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RT-PCR FOR ANTIOXIDANT GENES FROM EGYPTIAN GRAY MANGROVE *Avicennia marina* UNDER SALT STRESS TO NABQ PROTECTED AREA [182]

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ABSTRACT

RT-PCR was conducted for four genes implicated for salt tolerance, oxidative and osmotic stresses in Egyptian gray mangroves within Nabq protected area in South Sinai Governorate. The results showed over-expression of the mRNA of *ferritin* (*amFer1*) gene as very high expression, followed by increase in mRNA of *superoxide dismutase* (*amSOD1*) and *ubiquitin conjugation2* (*amUBC2*). At the same time gene expression of *catalase* (*amCAT1*) decreased.

Keywords: Antioxdant genes**,** *Avicennia marina*, Gray mangrove, RT-PCR, *amSOD1*, *amCAT1*, *amFer1*, *amUBC2* genes

INTRODUCTION

Mangroves (*Avicennia marina*) are largely restricted to the tropics and a few warm temperate regions between latitudes 30° north and 30° south, with the largest proportion found between latitudes 5° north and 5° south. About three-quarters of the worlds, mangroves are present in just 15 countries. Mangroves cover an estimated 15.2 million ha in 123 countries and territories **(FAO 2007).** This estimate, however, is 12.3% less than the most recent estimates by **Giri et al (2011).** Mangroves are most extensive in Asia (39%), followed by Africa (21%), North and Central America (15%), South America (12.6%), and Oceania (Australia, Papua new Guinea, New Zealand, South Pacific) (12.4%) **(FAO 2007).**

Mangroves are quite old, possibly arising just after the first angiosperms, around 114 million years ago **(Duke 1992).** *Avicennia* and *Rhizophora* were probably the first genera to evolve, appearing near the end of the Cretaceous period **(Chapman 1976)**. Though mangroves evolved in the tropics, one species; *Avicennia marina*, is found in temperate latitudes, particularly in the southern hemisphere **(Saenger 1998)**. Mangrove forests in Egypt are estimated to cover approximately 700 hectares, being the northern most mangroves in the Indo-Pacific-East. All mangroves in Egypt are now maintained in three main protectorates, one at Nabq (south Sinai Governorate), the others in Safaga and Wadi El Gamal (Red Sea Governorate).

Nabq Protectorate located in Egypt, South Sinai Governorate, over 600 kilometers square and 85 km long on the coast of the Red Sea. The mangrove stand at Nabq fronts the shoreline at the mouth of Wadi Kid.

Mangroves are adapted to their saline environment. Their root systems, seen as leafless branches sprouting from the ground around each tree, act as a barrier, keeping out most of the salts are the seawater. Salt not removed by the roots is exuded by the leaves and seen as salt crystals on the underside of each leaf **(Al-Mufti 2000).** Monospecific stands of *Avicennia marina* are found on shorelines at Nabq and Ras-Mohamed Protectorates. *Avicennia marina* is the only mangrove species growing in Ras-Mohamed and Nabq protectorates **(Al-Mufti 2000).**

Mangrove ecosystems, in general, are dynamic, undergoing changes on time scales of 100-100000 years **(Woodroffe 1992).** The plants must,

therefore, have some salinity tolerance. True mangroves (e.g. *Avicennia spp*. and *Rhizophora spp*.) tolerate higher salinity than do non-mangroves, but tolerance also varies among the true mangroves **(Kathiresan and Thangam 1990; Kathiresan et al 1996).**

A. marina possesses physical and structural attributes such as pneumatophores and salt glands. The salt tolerance of *A. marina* is due to three different mechanisms: (1) salt avoidance through the roots; (2) capacity to maintain normal metabolic activity in the presence of high intracellular salt levels and (3) secretion of penetrating ions using salt glands **(Waisel et al 1986).**

Tolerance to salt stress is a complex trait, which involves various aspects such as osmotic, ionic and oxidative stresses. Salt stress leads to dehydration and osmotic stress with the reduced availability of water resulting in stomata closure, reduced supply of carbon dioxide leading to a high production of reactive oxygen species (ROS) in the chloroplasts **(Tanaka et al 1999)**. The genes thought to be important for salt stress responses in these studies are ones that are involved in osmotic balance, oxidative protection (antioxidants), ion homeostasis (salt antiporters) and signal transduction. For instance, the presence of a large collection of ROS-scavenging enzymes of the catalase (CAT), ferritin (Fer), and superoxide dismutase (SOD), suggested that there is increased ROS production in plants under salt stress conditions and, therefore, these enzymes are needed to maintain redox homeostasis **(Zhang et al 2001).**

Superoxide dismutase (SOD) is an important antioxidant enzyme and is the first line of defence against oxidative stress in plants **(Parida et al 2004).** Catalases (CAT) are haem-containing tetrameric enzymes involved in the removal of H_2O_2 **(Cherian et al 1999).** The iron-storage protein ferritin, which is localized in the plastids in plants, plays roles during development and under stress conditions, due to its ability to buffer iron (Fe) fluxes, keeping this element temporary in a safe and bioavailable form. Ferritin (Fe) of leaves or nodules may serve as a preliminary pool for the building up of Fe containing proteins. Ferritin could also play a general role in stress response in plants. Concerning stress, it is more specifically involved in the buffering of Fe in the chloroplast during recovery of Fe deficiency and as an important component to protect plastids against Fe-mediated oxidative stress. Ubiquitin-conjugating is the crucial regulatory step for the selective protein degradation mechanism in the ubiquitin-26S proteosome pathway in plants ubiquitin conjugating enzymes were induced under salt, heat and heavy metal stress in different tissues in plants **(Jithesh et al 2006). Willekens et al (1995)** Relative mRNA expression levels could be used as indicators to study the role of individual genes of multigene family in each of these stresses. By monitoring PCR amplification in real-time, it is possible to measure the reaction during the exponential phase of growth, a time when none of the reagents are limiting and the reaction is most efficient. This allows real-time (Q-PCR) to achieve more sensitive detection, better reproducibility, and a wider linear dynamic range than conventional methods. Quantitative RT-PCR has been used extensively to study gene expression because of its high sensitivity and reproducibility **(Taylor and Harrier 2003).** Generally two quantification types in real-time RT-PCR are possible; (i) a relative quantification based on the relative expression of a target gene versus a reference gene to investigate the physiological changes in gene expression, the relative expression ratio is adequate for most purposes and (ii) an absolute quantification, based either on an internal or an external calibration curve **(Morrison et al 1998; Pfaffl 2001).** Furthermore, a normalization of the target gene with an endogenous standard is recommended. Therefore, mainly non-regulated reference genes or housekeeping genes like glyceraldehyde-3-phosphate dehydrogenase (G3PDH or GAPDH), albumin, actins, tubulins, cyclophilin, 18S rRNA or 28S rRNA are used **(Marten et al 1994; Xiao et al 2016).** The actin gene was often used to normalize the quantification of expression **(Thomas et al 2003).**

Mangroves inhabiting the intertidal zones suffer from diverse stresses such as high salinity, hypoxia, ultraviolet radiation, nutrition deficiency and so on. These primary stresses may lead to secondary oxidative stress, resulting in accumulation of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical and singlet oxygen ($^1O_2$) **(Parida et al 2004).** These cytotoxic reactive oxygen species can seriously disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids **(Parida et al 2004).** Mangroves with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to this oxidative damage **(Takemura et al 2000; Parida et al 2004).** In these cases, induction of antioxidant enzymes was shown to protect halophytes against ROS, thus preventing lipid peroxidation during salt stress. This suggests that these antioxidant en-

zymes are essential components of an adaptive defence mechanism against salt stress in halophytes. Some of the major antioxidant enzymes involved in scavenging are superoxide dismutase SOD, catalase (CAT), ferritin (Fer) and POX **(Fang et al 2005).** The activities of the antioxidative enzymes such as catalase (CAT), ferritin (Fer), guaiacol peroxidase (POD), and superoxide dismutase (SOD), increase under high salinity and a correlation of these enzyme levels and salt tolerance exists in mangroves **(Parida et al 2004; Takemura et al 2000)**. There are several reports of up-regulation of antioxidative enzymes and their corresponding genes in mangroves under salinity. In *Avicennia marina*, the activities of the antioxidant enzymes, superoxide dismutase (SOD) and catalase, show an immediate increase after the plants are transferred from water to high salinity **(Takemura et al 2000).** Salt treatment preferentially enhanced the content of H_2O_2 as well as the activity of ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and superoxide dismutase (SOD), whereas, it induced the decrease of catalase (CAT) activity **(Parida et al 2004).** It has also been reported that in the salt-secreting mangrove *Avicennia marina*, there is a linear increase of salt secretion of leaf with increase in period of salt treatment and a concomitant decrease in antioxidative enzymes such as CAT, POX and POD **(Mishra and Das 2003)**. These genes can be categorized into five major groups such as (1) osmolyte biosynthesis, (2) reactive oxygen scavenger, (3) chaperones, (4) transporters and (5) signaling components **(Miyama and Hanagata 2007).**

Superoxide dismutases constitute the first line of defence against ROS and catalyses the dismutation of the superoxide radical into hydrogen peroxide **(Salin, 1987)**. Catalases are tetrameric heme-containing enzymes that convert hydrogen peroxide to water and oxygen, protecting the cell from the damaging effects of hydrogen peroxide accumulation **(Sanchez-Casas and Klessig 1994)**. The balance between SOD and CAT or APX activities in cells is crucial for determining the steady-state level of superoxide radicals and hydrogen peroxide and counteracting the deleterious effects of ROS **(Bowler et al 1991; Apel and Hirt 2004).** Fe^{2+} plays a crucial role in oxidative stress, especially when there is an overproduction of superoxides Ferritin could sequester free iron to reduce this oxidative threat **(Harrison and Arosio 1996)**.

The objective of this study was to assess the potentials of Egyptian mangrove as a rich source of some antioxidant genes.

MATERIALS AND METHODS

Leaves of the Egyptian gray mangrove (*Avicenna marina*) were collected from the Nabq Protectorate in the South Sinai Governorate and were reserved and placed on dry ice (-70°C).

Total RNA isolation and purification

Total RNA was extracted from leaves of Egyptian gray mangrove (*Avicenna marina*) by using Direct-zol™ RNA MiniPrep (Zymo Research, USA). This protocol consists of two parts: sample preparation and RNA purification (catalog Nos.R2050).

Sample preparation

A100 mg leaf tissue was ground in liquid nitrogen, then immediately was transferred to a tube containing 600 µl TRI Reagent® and was homogenized. To remove particulate debris from homogenized tissue, centrifuged (10000 x g for 1 minute) and transferred the supernatant into an RNase-free tube.

Reverse transcription (RT- PCR) analysis

The protocol of (RT- PCR) was optimized to SensiFAST™ Probe Lo-ROX Kit to generate first-strand of cDNA for use in two-steps reverse transcriptase PCR (RT-PCR). All components were mixed, briefly centrifuged after thawing and kept on ice.

Quantitative RT- PCR analysis

Real-time PCR was performed with Fast SYBR Green® LO- Rox master mix (Bio Line, UK). Real-time PCR (qPCR) reaction was applied according to **Schmittgen and Livak (2008)** as follows:

Fast SYBR Green master mix 10 µl, Forward primer (5 pmol/µl) 0.8 µl, Reverse primer (10 pmol/µl) 0.8 µl, Template cDNA (25 ng/µl) 2 µl and dd H_2O up to 20 µl were added. Reactions were run on a Stratagene Mx3000p (Agilent Technologies, Santa Clara, CA) condition was 10 min at 95°C, 40 cycles of 5s at 95°C, 45s at 60°C, 20s at 72°C and 4ºC (infinite).

PCR products were examined by melt curve analysis. The reaction was achieved with five primer pairs for the five genes **(Table 1)**, each reaction was repeated three times for cDNA sample **(Schmittgen and Livak 2008).** Actin gene was used as a reference gene for data normalization. Analyses were made using the MxPro Mx3000P v3.00 software (Agilent Technologies, Santa Clara, CA).

Table 1. Primer pairs for amplifying of *amSod1, amCat1, amFer1, amUbc2* and *actin* genes

Gene	Primer sequences Forward	Primer sequences Reverse
amSod1	Forward 5'-ATCATTACCCAGTCGCTTGT- 3'	Reverse 5'-AGCAAAGATGATGTGGGAAC-3'
amCat1	Forward 5'-GAGAATGGAGGCAACGTTTA-3'	Reverse 5'-TGTGTGCATCAAGAAGTTCG-3'
amFer1	Forward 5'-ATCTCTATCCGTGGTTTGCC-3'	Reverse 5'-GGATTCACAGCTCCATCAAAT-3'
amUbc2	Forward 5'-TCCCTTACTAGACGGTTGG-3'	Reverse 5'-AGTGACGCGTTCCTTACA-3'
actin	Forward 5'-GCCGTGCTTTCTCTTTATGC -3'	Reverse 5'-CTCTCTGGAGGAGCAACCAC -3'

RESULTS AND DISCUSSION

Quantitative PCR (qPCR) results were validated and confirmed for the four mangrove genes regulation, abiotic stresses (salt, osmotic, ionic and oxidative) *(amFer1, amSod1, amUbc2 and amCat1)* respectively. Transcript levels of the four genes were determined by RT- PCR using the Egyptian Gray Mangrove (*Avicenna marina*).

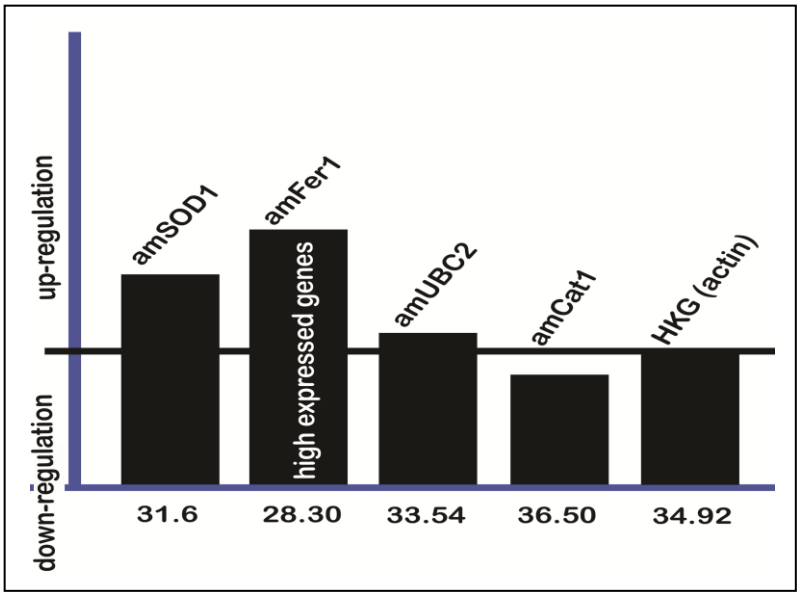

Fig. 1. Chart of fold Amplification plots and standard curve for *amcat1*, *amsod1*, *amfera*, *amUBC2* and HKG (*actin*) genes using real-time PCR assay of Egyptian Gray Mangrove (*Avicenna marina*)

Our choice depended on the expression fold change of genes, *amFer1, amSod1, amUbc2 and amCat1* at transcript levels as the ratio between the initial numbers of molecules in the target genes and the *actin* gene for normalization (housekeeping gene in qPCR) as shown in **Figure (1)**.

The results **(Figure 1)** showed that the expression level *amCat1* gene expression was down-regulated, *amSod1* gene expression was up-regulated, *amUBC2* gene expression slightly was up-regulated. *amFer1* gene expression is up-regulated (high expressed gene).

Wang et al (2004) found that linked levels during stress conditions were positively correlated with reduced levels of H_2O_2 lipid peroxidation and increased antioxidant enzyme activity.

While total SOD activity increased in response to salinity stress in leaf tissues of *A. marina* **(Cherian et al 1999)**. The decrease in CAT activity and the concomitant increase in SOD activity suggested that this is an adaptation to remove the excess H_2O_2 generated due to increase in SOD activity. It has been observed that cytosolic CAT transcripts as well as cytosolic CAT activities decreased after salt stress. The induction of CAT clearly showed that this enzyme plays a critical role in controlling increase of H_2O_2 concentration in plant cells during the initial salt-induced oxidative stress **(Wang et al 2004)**.

Jithesh et al (2006) assess of the role of ferritin in response to salt mediated oxidative stress in *A. marina*, increased Fer1 transcript levels was observed in leaf tissues within salt stress treatment. This suggested that Fer1 transcript accumulation is an immediate and short-term response to salt stress. Ferritin was shown to be an important constituent of the oxidative stress response in halophytes and may participate in the defence of chloroplasts against oxidative stress. They studied the expression of antioxidant genes such as Cu–Zn SOD (*amSod1*), catalase (*amCat1*) and ferritin (*amFer1*) in response to salt, iron, hydrogen peroxide, mannitol and light stress by mRNA expression analysis in *A. marina*. In response to NaCl stress, *amCat1, amFer1, amSod1* and *amUBC2* mRNA levels were induced by salt, oxidative, iron stress and by direct H_2O_2 stress treatment, thus confirming their role in oxidative stress response**.** Ubiquitin-conjugating 2 (*Ubc2*) mRNA levels were reported to be up-regulated by salt stress, which is a composite stress including oxidative, osmotic and ionic stresses **(Parani et al 2002).**

CONCLUSION

Crops like wheat, maize and rice enhance their abiotic stress tolerance; while it was evident from the aforementioned results that mangrove possesses a rich pool of genes implicated in abiotic stress tolerance. Such genes could be intensively characterized further to be utilized in the production of genetically engineered economic crops.

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RT-PCR للجينات المضادة للأكسدة في المانجروف الرمادي المصري *Avicennia marina* تحت ظروف الاجهاد الملحي لمحمية نبق

[182]

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الموجـــــــز

تم عمل تفاعل البلمرة المتسلسل في الوقت الفعلي لعدد اربع جينات خاصة بتحمل الملوحة والاكسدة والاسموزية للمانجروف الرمادي المصري داخل محمية نبق بمحافظة جنوب سيناء والجينات محل الدراسة هي الكتاليز والسوبر أكسيد ديسميوتاز والفيريتين واليوبيكيتين وقد تحصلنا علي النتائج التالية وهي زيادة كبيرة جدا في التعبير الجيني لجين الفيريتين مع زيادة في التعبير الجيني لجين السوبر أكسيد ديسميوتاز وكذلك زيادة في التعبير الجيني لجين اليوبيكيتين وصاحب ذلك انخفاض ملحوظ في التعبير الجيني لجين الكتاليز. وتؤكد النتائج علي ان الجينات محل الدراسة هي جينات فعالة ولها دو هام في تحمل الاجهاد الملحي والتاكسدي والاسموزى في نبات المانجروف الرمادي المصري تحت ظروف البحر الحمر داخل محمية نبق.

الكلمات الدالة: جينات مضادات الأكسدة، *Avicennia marina*، أشجار المانجروف الرمادي، RT-PCR – *amCAT1* – *amSOD1* – *amFer1* – – *amUBC2*

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