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Article

Molecular Response of Dodonaea Viscosa Chloroplast Genes to Environmental Stress Factors

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Abstract: *Dodonaea viscosa* plant is characterized by a highly stress-tolerant and provides an important plant for studying chloroplast gene responses to environmental stresses. The aim of this study was to discuss the differential expression of plastid genes (clpP, ccsA, rpoC1, and ycf1) at various sites in Salahaddin Governorate, Iraq, which characterized by different levels of pollution from vehicle traffic, wastewater and industrial activities. Using (qRT-PCR) a significant increase in the gene expression levels of all four genes was observed in plants taken from site near the Baiji oil refinery, indicating robust transcriptional reprogramming in response to chemical-oxidative stress. The increased of the clpP gene expression show activation of the proteolytic system in chloroplasts to mitigate protein damage, while the increased expression of the ccsA gene indicated adaptive enhancement of the system of electron transport. The increased expression of the rpoC1 gene reflected a stress-induced modification of chloroplast encoded RNA polymerase, while the expression of the ycf1 gene was associated with pollutant-induced oxidative stress and chloroplast membrane regulation. while the samples from unpolluted sites showed expression levels close to basal and intermediate responses were observed in sites affected by wastewater. The increased expression of the rpoC1 gene reflected a stress-induced modification of chloroplast encoded RNA polymerase, while the expression of the ycf1 gene was associated with pollutant-induced oxidative stress and chloroplast membrane regulation. while the samples from unpolluted sites showed expression levels close to basal and intermediate responses were observed in sites affected by wastewater. These results demonstrate that these genes in D. viscosa function as molecular marker sensitive to environmental changes and pollution. Furthermore, the results underscore the significant potential of this species as a bioindicator plant for monitoring environmental degradation and suggest that chloroplast gene expression can provide early warnings of ecosystem-level impacts. Future studies recommend expanding the gene pool and integrating environmental data to develop more robust bioindicator systems for plant-based environmental monitoring.

Keywords: qRT-PCR, Gene Expression, Stress Response, Plant Bioindicator, *Dodonaea viscosa, cDNA*

Introduction

Dodonaea viscosa is a shrub.. flowering plant and rarely a small tree with erect twiggy branches belonging to the Sapindaceae family which comprises 150 genera and 2000 species and it is characterized by the ability for affording harsh climatic conditions such as nutrient deficiency in the soil, drought, and high temperatures, Given these features it is widely used in afforestation projects, green belt creation, desertification control, and soil stabilization in environmentally degraded areas[1]. The *Dodonaea viscosa* plant is characterize by highly efficient photosynthetic system in chloroplasts which contain many specific genes, these genes play several important roles such as sensing environmental stresses and regulating cellular responses [2]. Some Genes in the chloroplast genome are respond to environmental changes such as temperature and pollution, like genes that associated with the plastid encoded RNA polymerase (PEP) enzyme such as the rpoC1 gene and it has been observed that the transcription rates of these genes are significantly affected by high temperatures, leading to noticeable changes in their mRNA levels [3]. One of the most important genes in plastids is clpP which it is responsible for producing a hydrolytic enzyme that break down damaged proteins in the plastid. Therefore, the presence of this gene is essential to prevent the accumulation of unwanted proteins in plant cells under conditions of oxidative stress resulting from high temperatures or pollution. Consequently, this gene is a highly valuable indicator of cellular challenges [2]. It is a fundamental subunit within the Clp protein complex of the plastid, contributing to the maintenance of protein homeostasis by breaking down degraded proteins. This enables the plant to survive under conditions of heat, oxidative, and photostress [4].

Any disruption in the Clp system (like a defect in ClpP gene) will lead to protein imbalance in chloroplasts and then activates the response to unfolded proteins in chloroplasts (cpUPR). This increases the plant sensitivity to stress. Cell recovery in such cases depends on increased activity of molecular chaperones such as ClpB3 which contribute to the refolding of damaged proteins, thus restoring protein balance within the chloroplasts [5],[6]. Some studies have shown the efficiency and integrity of the Clp system are crucial factors in a plant ability to adapt to heat and drought as significant changes in Clp protein concentration activate multiple stress response pathway [7],[4].

ccsA gene is considered one of genes that involved in the assembly of cytochromes which are necesary for electron transport and it is sensitive to any environment chemical change such heavy metals, gaseous pollutants, and other factor that negatively affect on the efficiency of photosynthesis [8]. The ycf1 gene is considered a large gene in the chloroplast genome and its function is not yet fully understood, but several studies have shown that this gene has a high response to environmental stresses such as heat, drought, and pollutant and it is believed to play important role in the transport of substances and the regulation of ionic balance within the chloroplast [8]. On other hand, studies have demonstrated a type of communication between plastid and nuclear genes known as retrograde signaling, which enables chloroplasts to influence nuclear gene expression under various stress conditions. Thus, any change in plastid gene expression levels is indicative of the plant overall response to environmental stresses [2]. Given the importance of these genes and the accelerating pace of climate change resulting from increased industrial and thermal pollution and other climate-altering factors, studying the four genes (clpP, ccsA, rpoC1, and ycf1) in *Dodonaea viscosa* is crucial for assessing its ability to adapt to these varying environmental conditions. Changes in the gene expression levels of these genes can be studied or detected using real-time polymerase chain reaction (PCR), a sensitive and efficient technique for assessing plant stress responses at the cellular level [2].

Materials and Methods

1. Plant Sample Collection

The *Dodonaea viscosa* plant leaves included in the study were collected from four diverse locations in Salah Al-Din Governorate, Iraq, which represent varying levels of environmental pollution, as follows:

Site A: represent plant samples grown on the side of a road with high traffic density (i.e., with a high level of exposure to emissions from vehicle smoke).

Site B: Surrounding sewage pumping stations (level of exposure to sewage pollutants).

Site C: Near the Baiji oil refinery (level of exposure to industrial emissions).

Site D: An indoor garden on a university campus, far from roads and pollution sources (unpolluted

site, used as a control group).

Fresh and healthy leaves were collected using sterile gloves to avoid contamination and immediately after collection samples were placed in RNase-free tubes, flash-frozen in liquid nitrogen, and then stored at -80°C until RNA extraction.

2. RNA Extraction

Total RNA extracted from 100 mg of frozen leaf tissue using TRIzol™ reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA concentration and purity were determined using a NanoDrop™ device (Thermo Scientific). Samples were considered suitable for subsequent applications if the A260/280 absorbance ratio was between 1.8 and 2.1.

3. cDNA Synthesis

One microgram of RNA was converted to complementary DNA (cDNA) using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and Oligo(dT) primers, according to the manufacturer's instructions. The resulting cDNA was stored at -20°C until use in quantitative polymerase chain reaction (qPCR) analysis.

4. Quantitative Polymerase Chain Reaction (qPCR)

The relative expression levels of four selected chloroplast genes (clpP, ccsA, rpoC1, and ycf1) were determined using qPCR. Primers specific to each gene were designed using the NCBI Primer-BLAST tool and were commercially available. The primer sequences are shown in Table 1.

Table 1. Primer sequences that used in this study

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	Product size (bp)
clpP	TTTATGAGGCACCAACGTCA	GGGCTTGCCTGTTCTTTGTA	101
ccsA	AAAGGAAACGTGGGCATTTA	GAAAGCCCATGGAAGCTACA	113
rpoC1	TCAACAAATAAGCGCTTGGA	GGCCCAAAAATTCTTTCACA	138
ycf1	ATCAATTCGGTCGTTGTGGT	CCAGTTGTTGCGGATACCTT	134
16srRNA (Housekeeping)	CGGTATCTGGGGAATAAGCA	GATTTGACGGCGGACTTAAA	129

Each qPCR reaction (total volume 20 μL) contained: 10 μL of SYBR™ Green Master Mix (Applied Biosystems), 0.5 μL of each primer (10 μM), 2 μL of cDNA template, and 7 μL of nuclease-free water. The thermal cycle was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) under the following conditions: Initial unfolding at 95°C for 5 minutes, 40 cycles of: 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds, Melt curve analysis from 60–95°C to confirm specificity. The 16S rRNA gene was used as an internal reference gene (housekeeping gene) for normalization.

5. Data Analysis

Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with samples from the uncontaminated site (Control, Site D) used as calibration samples. All reactions were performed in three bioreplicators per site. Statistical differences between collection sites were assessed using the Independent Samples t-test via SPSS version 26. A p-value less than 0.05 was considered statistically significant.

Result and Discussion

Real-time PCR analysis shows difference in the amplification of the studied genes.The amplification curves as have shwon in figure 1 exhibited the typical shape, including a baseline phase and an exponential phase and a steady-state phase reflecting efficient and consistent amplification in all reactions. Differences in Ct values between samples indicate varying expression levels of the target genes betwen different sites. To ensure standardization of experimental conditions, all reactions were performed within a single run. The observed amplification patterns reflect the reliability of the

resulting gene expression data, thus confirming the validity and reliability of the results.

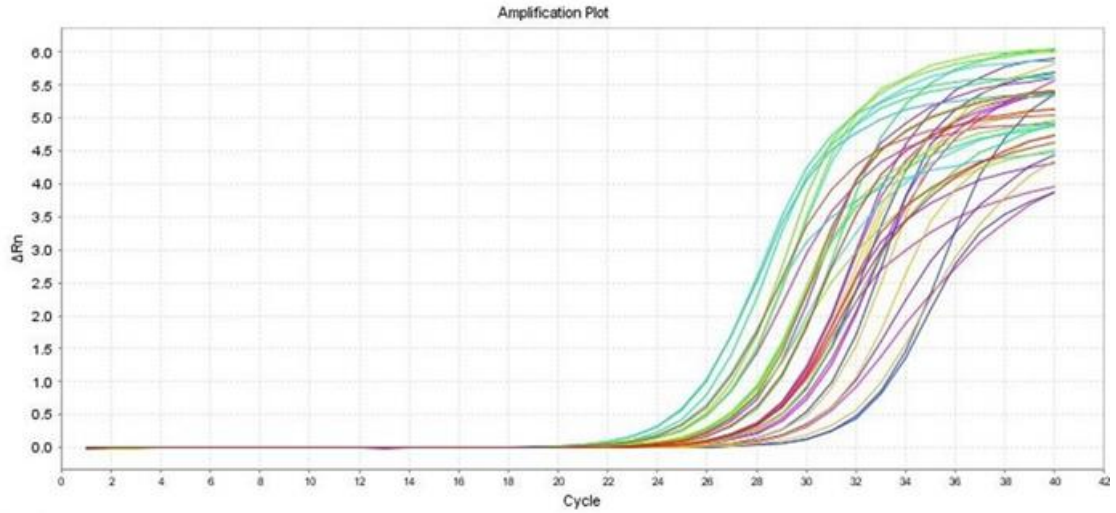

Figure 1. Real-timePCR amplification curves for the genes studied in this study.

The results of the ClpP gene expression analysis revealed several clear differences between the selected study sites (Table 2). Plants at site (A) showed an average expression level of 3.80 ± 0.36, significantly higher than the control group, with a statistically significant difference (P = 0.005). Samples at site (B) showed a relatively lower expression level (3.16 ± 0.05), but this decrease remained highly statistically significant when compared to the control group (P < 0.001). There is a significant increase in ClpP gene expression at site (C), reaching 22.80 ± 0.88, with very strong statistical significance (P < 0.001). This reflects the fact that different environmental conditions directly influenced the expression of this gene (ClpP) at the study sites.

Table 2. The level of ClpP gene expression in *Dodonaea viscosa* at different locations

Site	Mean 2^	Std. Deviation	Std.Error mean	p. Value
A	**3.8000**	**0.36056**	**0.20817**	**0.005**
B	**3.1667**	**0.05774**	**0.03333**	**< 0.001**
C	**22.8000**	**0.88882**	**0.51316**	**< 0.001**

The results of the ClpP gene showed that plants from the Baiji refinery site had a lot more of it than plants from the control site. This means that the plants in this area can handle oxidative stress, like pollution from heavy metals and particles [9]. The proteolysis system in chloroplasts is very important because it helps the cell deal with proteins that are broken. This system is important because it keeps the plastid's protein levels in check. This system will remove damaged proteins when plants are exposed to oxidative stress, like pollution or heat. This will lead to more expression of the ClpP gene[10].

The gene expression levels for the ccsA gene were different significantly between the sites (Table 3). Plants at site A showed an average expression of 2.37 ± 0.12 with a significant difference (P = 0.002), and samples at site (B) showed a higher expression level (3.57 ± 0.21), with a significant difference compared to site A (P = 0.0022). Plants that collate from the site C showed a significant increase in ccsA gene expression (14.67 ± 1.60), with a very high significance (P = 0.000122). This result indicates that ccsA gene expression was affected by environmental conditions, especially in areas exposed to industrial pollution sources.

Table 3. The level of ccsA gene expression in *Dodonaea viscosa* at different locations.

Site	Mean 2^	Std. Deviation	Std.Error mean	p. Value
A	**2.3667**	**0.11547**	**0.06667**	**0.002**
B	**3.5667**	**0.20817**	**0.12019**	**0.002185**

C	**14.6667**	**1.60104**	**0.92436**	**0.000122**

The results have shown a significant increase in ccsA gene expression in plants from site C compared to the sample in control site. The reason for this increase is the plants' response to oxidative stress caused by pollution, including heavy metals and airborne substances.

CcsA gene in chloroplast DNA plays an important role in associating heme groups with cytochrome c so in this case it is considered an essential part of the electron transport in chloroplasts. Some studies have indicated that the expression of this gene increases when plants exposure to oxidative stress. This increase of gene expression represent an adaptive response, therefore the plant able to perform photosynthesis correctly when it grows in unsuitable environmental conditions., [11] they have proven that ccsA expression increases in plants exposed to environmental stress making the plant capable of tolerating stress. ccsA is very important for keeping electron transport going in the chloroplast because it is the main protein that binds heme to cytochrome c. [2] discovered that ccsA transcripts elevate in reaction to oxidative stress, representing an adaptive mechanism to sustain photosynthetic efficacy in polluted or adverse conditions.

There are clear differences in the expression levels of the rpo gene between plants collected from different sites (Table 4). The average gene expression level at site A was 0.9167 ± 0.16073, and this decrease was not statistically significant when compared to the control group (p = 0.464). This means that pollution from traffic activity did not change the expression of genes at this site. At Site B, on the other hand, there was a relative increase in gene expression, with a mean value of 1.4333 ± 0.15275. This rise was statistically significant (p = 0.039), which means that rpo gene expression was likely turned on in a meaningful way at this site. The highest level of rpo gene expression was found at site C, with a mean value of 5.4333 ± 0.65064 and a very high p-value of 0.000295.

Table 4. The level of ccsA gene expression in *Dodonaea viscosa* at different locations.

Site	Mean 2^	Std. Deviation	Std.Error mean	p. Value
A	**0.9167**	**0.16073**	**0.09280**	**0.464**
B	**1.4333**	**0.15275**	**0.08819**	**0.039**
C	**5.4333**	**0.65064**	**0.37656**	**0.000295**

The results revealed that the expression of the rpo gene differed among the sites. In site A the mean expression level (0.9167 ± 0.1607) and did not show a statistically significant difference compared to the control group (P = 0.464). This indicate that the plants in this location were not exposed to Enough environmental stresses to stimulate plastid response pathways such as oxidative stress. While plants from site B showed a moderate but statistically significant increase in rpo gene expression (1.4333 ± 0.1527, P = 0.039), this increase reflects the effect of wastewater-associated environments which typically contain a mixture of metal ions, nutrients, and organic compounds. These conditions can induce mild oxidative stress within chloroplasts, prompting cells to modify gene expression programs as part of an adaptive response to environmental challenges. The most difference occurred was observed at Site C, the mean expression level was 5.4333 ± 0.6506 (P = 0.000295). This substantial upregulation indicates a robust response to industrial pollutants, including heavy metals and polycyclic aromatic hydrocarbons (PAHs). Such contaminants are known to promote the accumulation of reactive oxygen species (ROS) and to drive extensive reprogramming of plastid gene transcripts, including components of the plastid-encoded RNA polymerase.

ycf1 gene expression levels differed between the study sites, in site A the mean expression level was 2.10 ± 0.82, with no statistically significant difference compared to the control group (p = 0.145), this may be a result of the environmental conditions at this location having little effect on gene expression. At site B the average gene expression levels increased to 5.53 ± 2.22, but these increase was not statistically significant (p = 0.072). Although not statistically significant that suggests a moderate increase in gene expression, which may reflect the plants' adaptive response to environmental stressors such as metals and organic compounds found in wastewater. While in Site C the highest gene expression for ycf1 gene was (10.97 ± 0.49) with a highly significant difference (p = 0.001). These results show that the plants responded to heavy industrial pollution, such as heavy metals and PAHs.

These pollutants can increase the production of reactive oxygen species (ROS) and trigger widespread changes in how plastid genes are expressed. This seems to be a way for the plant cells to protect themselves and adapt to stressful conditions.

Table 5. The level of ycf1 gene expression in *Dodonaea viscosa* at different locations.

Site	Mean 2^	Std. Deviation	Std.Error mean	p. Value
A	**2.1000**	**0.81854**	**0.47258**	**0.145**
B	**5.5333**	**2.22336**	**1.28366**	**0.072**
C	**10.9667**	**0.49329**	**0.28480**	**0.001**

The high levels of the ycf1 gene at the sites studied show that *Dodonaea viscosa* can adapt to different environmental stresses. The average expression at site B was 5.53 ± 2.22 (p = 0.072), which is a fairly high level. This is probably because the area has been exposed to moderate levels of pollutants like metals and organic compounds that can be found in wastewater. These stresses cause mild oxidative stress in the chloroplasts, which causes the reprogramming of plastid gene transcripts, such as ycf1, as a way for the plant to adapt [12]. This means that ycf1 might be a temporary gene that is sensitive to moderate changes in the environment. It could be an early sign of how well the plant can adapt before it gets really stressed.

the highest expression level was observed in site C (10.97 ± 0.49, p = 0.001), This is because the severe exposure to industrial pollutants, like heavy metals and polycyclic aromatic hydrocarbons (PAHs), which are increase the levels of (ROS). These environmental stressors induce robust gene responses, therefore, to reprogramming of chloroplast gene transcription and this will lead to cell protection [13].

Many Studies have reported that the gene ycf1 is involved in plant defense strategies against oxidative stress including enhancing plastid membrane function and regulating Ion homeostasis, thus enhancing the plant's ability of *D. viscosa* in polluted environments, while in the site A result with no significant increase in gene expression level (2.10 ± 0.82, p = 0.145), this is because the absence or low intensity of environmental stressors and, Therefore, there is no need for a significant gene response[13].This indicates that plants growing in an unpolluted environment are characterized by low levels of gene expression in plastids, reflecting the stability of normal cellular activity without the need to resist stress.

In *D. viscosa* plant the ycf1 gen expression is affected by the pollution with wastewater consistent with previous studies showing that plastid genes like ycf1, are sensitive to the pollutants. It has been observed that the RNA editing efficiency of both ycf1 and clpP increases in abiotic stress especially on their role in adapting to environmental pressures [14]. Furthermore, [15] indicated that plastid gene expression is affected by stresses induced by salinity, heat, and heavy metals, factors common in wastewater discharge areas. Studies have proven that irrigation with polluted or industrial wastewater induces reprogramming of gene expression patterns and activates stress-responsive genes such as ZAT12 [16], [17]. The accumulation of heavy metals in soil, such as cadmium and lead, has also been linked to altered gene expression associated with plastid function and photosynthesis[18]. Therefore, it can be considered *D. viscosa* as bioindicator, as the expression of the ycf1 and clpP genes reflects environmental changes and pollutants in the ecosystem surrounding this plant.

The study results showed that the gene expression of four genes (ClpP, ccsA, rpo, ycf1) in *Dodonaea viscosa* was significantly affected by surrounding environmental conditions and the degree of industrial pollution at different locations. Sites with industrial pollution (Site C) recorded the highest gene expression level for all genes compared to control sites such as the site A. Sites with partial pollution like Site B showed moderate gene expression level reflecting the plants' response to moderate environmental stresses. A clear relationship was observed between the intensity of pollution and the degree of gene expression indicating that the plant gradually reprograms its plastid gene transcripts according to the level of environmental stress.

This progressive change in gene expression suggest that plant reprogram plastid gene transcription to adapt to environmental stresses particularly oxidative stress caused by heavy metals

and particulate matter and organic compounds in industrial wastewater. For example, high ClpP gene expression reflect the activation of the chloroplast's protein quality control system for managing damaged proteins, while ccsA and ycf1 high gene expression indicates enhanced photosynthetic efficiency and improved electron transport chain performance under stress conditions.The change in rpo expression reflect plastid transcription reprogramming in response to oxidative stress ensuring the continuity of essential plant functions.

Conclusion

These results reveal the critical effect of environmental pollutants on the transcriptional characteristics of chloroplast genes (D. viscosa) (clpP, ccsA, rpoC1, and ycf1), which was at the highest in D. viscosa near the Baiji oil refinery and suggested the activation of molecular defense responses against industrial oxidative stress in ever-higher levels. The significant upregulation of these genes indicates that proteolytic activity, electron transport adaptation, and transcriptional reprogramming are enhanced by chloroplasts to aid the plant in preserving cellular homeostasis in response to pollution. The sensitivity of chloroplast gene expression to environmental perturbations and the potential suitability of this species D. viscosa as a bioindicator for ecosystem health and pollution is emphasized by these results. These findings have important implications for environmental monitoring and plant stress biology because molecular markers based on plastid genes may serve as an early warning of habitat degradations. In order to further develop plant-based biomonitoring systems as well as a deeper understanding of plant resilience to anthropogenic stressors, future research should increase the scope of the genetic analysis, integrate specific measurements of environmental pollutants, and explore long-term mechanisms of adaptation.

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