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Correlation between the Chlorophyll Contents and duf538 Gene Expression Level in Celosia cristata

Ashraf Gholizadeh*

Research Institute for Fundamental Sciences (RIFS), University of Tabriz, Iran

***Corresponding author:** Ashraf Gholizadeh, Research Institute for Fundamental Sciences (*RIFS*), University of Tabriz, Iran, Tel: 9841133393924; E-mail: aghz_bioch@yahoo.co.in

Abstract

Proteins containing domain of unknown function 538 (DUF538) were frequently detected as stress-induced elements in plants. They were very recently suggested as a group of chlorophyll binding proteins and hydrolytic enzymes that most possibly degrade chlorophyll molecules. To enhance our information in plant system of DUF538 protein superfamily, our efforts were made to unravel the relative expression patterns of Celosia DUF538 transcript using real time reverse transcription and polymerase chain reaction (RT-PCR) with template materials collected from the drought stressed leaves having different scores of chlorosis. The results indicated that duf538 transcript is consistently detectable in all tissues except for the highly chlorotic died samples. In comparison with non-stressed green tissues, the expression level of duf538 gene was detected up to 4.3 folds increase in stressed yellow color tissues. The results suggested the duf538 gene product may be induced as hydrolytic enzyme to degrade chlorophyll molecules as defense response of drought stress challenged plants.

Keywords: Celosia, Drought stress, duf538, Gene expression, Real-time PCR.

Introduction

Protein superfamily containing domain of unknown function 538 has been frequently detected as stress-induced proteins in plants. The protein members of this superfamily have been distributed in wide ranges of monocotyledonous and dicotyledonous species [1,2]. They have been estimated to have molecular weights of about 19-21 kDa encoding around 170 amino acids. The three dimensional petal-like structure of *Arabidopsis thaliana* DUF538 protein has been determined to possess a protein structure dominated by ß-strands (PDB ID: D1ydua1). Several hundreds of DUF538 deduced amino acid sequences have been deposited as hypothetical proteins in universal databases. But, none of the members of this protein superfamily has been found in algae and cyanobacteria, so far [2]. DUF538 domain containing proteins have been mostly identified using genome annotation tools and also been cloned as inducible genes from plants challenged with various environmental stresses such as nutrient deficiency (GenBank, BFT415717), crown gall (GenBank, BG131101), and mixed elicitors (GenBank, AW040635) from tomato, and mild drought from Populus (GenBank, CU232042) and Celosia (GenBank, AJ535713) plants without real time analysis and comparison of its expression level [3]. On the basis of the high

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phosphorylation potential, DUF538 proteins have been predicted to play important as regulatory element in different stress-challenged plants [4]. Then after, by using a plant tissue abrading material, it has been revealed that the exogenously applied fusion form of a DUF538 protein activates the redox system of the plant cells [5].

In a recent study, DUF538 proteins have been suggested to act as bactericidal factors and affect the bacterial growth rates possibly through the binding to lipopolysaccharide molecules on the outer leaflet of the bacterial membranes as like as bactericidal permeability increasing proteins in mammalians innate immune system [6]. Besides this, by using the bioinformatics tools, DUF538 tertiary structures was predicted to be similar to the esterase-type hydrolases or lipolytic enzymes of which carboxyesterease type B, acyl-peptide hydrolase, entrochelin esterease and peroxisomal long chain acyl-coA hydrolase were detected as the best matches [7]. Very recently, the photoconvertible water soluble chlorophyll binding protein 1 (WSCP1) of *Chenopodium album* was found to be a structural signature of DUF538 superfamily [2]. This research work was then followed by the investigations on the chlorophyll hydrolyzing activity of the DUF538 domain containing proteins. With the most probability, DUF538 proteins were reported as chlorophyll degrading enzymes which involve in the chlorophyll breakdown process, *in vitro* [8]. Keeping all these information in view, DUF538 members may be suggested as a common chlorophyll degrading stress-related strategy in plant system. Therefore, as a part of our studies to increase our knowledge regarding the possible roles of DUF538 protein superfamily in plants, our attempt was made to investigate the real time-based expression profile of DUF538 gene in the non-stressed non-chlorotic and drought-stressed challenged chlorotic leaf tissues of *Celosia cristata* plants. A correlation between chlorophyll contents and the level of *duf538* gene expression was detected and discussed.

Materials and Methods

Chemicals

Chlorophylls were extracted and detected by Moran reagents. Trizol reagent (Cat. No. RN7713C; RNX^{TM}; CinnaGen) was used for total RNA isolation. mRNA purification kit was provided by QIAGEN, USA (Cat. No.70022). AcessQuick^{TM} RT-PCR System for cDNA synthesis was purchased from Promega (Cat. No. A1701). SYBR Green dye used for real-time detection of gene expression was from Bio Rad Company. Fermentas DNA extraction kit (Cat. No. K0513) was used for the purification of the PCR product from the agarose gel. Plasmid vector pGEM-T easy (Cat. No. A1360; Promega) was used for PCR product cloning All of the other chemicals used in this research work were of molecular biology grades.

Plant materials

The seeds of *Celosia cristata* were taken from our Laboratory stock. Test plants were allowed to grow under normal laboratory conditions of day to night period and humidity. In order to collect the leaf materials, test plant was well watered until sixth leaf stage and then after the water was withheld till the plant was visibly welted. Drought stress conditions were continued for 4 weeks. Experimental materials were collected from the leaf tissues having three different chlorotic scores before and after stress treatment. The test samples were include non-stressed green tissues, mild stressed yellow colored leaves, highly stressed yellow colored leaf tissues and died dried leaves.

Chlorophyll extraction and estimation

The chlorophyll content of the test leaf extracts were detected according to method of Moran (1982) with some modifications. The harvested fresh leaf materials (about 200 mg) were chapped and treated with 4 ml of ethanol and DMF (at

the rate 1/100) at 4°C for 48 h. DMF was then removed from the mixture by using a rotary evaporator. The dried sample was dissolved in 100% methanol and it's Chlorophyll (*a*, *b*, pchl and total) contents were quantified according to the following equations:

Ca = 12.65A664 – 2.99A647 – 0.04A625

Cb = –5.48A664 + 23.44A647 – 0.97A625

Cp = –3.49A664 – 5.25A647 + 28.3A625

CT = Ca + Cb + Cp

Where: Ca, Cb, Cp and CT represent the concentrations of *a*, *b*, proto and total chlorophylls.

Total RNA isolation and mRNA purification

Total cellular RNA of each sample was separately isolated from test materials using Trizol reagent, separately. For this, about 0.2 g of each material was fine powdered using liquid N_2 and 2 ml of Trizol reagent was added to homogenize it at room temperature (RT). 200 µl of chloroform was added to the mixture, mixed for 15 s, incubated on ice for 5 min and centrifuged at 13000 g for 15 min. The upper phase was transferred to another tube and RNA was precipitated with an equal volume of isopropanol. The pellet was washed in 1 ml of 75% ethanol, dried at RT and dissolved in 30 µl RNase-free water. The integrity of the RNA was tested on 1% non-denaturing agarose gel using TBE running buffer. Poly (A^+) RNA was purified from the total cellular RNA using oligo dT-columns according to the protocol of the provided kit. The integrity of the purified mRNA was also analyzed by electrophoresis using 1% non-denaturing agarose gel. The quantity of the RNA in each starting material was measured spectrophotometrically [9].

cDNA synthesis by RT-PCR

Specific primers used for the cDNA amplification were designed based on already reported DUF538 transcript sequence [3]. The primer sequences were designed by online internet-based Primer3 software. In order to analyze the expression pattern of DUF538 gene, the RT-PCR reactions were separately performed using one-step AcessQuick™ RT-PCR System. About 0.5 µg of each mRNA sample was mixed with 25 µl Master Mix (2x) and 1 µl of correspondent primer set. The mixtures were adjusted to a final volume of 50 µl using nuclease-free water. The reaction mixtures were incubated at 45°C for 45 min and preceded with PCR cycling. PCR was carried out after a pre-denaturation stage at 95°C for 3 min in 25 cycles. The nucleotide sequence of the used primer set and the details of PCR steps were performed as our previous work [5] and presented in Table 1.

Table 1. Primer set & PCR amplification steps

Primer sequences	
Left primer: 5´CGACCTTCATTAATGGACC3´	
Right primer: 5´ACTCATCCAAGCTCGCAAA3´	
PCR amplification steps	
Denaturation:	95°C / 1min
Annealing:	55°C / 1min
Extension:	72°C / 1.5min
Final Extension:	72°C / 10min

Primer sequences were designed by Primer3 software at: http// www.primer3plus.com/

In the next step, the amplified products were extracted from the agarose gel, cloned in pGEM-T easy cloning vector [9]. The cloned fragments proceeded for the sequencing in Microsynth DNA sequencing center at Switzerland.

Real time-based gene expression analysis

The expression level of the DUF538 transcripts in test samples were analyzed by real-time RT-PCR using iQ SYBR Green super mix (Bio Rad) on Miniopticon Real Time PCR Detection System (Bio Rad). Experiments were performed in three replicates and the mean values of the data were presented. All the expression data were normalized by adjusting the expression level of actin gene in test samples and the relative fold expression was calculated using the $2^{-\Delta\Delta Xt}$ value [10]. Each experiment was carried out with three replications using the same starting materials and experimental conditions. Data points on the graphs represent the mean values ± SD of replicates.

Sequence analysis of the isolated cDNAs

The nucleotide sequences of the amplified cDNAs were analyzed by computing at BLAST (Basic Local Alignment Search Tool) at http://www.ncbi.nlm.blast.com/ and CLASTALW sequence alignment software at http://www.genome.jp/ and *Expasy* proteomic tools at http://www.expasy.org/tools/

Results and Discussion

DUF538 proteins are recently known to have the structural and functional signatures of bactericidal proteins in the immune system of mammalians [6] and the type 1 water soluble chlorophyll binding proteins in the photosynthetic apparatus of plants [2]. Our survey revealed that both the bactericidal and chlorophyll binding proteins belong to stress-related proteins that are able to bind the lipid compounds or their derivatives [11,12]. Very recently, DUF538 protein superfamily was found to be a member of hydrolase enzymes that degrades chlorophyll molecules, *in vitro*. It was reported that DUF538 proteins like to chlorophylases contain the hydrolase fold characteristic of enzymes that catalyze ester hydrolysis [8]. The computational analysis revealed that DUF538 superfamily belongs to carboxyesterases (EC 3.1.1.1). Considering chlorophyll structure as an aromatic carboxylic ester, the carboxyesterase activity towards chlorophyll molecules for DUF538 proteins could be acceptable. Following these computational and *in vitro* experimental findings, our attempt was made to analyze the real time expression pattern of *duf538* gene in green and chlorotic leaf materials to identify the possible correlation between *duf538* gene activity or its deduced product and the chlorophyll contents of the leaves. In compare to the green control sample, the chlorophyll contents of the test samples were assessed to be 70%, 30% and 30% and 6% in Lowly, Mildly, highly stressed leaf samples and dried died sample, respectively (Figure 1). Parallel to the chlorophyll assays, the presence and the level of *duf538* gene expression was analyzed in the same three differently scored drought-stressed chlorotic leaves as well as the non-stressed green leaves and dried died leaf materials of Celosia plant using real-time gene expression method. All data were normalized by actin expression and related to the green sample as control using $2^{-\Delta\Delta X\tau}$ values. The results showed that parallel to a decrease in chlorophyll content in each test sample, an increase is detectable in the expression level of *duf538* (Figure 2). The expression levels of *duf538* gene showed 2.4, 3.5 and 4.3 folds increase when the chlorophyll contents of the chlorotic leaf samples decreased to the levels of 70%, 50% and 30%, respectively. This experiment result indicated that DUF538 protein superfamily might play an important role in chlorophyll breakdown process *in vivo*. The relevance of the *duf538* gene expression level to the stress severity and chlorophyll content in test leaf materials reveals that DUF538

members might be induced to act as defense mechanism through the degradation of chlorophyll molecules under stress conditions, *in vivo*.

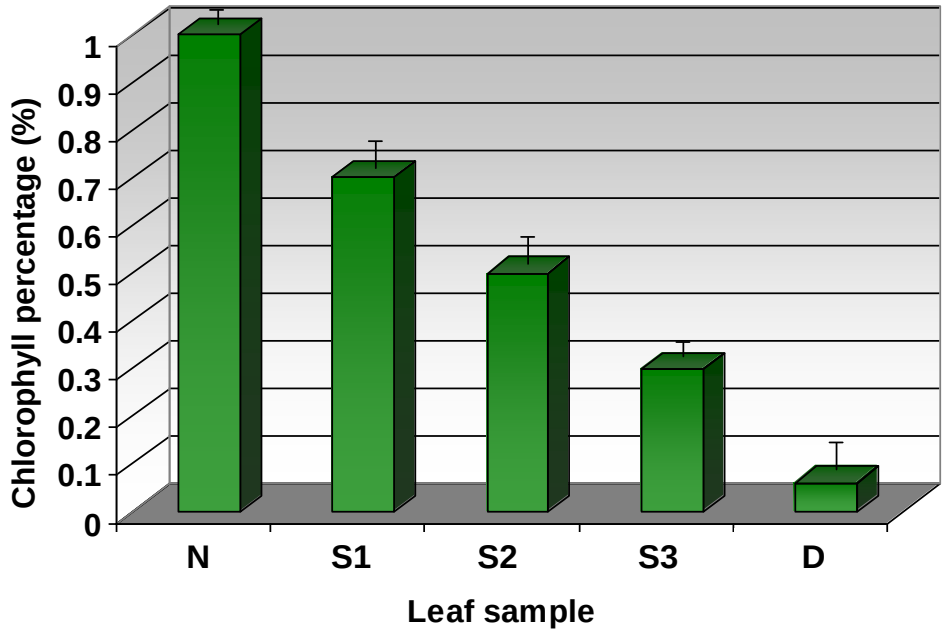

Figure 1: Chlorophyll assessment. The chlorophyll contents of the test samples were assessed by using Moran method and expressed as the percentage.

N: Non-stressed leaf sample; S1: Lowly stressed leaf sample; S2: Mildly stressed leaf; S3: highly stressed leaf sample D: Died leaf sample. Data presented as the mean values of three replicates ± SD

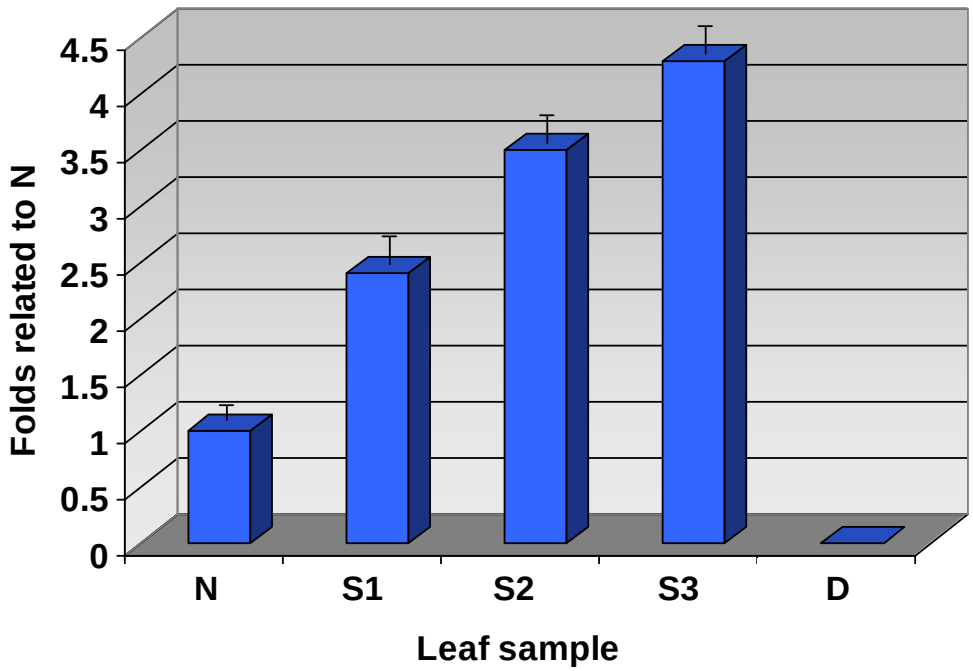

Figure 2: Real-time based expression analysis of *duf538* transcript. Expression level of *duf538* gene was analyzed in three differently scored chlorotic leaf tissues of Celosia plant grown under drought stress by using iQ SYBR Green dye.

N: Non-stressed leaf sample; S1: Lowly stressed leaf sample; S2: Mildly stressed leaf; S3: highly stressed leaf sample D: Died leaf sample. Data presented as the mean values of three replicates ± SD.

The gene expression of a DUF538 protein had been initially identified from mild drought-stressed Populus leaves (GenBank acc. no. CU232042). Then after, the expression of Celosia leaf *duf538* gene had been examined under drought stress conditions by using basic RT-PCR method through a time-course experiment [3]. However, the results of the present work by using real time detection method with regard to chlorophyll contents of the test samples clearly revealed that there is a reverse correlation between *duf538* gene activity of the leaves and their chlorophyll contents, *in vivo* (Figure 2). Despite chlorophyll biosynthetic pathway, our information about its catabolism is still very low. The relevance of the chlorophyll breakdown to the leaf senescence, fruit ripening and stress responses has been massively reported in plants. But, whether this process is only carried out via the hydrolytic activity of chlorophyllase enzyme is not yet clear. Chlorophyllase (EC 3.1.1.14), is the first enzyme in the chlorophyll degradation pathway [13,14]. Its relevance to all chlorophyll breakdown processes is unclear. Fang group has already reported that the loss of chlorophylls in senescing leaves is not directly related to the activity of Chlorophyllase [15]. It has been also shown that plant leaf chlorosis elicited by *Diuraphis noxia* feeding is different from the activity of chlorophyllase [16]. Besides these, it has been suggested that *Arabidopsis thaliana* chlorophyllases are not required for senescence-related chlorophyll breakdown *in vivo* [17]. This controversy reveals that the chlorophyll catabolism is still obscure process in plant system and needs to be more identified.

Based on our present experimental data along with the considering of DUF538 as a common stress-related protein in photosynthesizing organisms, a different type of catabolic pathway with regard to DUF538 activity may be proposed for the chlorophyll degradation in stress-challenged plants. This suggestion will open a new gate for functional studies on DUF538 protein members with regard to the chlorophyll breakdown processes of plants, *in vivo*.

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References

1. Gholizadeh A (2011) Heterologous expression of stress-responsive DUF538 domain containing protein and its morpho-biochemical consequences. Protein J 30: 351-358.

2. Takahashi S, Yoshikawa M, Kamada A, Ohtsuki T, Uchida A, et al. (2013) The photoconvertible water-soluble chlorophyll-binding protein of *Chenopodium album* is a member of DUF538, a superfamily that distributes in Embryophyta. J Plant Physiol 170: 1549-1552.

3. Gholizadeh A, baghbanKohnehrouz B (2010) Russ J Plant Physiol 57: 247.

4. Nakagami H, Sugiyama N, Mochida K, Daudi A, Yoshida Y, et al. (2010) Large-scale comparative phosphoproteomics identifies conserved phosphorylation sites in plants. Plant Physiol 153: 1161-1174.

5. Gholizadeh A (2011) Heterologous expression of stress-responsive DUF538 domain containing protein and its morpho-biochemical consequences. Protein J 30: 351-358.

6. Gholizadeh A, Kohnehrouz SB (2013) DUF538 protein super family is predicted to be the potential homologue of bactericidal/permeability-increasing protein in plant system. Protein J 32: 163-171.

7. Gholizadeh A (2014) Prediction of tertiary structure homology between bactericidal/permeability increasing protein of innate immune system and hydrolase enzymes Int J Biosci 5: 1-6.

8. Gholizadeh A (2016) DUF538 protein superfamily is predicted to be chlorophyll hydrolyzing enzymes in plants. Physiol Mol Biol Plants 22: 77-85.

9. Ausubel FM, Brent R, Kingston RE, Moore DD, Seiolman JG, et al. (1991) Current protocols in molecular biology, John Wiley & Sons, New York.

10. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) method. Methods 4: 402-408.

11. Raetz CR, Whitfield C (2002) Lipopolysaccharide endotoxins. Annu Rev Biochem 71: 635-700.

12. Horigome D, Satoh H, Itoh N, Mitsunaga K, Oonishi I, et al. (2007) Structural mechanism and photoprotective function of water-soluble chlorophyll-binding protein. J Biol Chem 282: 6525-6531.

13. Hörtensteiner S, Kräutler B (2011) Chlorophyll breakdown in higher plants. Biochim Biophys Acta 1807: 977-988.

14. Chairat B, Nutthachai P, Varit S (2013) Effect of UV-C treatment on chlorophyll degradation, antioxidant enzyme activites and senescene in Chinese kale (*Brassica oleracea* var. alboglabra) Int. Food Res. J 20: 623-628.

15. Fang Z, Bouwkamp JC, Solomos T (1998) Chlorophyllase activities and chlorophyll during leaf senescence in non-yellowing mutant and wild type of *Phaseolus vulgaris* L.J. Exp. Bot 49: 503-510.

16. Ni X, Quisenberry SS, Markwell JP, Heng-Moss P, Higley LG (2001) Entomo Exp et Appl 101, 159.

17. Schenk N, Schelbert S, Kanwischer M, Goldschmidt EE, Dörmann P, et al. (2007) The chlorophyllases AtCLH1 and AtCLH2 are not essential for senescence-related chlorophyll breakdown in *Arabidopsis thaliana*. FEBS Lett 581: 5517-5525.