ISOLATION AND IDENTIFICATION OF KEY GENES CONTROLLING SALT TOLERANCE IN GREY MANGROVE (*Avicennia marina*) AL-NABQ PROTECTORATE, EGYPT

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The salient properties of grey mangrove (*Avicennia marina*) as a typical halophyte and its wide spread geographical distribution has been documented (Duck, 2006; Duke, 2007; Tomlinson, 1986; Nguyen *et al*., 2015). Salt stress has toxic effects on plants and leads to several metabolic changes, like loss of chloroplast activity, decreased photosynthetic rate and increase photorespiration rate which then leads to an increased reactive oxygen species (ROS) production (Parida and Das, 2005), such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical and singlet oxygen ($^1O_2$) (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 enzymes are essential components of an adaptive defence mechanism against salt stress in halophytes like mangrove. Some of the major antioxidant enzymes involved in scavenging are SOD, CAT and POX (Fang *et al.*, 2005). Catalase enzyme catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS) (Parida *et al*., 2004; Negrão *et al.*, 2017). Superoxide dismutase is an enzyme that alternately catalyzes the dismutation (or partitioning) of the superoxide (O_2-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). (Jithesh *et al*., 2006; Negrão *et al*., 2017). Ubiquitination is known to regulate important functions in a wide variety of plant growth and developmental processes including photomorphogenesis, vascular differentiation, flower development, both phytohormone and light signaling, as well as biotic and abiotic stress responses (Jithesh *et al*., 2006; Negrão *et al*., 2017). 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 (Arbona *et al*., 2017; Negrão *et al*., 2017). Sequences of the internal transcribed spacer (ITS) regions of nuclear ribosomal (nr) DNA were used for phylogenetic studies at the inter and intrageneric levels in many angiosperm

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families (Hilu and Liang, 1997; Blaalid *et al*., 2013).

The ITS region between the rRNA encoding regions within eukaryotic genomes was correspond to just such locus (Marçon *et al*., 1999; Johan *et al.*, 2013). The structural features of rRNA have been used to redefine the universal phylogenetic tree which divides the living systems into bacteria, archaebacteria and eukaryotes (Woese and Fox, 1977; Blaalid *et al*., 2013). Sequence data along with the structural features of rRNA could even be more useful in solving the question of genetic relatedness among different species (Coleman and Mai, 1997). Two ITS regions separate the conserved 18S, 5.8S and 28S genes (Hillis and Dixon 1991). The intraspecific homogeneity and availability of highly conserved sequences flanking the variable regions, make the ITS sequences an excellent marker for species identification and phylogenetic inferences in closely related species. The fewer functional and selection constraints on the noncoding regions such as the ITS regions make them more useful for phylogenetic analysis as compared to protein coding regions such as Cytochrome oxidase gene which is highly conserved (Boskovic *et al.*, 2017). The highly conserved regions of ribosomal DNA can be used to construct universal primers that can be used with a variety of different species (Hillis and Dixon, 1991).

Protein non-coding mRNA regions are now known to play important roles in the regulation of gene expression. In particular, untranslated region (3'-UTR), the region between the stop codon and the start of the poly(A) tail, 3'-UTR of the vast majority of genes have been shown as important regulatory elements with a strong impact on the post-transcriptional regulation of gene expression. Together with the complex of different RNA-interacting factors, UTRs regulate mRNA stability export to the cytoplasm, sub-cellular localization and translation efficiency to influence the total amount of synthesized protein (Moore, 2005; Barrett *et al*., 2012; Pichon *et al.*, 2012). The 3'-UTR can contain sequences that attract proteins to associate the mRNA with the cytoskeleton, transport it to or from the cell nucleus, or perform other types of localization. In addition to sequences within the 3'-UTR, the physical characteristics of the region. The 3'-UTR of mRNA has a great variety of regulatory functions that are controlled by the physical characteristics of the region. Sequences within the 3'-UTR also have the ability to degrade or stabilize the mRNA transcript. Modifications that control a transcript's stability allow expression of a gene to be rapidly controlled without altering translation rates. The 3'-UTR also contains sequences that signal additions to be made, either to the transcript itself or to the product of translation (Hesketh, 2005; Mignone and Graziano, 2011; Barrett *et al.*, 2012; Pichon *et al.*, 2012).

The objectives of this study were to correctly confirm the species ID using ITS sequencing prior to the sequencing of the candidate genes amSOD1, amCat1,

amFer1 and amUBC2 under the extreme salinity conditions of the Red sea shores at Al-Nabq protectorate.

MATERIALS AND METHODS

Plant Materials

Grey mangrove (*Avicennia marina*) samples (fresh young leaves above sea water) were from Nabq Protectorate at the specific working locations of two Japanese projects; 1) Plant Conservation Efforts in Egypt and 2) Relationship between the Exclusive Invasion of Alien Vegetation and the Heterogeneity of Subsurface Zone in Arid Environment, Sudan.

Methods

DNA extraction and species ID confirmation

Total DNA was extracted from the fresh young leaves using Genomic DNA Purification Kit (#K0791, Thermo Scientific, Lithuania) according to the manufacturer protocol. Extracts were tested for quality using 1% agrose gel electrophoresis method and quantified using fluorometer device (Quantus, Promega, Inc. USA). Purified DNA was amplified using ITS primer pair (ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′; (White *et al*., 1990). The PCR reactions were performed at 50 µL mixture containing 10 µL of 5 × FastPfu Buffer, 5 µL of 2.5 mM dNTPs, 1 µL of each primer (5 µM), 1.5 unit of FastPfu Polymerase and 10 ng of template DNA. PCR-Reactions were carried out in a DNA thermocycler (Progene 30, Techne, Cambridge Ltd. Dux ford Cambridge, UK). The Thermal Cycler was programmed in three main steps as follows: One cycle at 94°C for 5min; 35 cycles at 94°C for 1 min, 40°C for 1 min, 72°C for 2 min; and one cycle at 72°C for 7min; then hold at 4°C.

RNA extraction and amplification

Total RNA was extracted from the dry-ice preserved young leaves and grounded in liquid nitrogen for further extraction using mortar and pestle. Grinded material was subject to lysis by TRIzol (Zymo Research) and RNA purification (Direct-zol RNA Miniprep, Zymo Research) following the manufacturer manual. Total RNA was visualized and quantified as previously mentioned, then were used as a template for cDNA synthesis using Bioline cDNA synthesis kit (Bioline, UK) using the kit protocol. When successful, cDNA was quantified using the Quantus fluorometer for further quantification. cDNA was used as a template for PCR reactions to amplify the four candidate genes for salinity tolerance in grey mangrove. The PCR reactions were performed in triplicates at 20 µL mixture containing 4 µL of 5 × FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase and 10 ng of template DNA.

3`UTR Sequencing and analysis

In molecular genetics, the three prime untranslated region (3'-UTR) is the section of messenger RNA (mRNA) that

immediately follows the translation termination codon. An mRNA molecule is transcribed from the DNA sequence and is later translated into protein. Several regions of the mRNA molecule are not translated into protein including the 5' cap, 5' untranslated region, 3' untranslated region, and the poly(A) tail. The 3'-UTR often contains regulatory regions that post-transcriptionally influence gene expression

PCR products were visualized using 1.5% agrose gel electrophoresis following the standard protocol. When successful, PCR product was subject to purification and sequencing using a private service company for sequencing (Macrogen, Inc.). Retrieved sequences were trimmed, filtered and assembled (forward and reverse directions) using Geneious V8. Clean consensus sequences were subject to BLAST tool to confirm the sequenced gene ID. The analyzed sequence along with the similar one on the NCBI database were aligned and analyzed for phylogenetic relationship using geneious tree builder tool.

RESULTS AND DISCUSSION

Species ID using internal transcribed spacer (ITS)

A DNA sample was amplified from mangrove by the ITS1 and ITS4 primers, the PCR product was a single band of the target size (545 bp). The BLASTn results confirmed that the wild grey mangrove from Al-Nabq protectorate is *Avicennia marina*; the query sequence was 99% identical to the "18S ribosomal RNA gene as a partial sequence; complete sequence of internal transcribed spacer 1 *(ITS1)*, 5.8S ribosomal RNA gene and internal transcribed spacer 2 *(ITS2);* and 26S ribosomal RNA gene as a partial sequence" of *Avicennia marina* accession number AF365978.1/MF063712.1 were both are identified equally by species, but may differ in the subsp., thus only the species name was reported (Fig. 1) while the subspecies was tested by phylogenetic analysis.

The constructed dendrogram for the phylogeny relationship between the used *Avicennia marina* and the other sub family *Avicennioideae* according to the ITS sequence in NCBI database appeared in Fig. (2). The alignment results showed that used *Avicennia marina* was known as subsp. marina which was closely related to *Avicennia marina* subsp. australasica with 99% affinity. Boskovic *et al.* (2017) stated that the fewer functional and selection constraints on the noncoding regions such as the ITS regions make them more useful for phylogenetic analysis as compared to protein coding regions such as Cytochrome oxidase gene which is highly conserved. Furthermore, The highly conserved regions of ribosomal DNA can be used to construct universal primers that can be used with a variety of different species (Hillis and Dixon, 1991).

Sequences of the internal transcribed spacer (ITS) regions of nuclear ribosomal (nr) DNA were used for phylogenetic studies at the inter and

intrageneric levels in many angiosperm families (Hilu and Liang 1997; Blaalid *et al*., 2013).

Sequencing of 3'-UTR genes amSOD1, amCat1, amFer1 and amUBC2

In molecular genetics, (3'-UTR) is the section of messenger RNA (mRNA) that immediately follows the translation termination codon. An mRNA molecule is transcribed from the DNA sequence and is later translated into protein. Several regions of the mRNA molecule are not translated into protein including the 5' cap, 5' untranslated region, 3' untranslated region, and the poly(A) tail. The 3'-UTR often contains regulatory regions that post-transcriptionally influence gene expression. These antioxidant enzymes are essential components of an adaptive defence mechanism against salt stress in halophytes like mangrove. Some of the major antioxidant enzymes involved in scavenging are SOD, CAT and POX (Fang *et al.*; 2005)

3'-UTR amUBC2 sequence

Comparing the isolated sequence from the *Avicennia marina* mangrove in Nabq protectorate and *Avicennia marina* Ubiquitin conjugating E2 found in a database of WebLogo 3 fonud that, There is no difference between them *Avicennia marina* Ubiquitin conjugating E2 the results appear in Fig. (3). (Hershko and Ciechanover, 1998) reported that. UBCs function was to conjugate ubiquitin to substrate proteins in the ubiquitin-proteasome pathway, which carries out the selective degradation of many short-lived proteins in eukaryotic cells. The conserved cysteine residue at the active site of UBCs is involved in ubiquitin thioester formation and transfer to substrate protein(s) (Haas and Siepmann, 1997).

The results confirmed that the variation in the 3'-UTR *amUBC2* Compared with the isolated sequence from the *Avicennia marina* mangrove in the Nabq protectorate with a five database fonud that Match ratio 100% with the *Avicennia marina* Ubiquitin conjugating E2. Which indicates the preservation of this important region without changes.

3'-UTR amCat1 sequence

The sequence alignment showed 3-UTR amCat1 difference between our sample (*Avicennia marina* in Nabq protectorate) and WebLogo 3.6.0 database. Sequentially, one nucleotide was substituted with G/T, as position number 137. The results confirmed that the variation in the 3'-UTR *amCat1* compared with the isolated sequence from the *Avicennia marina* mangrove in Al-Nabq protectorate with a five database fonud that Match ratio 99% with the *Avicennia marina* Cat1 one nucleotide was the only change. According to (Parida *et al.*, 2004; Negrão *et al*., 2017). Catalase (Cat) is involved in the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS), catalase has one of the highest turnover numbers of all en-

zymes; one catalase molecule can convert millions of hydrogen peroxide molecules to water and oxygen each second.

3'-UTR amSOD1 sequence

The sequence alignment showed 3-nucleotide difference between our sample (Nabq) and WebLogo 3 database. Sequentially, the nucleotide was substituted with C/G, A/G, A/C at position number 22, 126, and 147 respectively, the results appear in Fig. (5). The results confirmed that the variation in the 3'-UTR *amSOD1* compared with the isolated sequence from the *Avicennia marina* mangrove in Nabq protectorate with a five database fonud that Match ratio 98% with the *Avicennia marina SOD1*. Three nucleotide were substituted. Fang *et al*. (2005) stated that NaCl enhances thylakoid-bound SOD activity in the leaves of C3 halophyte *Suaeda salsa* L.

3'-UTR amFer1 sequence

Comparing the isolated sequence from the *Avicennia marina* mangrove in Al-Nabq protectorate with NCBI database, the matching ratio was found to be 99% with the *Avicennia marina* Fer1. The sequence alignment showed 3-nucleotide difference between our sample (Nabq) and T-coffee dataset. Sequentially, three nucleotides were substituted with C/G, C/G, and G/C as position number 60, 130, and 200, respectively (Fig. 6). The results confirmed that the variation in the 3'-UTR region is very limited because it is harboring the important regulatory region that controls the efficiency and half-life time of mRNA and thus is reflected on the gene expression. Jithesh *et al*. (2006) indicated that expression profiles of antioxidant genes to salinity, iron, oxidative, light and hyperosmotic stresses in the highly salt tolerant grey mangrove, *Avicennia marina* (Forsk).

Finally, the comparative genetic analysis of the *A. marina* grey mangrove trees in Al-Nabq protectorate showed that, the plant trees are *A. marina* subsp. australasia as shown by ITS sequencing, and the plants possess genetic differences from its peers on the genbank database (NCBI) for three of the studied key genes to salinity tolerance out of four: *amCat* genes (99%), *amFer* (99%) *amSOD* (98%) and *UBC2* (100%). It was evident that the variation in the 3'-UTR region is very limited because it is harboring conserved regulatory region that controls the efficiency and half-life time of mRNA and thus it is reflected on the gene expression.

The results confirm that these genes have important roles in controlling salt, oxidative and osmotic stress in the Egyptian gray mangrove plant under the red sea conditions within the Al-Nabq protectorate in South Sinai. These results would help geneticists and crop breeders as they could provide important bases for future investigations to functionally analyze and exploit the complex gene network behind its outstanding recourses. This could provide such genes to enhance abiotic stress tolerance through gene transfer to some strategic crops such as wheat, maize and rice.

SUMMARY

DNA barcoding by internal transcribed spacer (ITS) was obtained. DNA sample was amplified from mangrove by the ITS1 and ITS4 primers), the PCR product exhibited a single band of the target size (545bp). BLASTn search results of *Avicennia marina*; 18S ribosomal RNA geneas a partial sequence; complete sequence of internal transcribed spacer 1 (*ITS1*), 5.8S ribosomal RNA gene and internal transcribed spacer 2 (ITS2)); and 26S ribosomal RNA gene as a partial sequence, confirmed our results. Molecular characterization of the 3'-UTR-structured region of each of the five gene confirmed the high homologies of *amCat* genes (99%), *amFer* (99%) *amSOD* (98%) and *UBC2* (100%). It was evident that the variation in the 3'-UTR region is very limited because it is harboring conserved regulatory region that controls the efficiency and half-life time of mRNA and thus it is reflected on the gene expression. These genes have important roles in controlling salt, oxidative and osmotic stresses in the Egyptian gray mangrove plant under the red sea conditions within the Nabq protectorate in South Sinai.

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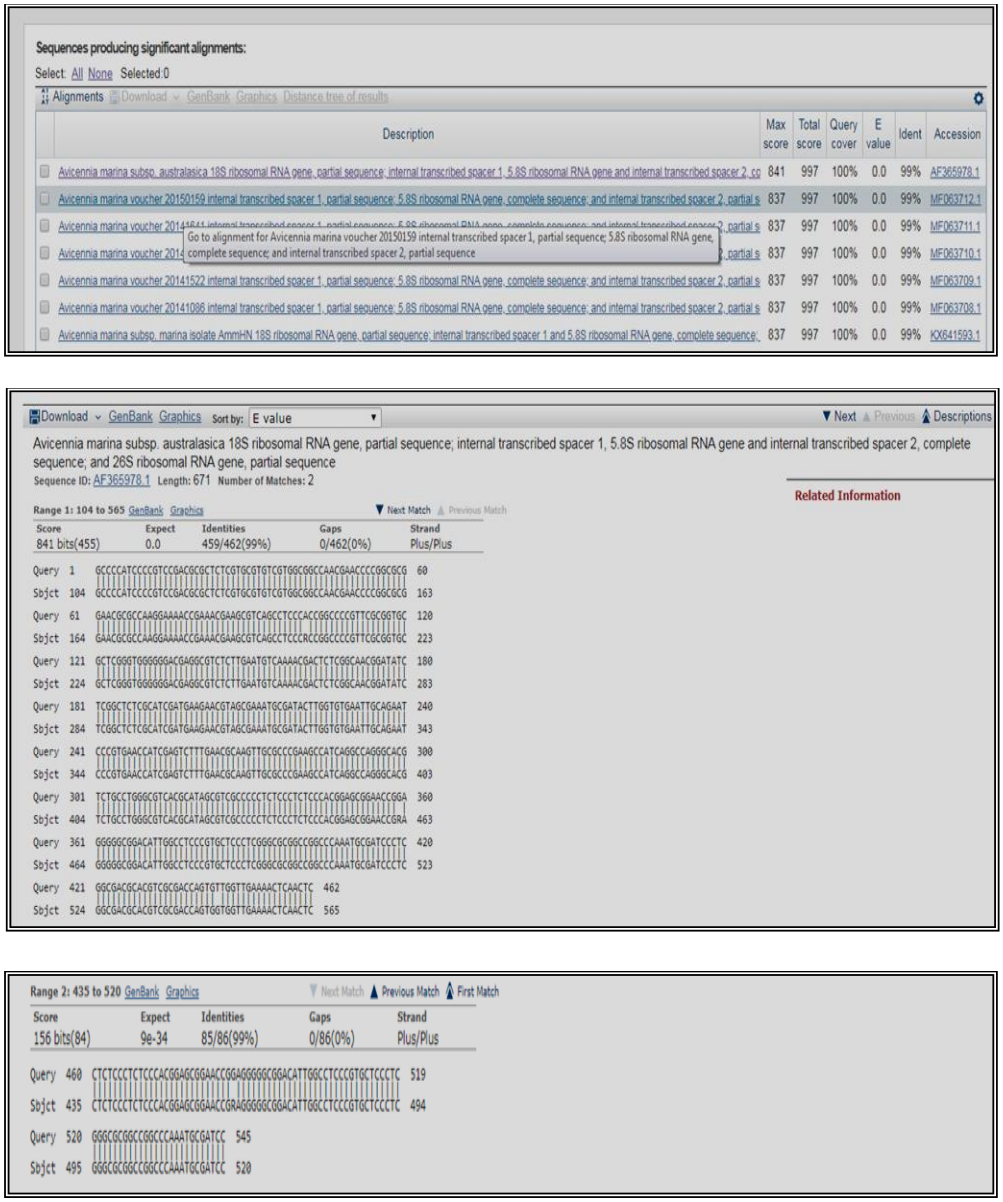

Fig. (1): The BLASTn results of *Avicennia marina* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence.

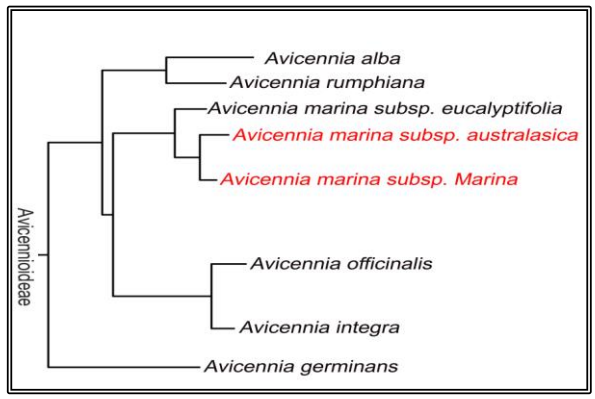

Fig. (2): Phylogenetic relationship based on sequence of *Avicennia* sp. in NCBI database.

Fig. (3): The multiple sequence alignment result as produced by WebLogo 3.

Fig. (4): The multiple sequence alignment result as produced by WebLogo 3.6.0. The change G/T was detected at position 137 bp.

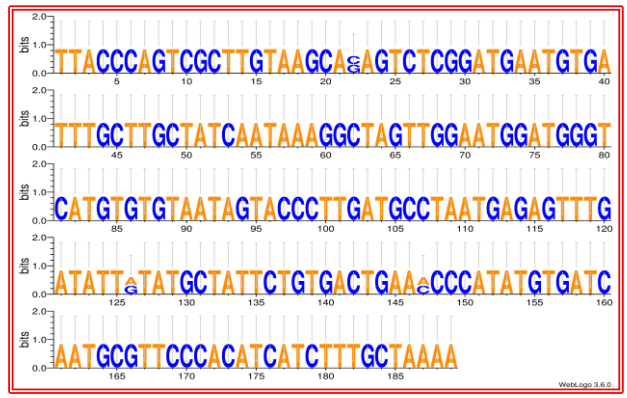

Fig. (5): The multiple sequence alignment result as produced by WebLogo 3. Three substitutions of C/G, A/G, A/C at positions number 22, 126, and 147 bp, respectively.

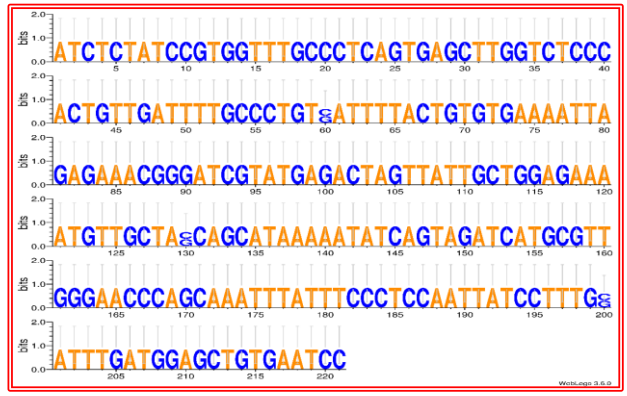

Fig. (6): The multiple sequence alignment result as produced by WebLogo 3. Three substitutions of C/G, C/G, and G/C at positions number 60, 130, and 200 bp, respectively.