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EXPRESSION OF SOME SALT TOLERANCE GENES ISOLATED FROM EGYPTIAN GRAY MANGROVE (*AVICENNIA MARINA*)

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SUMMARY

Mangroves are well-adapted halophytes that thrive in coastal saline environments. They live under difficult environmental conditions, such as high light intensity and external salt concentrations, as well as low-oxygen environments, such as water-logged muck, that are typically inappropriate for the survival of other plants. Salinity is a major abiotic factor that affects plant growth, productivity, and dispersal in tropical and semitropical intertidal areas. Furthermore, it affects approximately 20% of all cultivable land and 50% of all irrigated land on the planet. Mangroves have developed a sophisticated salt filtration mechanism and a complicated root structure to withstand salty water exposure and tidal movement. The expression patterns of five salt tolerance genes (*amFer1*, *amDhna*, *amSod1, amCat1*, and *amUbc2*) in the Egyptian gray mangrove (*Avicennia marina* Forssk.) grown under different environmental conditions in South Sinai protectorates (Nabq, Ras Mohamed, Safaga, and Wadi El-Gemal), Egypt, were investigated in this study. This study aimed to assess and examine the genetic behavior of mangroves in response to salinity by using quantitative real-time PCR. Findings revealed differences in the expression patterns of the investigated genes under various conditions, showing that salinity influences plant genetic response. Ferritin gene expression was high in all locations, indicating that ferritin represents an essential component of the mangrove response mechanisms.

Keywords: Mangrove (*Avicennia marina*), (*amFer1*, *amDhna*, *amSod1, amCat1*, and *amUbc2*), salt tolerance genes, quantitative real-time PCR

Key findings: The variations in the expression levels of five selected genes (*amFer1*, *amDhna*, *amSod1*, *amCat1*, and *amUbc2*) in the Egyptian gray mangrove were examined. Plant samples were collected from four sites in South Sinai (Nabq, Ras Mohamed, Safaga, and Wadi El-Gemal). The ferritin gene showed the highest expression level in *A. marina* Forssk regardless of collection site, reflecting its vital role in the response (adaptation) mechanism of *A. marina* to abiotic stresses.

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INTRODUCTION

Mangroves are a genus of tropical and semitropical trees and shrubs that grow in the intertidal zones of tropical and semitropical coasts worldwide (Hong *et al*., 2018). Approximately 80 species of mangrove trees have evolved morphological and physiological characteristics to adapt to harsh coastal climatic conditions, with their genetic diversity originating from 20 plant families (Duke *et al*., 1998; Duke, 2013). Mangroves are woody trees that serve as the primary vegetation between tidal and salty marshes in tropical and semitropical coastlines (Parida and Jha, 2010). Mangrove trees constitute a delicate ecology that is being endangered by rising sea levels (Cheng *et al*., 2015; Woodroffe *et al*., 2016), extreme climate disasters, increased UV radiation (Fei *et al*., 2015), and human activities, (i.e., oil spills, contamination, and deforestation) (Pan *et al*., 2019).

Mangrove trees are unique because they can grow well under extreme salt stress and freshwater conditions. Mangroves are possibly the only plant system with wide adaptations to extreme conditions (Rajalakshmi and Parida, 2012). Furthermore, they are well-adapted halophytes. They live in a harsh arid environment with light intensity and salt concentrations that increase from the outside; such an environment is typically unfavorable for the survival of different kinds of plants (Cheeseman *et al*., 1997). Mangrove forests are a vital ecosystem found in coastal locations. Mangroves are often beneficial to humans. However, their spread and diversity are increasingly threatened due to increasing sea levels and human actions. Furthermore, the excessive use of mangroves disrupts the natural balance of forces in the ecosystem, and the conversion of mangrove forests into ponds causes harm. As a result of settlements and firewood use, the rate of carbon absorption decreases, and the accumulation of global warming gases increases as garbage decomposes, resulting in a reduction in the rate of carbon absorption and an increase in the accumulation of global warming gases (Wardiatno *et al*., and Xie *et al*., 2020).

Mangrove trees cover approximately 525 ha of 28 different protectorates along Egypt’s Red Sea coastlines. One massive discontinuous section is found in the Gulf of Aqaba in the Nabq Protected area, and one small site in the Gulf of Suez's most southern part is located in the Ras Mohammed National Protectorate (Zahran and Willis, 2009).

Mangroves in Egypt are mainly monospecific, except for those in a few areas along the Egyptian–Sudanese border area, where *Rhizophora mucronata* (loop-root mangrove; Rhizophoraceae) grows alongside *Avicennia marina* (gray mangroves; Avicenniaceae). The Sinai Mangrove Protectorate is home to Egyptian mangroves and mangrove trees that grow on Egyptian land (PERSGA, 2004). Compared with *Rhizophora mucronata*, *A. marina* is more resistant and adaptable to salinity, low rainfall, and severe temperature conditions and thus has a larger global and local coverage (Egypt) (Afefe *et al*., 2019). Mangrove forests are one of the most important natural resources for corrosion mitigation, biological diversity support, and environmental purification (Alongi 2002; Lovelock *et al*., 2015; Wang, 2019).

Salinity is a critical abiotic factor that affects plant growth, productivity, and dispersion in tropical and semitropical intertidal areas because excessive Na^+ causes an osmotic imbalance in organisms (Zhu, 2001; Krauss *et al*., 2008; Munns and Tester, 2008). Salinity affects approximately 20% of the world’s cultivable land and 50% of its irrigated land (Tuteja, 2007). Salinity causes two forms of stress in plants: osmotic pressure (due to increased osmotic pressure) and ionic stress (due to increased amounts of harmful ions, such as Na^+ and Cl^-, resulting in ionic imbalance) (Flowers and Colmer, 2008). In this context, mangrove trees are essential halophytes that can develop in an increasingly saline environment. Several mangrove plants grow well at salinities ranging from 5% to 25% of normal seawater (Parida and Jha, 2010; Krishnamurthy *et al*., 2017). Salt tolerance in halophytes is primarily achieved via three mechanisms: i) the reduction in Na^+ flow, ii) the compartmentalization of Na^+ into vacuoles, and iii) the excretion of Na^+ via plasma membrane Na^+/H^+ antiporters (Taji *et al*., 2004). Furthermore, scientific consensus on how mangrove plants develop unique characteristics, such as viviparity, to survive/adapt to their habitat may be important for the protection, control, and administration of mangrove forest ecosystem resources (Hong *et al*., 2018). Viviparity is a biological phenomenon in mangrove plants and is defined as continuous development inside the maternal plant without an apparent quiescent phase (Sun *et al*., 1998; Vang *et al*., 2005).

Salinity, a combination of dehydration and osmotic pressure caused by an increase in Na^+ ion accumulation and a decrease in K^+ ion concentration, harms plant growth and development. Meanwhile, the production of reactive oxygen species, including superoxide anion (O_2^-), singlet oxygen (O_2^1), hydroxyl radicals (OH^-), and hydrogen peroxide (H_2O_2), causes oxidative damage as an early fast reaction to salinity (Abo-Doma and Azzam, 2007; Azzam *et al*., 2009; Paul and Roychoudhury, 2016; Azzam *et al*., 2021).

Quantitative real-time PCR (qRT-PCR), Northern blot analysis, and in situ hybridization are some of the techniques used for relative gene expression analysis. qRT-PCR is a reliable and sensitive gene expression measurement method with a broad quantification range (Bustin, 2002; Saddhe *et al*., 2018). Salinity tolerance is achieved through a set of genes that regulate several physiological, biochemical, and molecular processes (Flowers, 2004; Munns and Tester, 2008). SODs are enzymes that catalyze the change of reactive superoxide anions into H_2O_2, a dangerous ROS (Kim *et al*., 2015). SODs are categorized into three common types in accordance with their internal metal cofactor: Fe SOD, Mn SOD, and Cu/Zn SOD. Each of these types is coded by several genes. The cytoplasmic Cu/Zn SOD enzyme is encoded by the Sod1 gene (Tower, 2015; Rossatto *et al*., 2017). Furthermore, increased salt stress may inhibit photosynthesis in addition to CO_2 fixation, resulting in cellular damage. Additionally, increased NaCl stress causes a reduction in the expression pattern of genes related to the antioxidant agent *Catl1* (catalase) and *Fer1* (ferritin) in the mangrove species *A. marina*, thus inhibiting protein synthesis. Moderate salt concentrations (≈8.7–17.5 psu) encourage the growth of most mangrove plants, whereas high salt concentrations (above 35 psu) or the absence of salt restricts growth (Lopes *et al*., 2019).

The ubiquitin–proteasome pathway has been implicated in the intermediate proteolysis of cellular proteins through the covalent attachment of ubiquitin. Ubiquitin-activating enzyme, ubiquitin-conjugating enzyme, and ubiquitin-protein ligase are the three enzymes involved in this process as reported by Parani *et al*. (2002). Furthermore, previously discovered stress-related proteins, such as the ubiquitin-conjugating enzyme, have been reported (Gao *et al*., 2017). Finally, dehydrins play an essential function in increasing tolerance to abiotic stressors, such as salinity, water molecule adsorption, and osmotic regulation (Nylander *et al*., 2001; Zhang *et al*., 2021).

This study was performed to detect and examine the expression of five genes (*Sod1*, *Cat1*, *DHNAs*, *Fer1*, and *Ubc2*) related to salt, oxidative, and osmotic stresses in the Egyptian gray mangrove plant under the conditions of different protectorates in the Sinai Peninsula by using the qRT-PCR technique. This study would help geneticists and plant breeders provide an essential basis for future investigations on the functions of genes, their pathways within cells, and functional interrelations between genes in physiological processes. Moreover, those genes could emerge to enhance abiotic stress tolerance via gene transfer into

Table 1. List of primer sequences.

No.	Gene	Primer sequence	
1	*amSod1*	Forward	5'–ATCATTACCCAGTCGCTTGT– 3'
		Reverse	5'–AGCAAAGATGATGTGGGAAC–3'
2	*amCat1*	Forward	5'–GAGAATGGAGGCAACGTTTA–3'
		Reverse	5'–TGTGTGCATCAAGAAGTTCG–3'
3	*amFer1*	Forward	5'–ATCTCTATCCGTGGTTTGCC–3'
		Reverse	5'–GGATTCACAGCTCCATCAAAT–3'
4	*amUbc2*	Forward	5'–TCCCTTACTAGACGGTTGG–3'
		Reverse	5'–AGTGACGCGTTCCTTACA–3'
5	*am DHNA*	Forward	5′– GTCTTCGGAGGACGATGG 3′
		Reverse	5′–CCATCGTCCTCCGAAGAC–3′
6	*Actin*	Forward	5'–GCCGTGCTTTCTCTTTATGC –3'
		Reverse	5'–CTCTCTGGAGGAGCAACCAC –3'

some economic crops, including wheat, maize, and rice.

MATERIALS AND METHODS

The leaves of Egyptian gray mangroves in South Sinai protectorates (Nabq, Ras Mohamed, Safaga, and Wadi El-Gemal), Egypt, were collected, then placed on dry ice, and stored at −80 °C until use.

Isolation and purification of total RNA

Direct-zol^{TM} RNA MiniPrep from Zymo Research, USA was used to isolate total RNA from the leaves of the Egyptian gray mangrove. This protocol has two phases: sample preparation and RNA purification (catalog Nos. R2050).

Sample preparation

A total of 100 mg of leaf tissue was crushed in liquid nitrogen and homogenized immediately in a tube containing 600 µl of TRI Reagent®. The tubes were centrifuged at 10 000 × *g* for 1 min to remove particle debris from the homogenized tissues. Then, the supernatant was transferred into an RNase-free tube (Elatawy *et al*., 2018).

RT-PCR analysis

The protocol of the SensiFAST™ Probe Lo-Rox Kit was optimized to generate the first strand of cDNA for use in two-step qRT-PCR. All components were mixed and centrifuged after thawing and kept on ice.

Real-time PCR

Fast SYBR Green® LO- Rox master mix was used for real-time PCR (Bio Line, UK). The qRT-PCR volume was prepared in accordance with Schmittgen and Livak (2008) as follows: ddH_2O was added to 10 µl of fast SYBR Green master mix, 0.8 µl of forward primer (5 pmol/µl), 0.8 µl of reverse primer (10 pmol/µl), and 2 µl of template cDNA (25 ng/ µl). The reactions were carried out on a Strata gene Mx3000p system (Agilent Technologies, Santa Clara, CA) for 10 min at 95 °C, then was followed by 40 cycles of 15 s at 95 °C, 60 s at 60 °C, 20 s at 72 °C, and 4 °C (infinite). Melting curve analysis was used to evaluate PCR products. The reaction was conducted with the primer pairs *amFer1*, *amSod1*, *amUbc2*, *amCat1*, and *amDhna* (Table 1), and each cDNA sample was tested three times. The actin gene was utilized as the reference gene for data normalization. MxPro Mx3000P v3.00 software was used to acquire the Ct values (Agilent Technologies, Santa Clara, CA).

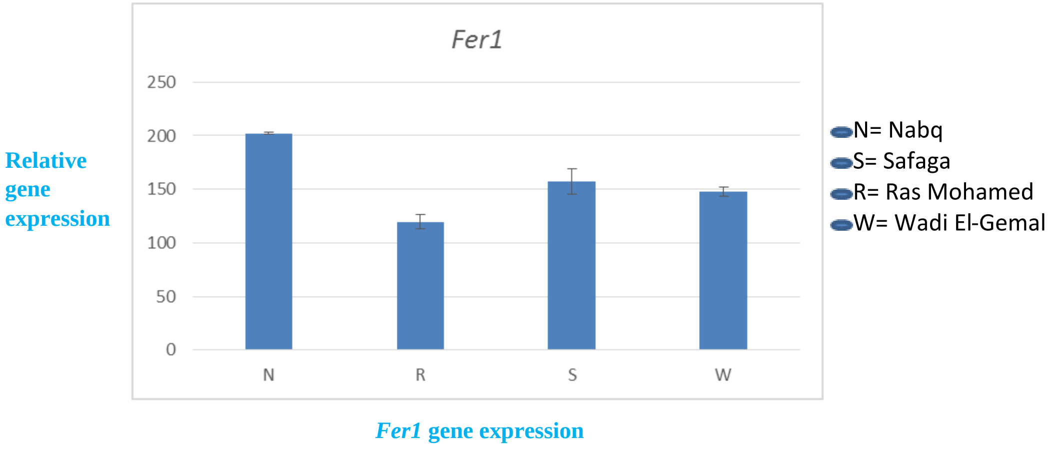

Figure 1. Diagram showing the real-time PCR analysis of the transcript levels of the abiotic stress response gene *amFer1* in Egyptian gray mangroves collected from four protectorates in the South Sinai Peninsula.

Data analysis from real-time PCR

The following formula was applied to compute the relative difference in gene expression = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta_{Ct}$ = Δ_{Ct} control – Δ_{Ct} treatment, and Δ_{Ct} = (Ct genes – Ct Actin gene) according to Livak and Schmittgen (2001) and Schmittgen and Livak (2008).

RESULTS

Gene expression pattern in Egyptian gray mangroves in response to salt tolerance

Mangroves are physiologically fascinating as prospective stress tolerance models and as sources of alternative ideas for physiological strategies that are applicable at the ecosystem level (Datta *et al.*, 2009). The genetic response of mangroves to salt stress in different locations was confirmed by using qRT-PCR to explore the effect of abiotic stresses on five salt-tolerance genes (*amFer1*, *amDhna*, *amSod1*, *amCat1*, and *amUbc2*) in Egyptian gray mangroves grown in South Sinai. In the same context, selection depended on the fold change in the expressiveness of genes at the transcript level. The ratio between the original numbers of molecules in the target genes and the actin gene was used (reference gene in qRT-PCR). The expression patterns had changed in response to salt stress, revealing that *Fer1* expression was up-regulated (202.25%) in samples from the Nabq protectorate but was down-regulated in samples form the Ras Mohamed protectorate (119.39%). Moreover, *Fer1* showed moderate gene expression (156.86% and 147.6%) in samples from the Safaga and Wadi El-Gemal protectorates, respectively (Figure 1). In addition, the *amDhna* gene exhibited a moderate expression level (373.37%) in samples from the Nabq protectorate and a significant increase (562.30%) in samples from the Ras Mohamed protectorate. By contrast, the expression pattern of the *amDhna* gene decreased in samples from the Safaga and Wadi El-Gemal protectorates (42.32% and 71.76%), respectively (Figure 2).

Furthermore, the expression pattern of the *Sod1* gene in mangrove plants exposed to abiotic stressors in the Nabq Protectorate significantly increased (42.93%) but remarkably decreased in samples from Ras Mohamed (8.30%), Safaga (1.23%), and Wadi El-Gemal (1.12%) (Figure 3). The *Cat1* gene displayed an expression level of 2.20% in samples from the Safaga protectorate and diminished sequentially to 0.705%, 0.344%, and 0.253% in samples from the Nabq Protectorate, Wadi El-Gemal, and Ras Mohamed, respectively (Figure 4). Finally, the expression levels of the *Ubc2* gene in response to abiotic stresses had significantly increased to 10.09% in the samples from the Safaga Protectorate. At the same time, they showed a marked decrease in samples from Nabq, Ras Mohamed, and Wadi El-Gemal (2.630%, 1.245%, and 2.895%), respectively (Figure 5). In addition, *Fer1*, *Dhna*, *Sod1*, *Cat1*, and *Ubc2* had nearly the same pattern of responses in the samples from the Nabq Protectorate and Ras Mohamed. On the other hand, *Fer1, Ubc2*, and *Cat1* had slightly different response patterns in the mangroves from Safaga*.* The obtained results may be attributed to variations in salinity between the sites of plant collection.

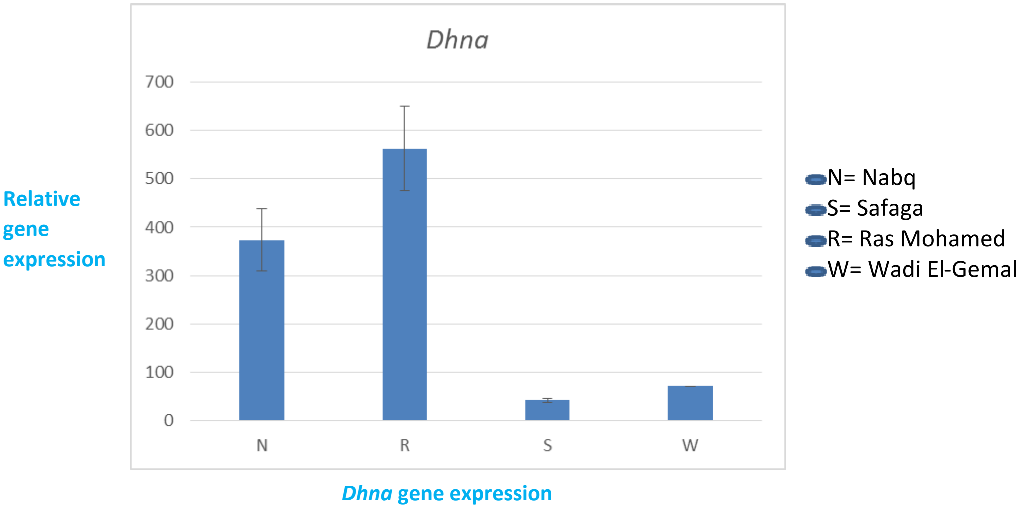

Figure 2. Diagram showing the real-time PCR analysis of the transcript levels of the abiotic stress response gene *amDhna* in Egyptian gray mangroves collected from four protectorates in the South Sinai Peninsula.

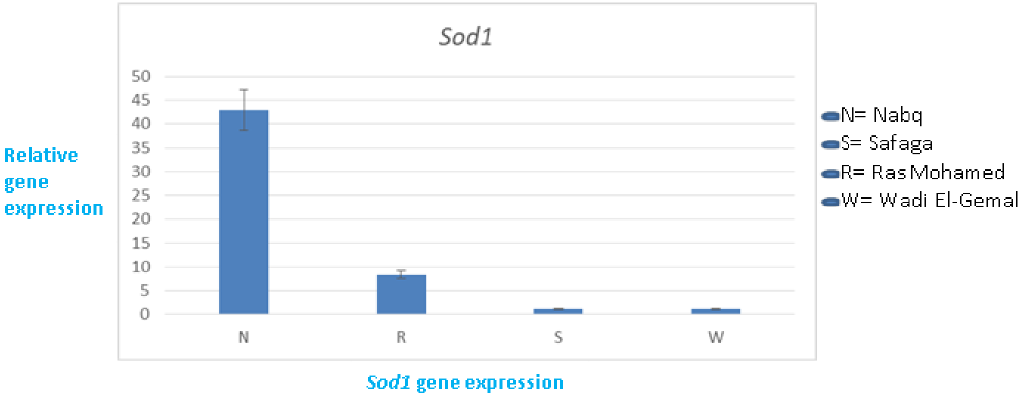

Figure 3. Diagram showing the real-time PCR analysis results for expression levels of the abiotic stress response gene *amSod1* in Egyptian gray mangroves collected from four protectorates in the South Sinai Peninsula.

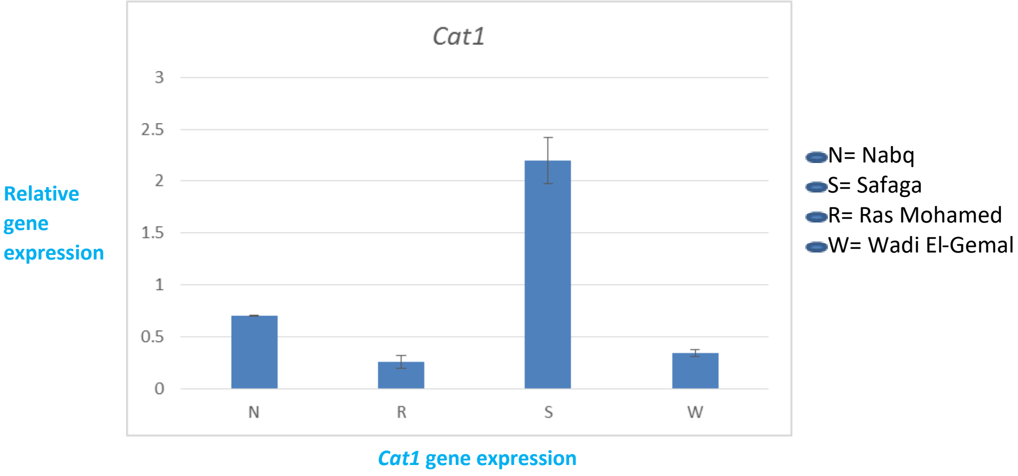

Figure 4. Diagram showing the real-time PCR analysis results for the expression levels of the abiotic stress response gene *amCat1* gene in Egyptian gray mangroves collected from four protectorates in the South Sinai Peninsula.

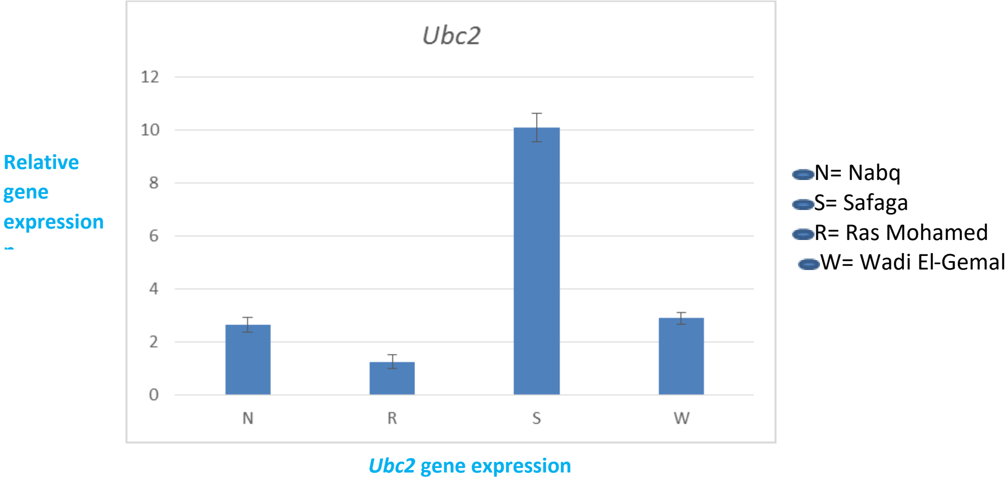

Figure 5. Diagram showing the real-time PCR analysis results for expression levels of the abiotic stress response gene *amUbc2* in Egyptian gray mangroves collected from four protectorates in the South Sinai Peninsula.

DISCUSSION

Drought, salinity, strong light strength, high temperature, cold, and heavy metals are obstacles to plant growth and development. Plants respond to stress through various physiological, behavioral, biochemical, or molecular pathways, such as gene expression alterations, cellular metabolism, growth, extensive cellular damage, inhibiting photosynthesis, osmotic impact, ion toxicity, and nutritional imbalance (Gull *et al*., 2019; Forghani *et al*., 2020). Given that *A. marina* grows in highly saline habitats with the highest silt value (El-khouly and Khedr, 2007), the obtained results could be attributed to differences in salinity among the plant collection sites. In addition, the diversity of environmental stresses in different habitats has a considerable effect on the biological responses of plants. For example, variations in habitat significantly affects a plant's physiological behavior and biochemical expression levels (Datta *et al*., 2009). Mangroves have evolved diverse mechanisms to cope with high salinity during lengthy periods of acclimation. These mechanisms include morphological (Halliwell and Gutteridge, 1985), physiological (Hasegawa *et al*., 2000), and molecular (Hassanein, 1999) mechanisms.

Furthermore, abiotic stress factors cause oxidative stress by forming ROS, damaging cellular proteins, lipids, and DNA, thus resulting in cell death (Jithesh *et al*., 2006a). Plants have developed mechanisms of defense, including nonenzymatic and enzymatic antioxidant processes, to counteract the harmful effects of ROS. These mechanisms include low-molecular-weight antioxidant compounds, including ascorbic acid; carotenoids; and nonenzymatic components, such as glutathione (Alscher and Hess, 1993). The antioxidant enzymatic pathway is composed of enzymes that effectively eliminate free radicals, such as superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase. Antioxidative enzymes are essential components of a plant's antioxidative system because they are primarily responsible for scavenging ROS (Allen, 1995; Jithesh *et al*., 2006a).

Many oxidation processes require *SOD* genes, which offer complete defense against ROS in plants (Mueller *et al*., 2005). The gene expression patterns of *Sod1* and *Fer1* mRNA are also altered. Consequently, H_2O_2, which is thought to be an intermediary molecule for antioxidant responses to different stressors, activates all three genes simultaneously, confirming that the *Sod1*, *Cat1*, and *Fer1* genes function in the antioxidative pathway (Jithesh *et al*., 2006a). Environmental stressors, including high salinity, ionic tolerances, oxidative drought, and heavy metal toxicity, affect relative gene expression and *SOD* enzyme activity (Schützendübel and Polle, 2002; Atkinson *et al*., 2013). Zang *et al*. (2017) reported that TaFER-5B is believed to have a critical function in shielding cells from heat stress and ROS. *TaFER-5B* overexpression also improves resistance to salt, drought, oxidative, and excess iron stress. However, little is known about the roles of ferritin genes in heat tolerance and other abiotic stresses.

Feng *et al*. (2016) reported that the response to abiotic stressors alters the majority of *Slsod* expression. In addition, under salt treatment, *SlSod1* is the only gene that showed substantial up-regulation among the nine *SlSod* genes, indicating that SlSOD1 function is related to abiotic and biotic stressors (Duan *et al*., 2008). Certain plants create various detoxification systems by activating antioxidative genes, such as *Sod*, *Pod*, *Cat*, *Apx*, and *Gpx*. Saline stress enhances *Gpx* and *Cat* gene activities in several vegetative varieties. *Cat* and *Pod* activities are also increased in salinity-stressed plants. SODs are also the best researched antioxidant enzymes in halophyte plants, such as *A. marina*, and have been linked to salinity tolerance (Ben Hamed *et al*., 2020).

AoDHN1 is up-regulated in growing *Escherichia coli* cells due to abiotic stresses, ionic tolerances, oxidative stresses, and some functional perceptions, as well as in *Avicennia* (Jyothi-Prakash *et al*., 2014). Under abiotic stress, *E. coli* cells expressing *AoDHN1* grew considerably faster than control cells lacking *AoDHN1*, demonstrating that the dehydrin gene helps mangroves reduce abiotic pressures. Salt stresses also enhance the expression of the *Fer1* gene in the leaves of some halophytic plants, as *Mesembryanthemum crystallinum* (Paramonova *et al*., 2004). Jithesh *et al*. (2006b) reported a transient increase in the ferritin gene (*Fer1*) in *A. marina.* Gene expression was detected on the basis of the change in leaf color in response to salinity.

Finally, the *GmUbc2* gene regulates stress-sensitive genes and plays an essential role in salt and drought stress response. The alteration of its mRNA patterns under salt and drought stress indicated that *GmUbc2* is involved in plant tolerance to abiotic stressors. Comparative gene expression studies have shown that *GmUbc2* is also involved in the regulation of ionic tolerance, osmolyte production, and cellular oxidative responses during stress (Zhou *et al*., 2010). The *amCat1* gene was down-regulated relative to the *amSod1* gene, which was up-regulated; meanwhile, the *amUBC2* gene was moderately expressed. Moreover, the results showed that the *amFerI* gene was a more up-regulated than the other three genes (Elatawy *et al*., 2018).

Additionally, the structural 3'-UTR regions of all four genes (*amCat*, *amFer*, *amSOD*, and *amUBC2*) revealed that the *amCat1* (99%), *amFe1r* (99%), *amSOD1* (98%), and *UBC2* (100%) genes were substantially similar. Furthermore, in Egyptian gray mangroves (*A. marina*) grown on South Sinai reserves under diverse environmental conditions, all four genes are important for controlling abiotic stresses, such as oxidative and osmotic stress (Abdel-Tawab *et al*., 2018). Meanwhile, the *Ubc1* and *Ubc2* genes are up-regulated in response to abiotic stresses, including salt stress, osmotic and ionic tolerances, and oxidative stress via modulating the *MYB42*-mediated expression of the SOS2 pathway (Sun *et al*., 2020).

CONCLUSIONS

Tolerance to abiotic stresses and the development of stress-tolerant crops require specific genes that respond to adverse growth conditions. Their responses are translated into biochemical and physiological pathways that help plants survive and acclimatize under extreme conditions. The five stress-related genes under study revealed variable expression across different locations, reflecting the genetic defense mechanisms of mangroves. Furthermore, among the four genes under investigation, the ferritin gene revealed significant expression levels in samples from all protectorates, showing that ferritin plays a critical role in the mangrove response mechanism.

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