setosa* by using degenerate primer combination. Line 1 is negative (without DHA template) control, line 2 is primer combination (dense band represents the primer coaction).

Table 2. Comparison of 18S rDNA small subunit partial sequence of *Ctenanthe setosa* with those from GenBank.

Resembled Species	Family	Percentage of similarity
Marantochloa atropurpurea	Marantaceae	99 %
Maranta bicolor	Marantaceae	99 %
Calathea loeseneri	Marantaceae	98 %
Strelitzia nicolai	Strelitziaceae	99 %
Phenakospermum guyannense	Strelitziaceae	99 %
Ravenala madagascariensis	Musaceae	99 %
Musa acuminata	Musaceae	98 %
Heliconia indica	Heliconiaceae	99 %
Orchidantha fimbriata	Lowiaceae	99 %
Orchidantha siamensis	Lowiaceae	99 %

also resembled *Strelitzia nicolai* (99 %) and *Phenakospermum guyannense* (99 %) belonging another family, Strelitziaceae from Zingiberales order. In addition it seemed like to *Ravenala madagascariensis* (99 %), *Musa acuminate* (98 %), *Heliconia indica* (99 %), *Orchidantha fimbriata* (99 %) and *Orchidantha siamensis* (99 %) from same order (Table 2). The small subunit ribosomal RNA is an invaluable tool in molecular evolution (De Peer and De Wachter, 1997). Percentages of similarities are very

TACGCCCGACGGTGACGGAGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAAC

GGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACGGG

GAGGTAGTGACAATAAATAACAATACCGGGCTCTTCGAGTCTGGTAATTGGAATGA

GTACAATCTAAATCCCTTAACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAG

CCGCGGTAATTCCAGCTCCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCG

TAGTTGGACCTTGGGTTGGGTCGGTCGGTCCGCCTCGCGGTGTGCACCGGTCGTCCC

GTCCCTTCTGCCGGTGATGCGTGCCTGGCCTTAACTGGCCGGGTCGTGCCTCCGGCG

CCGTTACTTTGAAGAAATTAGAGTGCTCAAAGCAAGCCCACGCTCTGGATACATTAG

CATGGGATAACATCACAGGATTTCGGTCCTATTGTGTTGGCCTTCGGGATCGGAGTA

ATGATTAAGAGGGACAGTCGGGGGCATTCGTATTTCATAGTCAGAGGTGAAATTCTT

GGATTTATGAAAGACGAACCACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATC

AAGAACGAAAGTTGGGGGCTCGAAGACGATCAGATACCGTCCTAGTCTCAACCATA

AACGATGCCGACCAGGGATCGGCGGATGTTGCTTTTAGGACTCCGCCGGCACCTTAT

GAGAAATCAAAGTCTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAA

AGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAA

CACGGGGAAACTTACCAGGTCCAGACATAGCAAGGATTGACAGACTGAGAGCTCTT

TCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTG

GTTCTAGTGGATCCCCCAGAAAGAGCTGAATCGTATC

Figure 3. 18S rDNA small subunit partial sequence of *Ctenanthe setosa*.

high and so these results have been pointed out that obtained sequence is true for *Ctenanthe setosa*.

Finally, total RNA content decreases during leaf rolling in *Ctenanthe setosa.* In addition, primers based on 18S rDNA small subunit partial sequence of *C. setosa* can be designed to use as a control marker in PCR studies in the near future.

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