Identification, Characterization and Evolutionary Patterns of Abiotic Stress Related Gene(s) in *Moringa Oleifera*

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Abstract

The drumstick tree (*Moringa oleifera*, Lam) is a perennial crop that has gained popularity in certain developing countries for its high-nutrition content and adaptability to arid and semi-arid environments. The genome of *Moringa oleifera*, a fast-growing stress-tolerant orphan crop with highly valuable agronomical, nutritional and pharmaceutical properties has recently been reported. The present study was conducted to study the seedling growth, morphological and molecular level changes of *Moringa* under saline soil conditions. Abiotic stress-related genes were induced in *Moringa oleifera* and their response to different stress conditions were studied. Plants of *Moringa oleifera* were collected extraction of DNA was done by using the CTAB buffer method. PCR was done for the amplification of abiotic stress-related genes. Bioinformatics analysis and phylogenetic analysis was performed to check the genetic diversity of the abiotic stress-related gene in *Moringa oleifera*. The potential candidate of abiotic stress-related genes were identified and characterized in *M. oleifera* including WARKY, MoTPS & MoTPP genes. The abiotic stress-responsive genes were also investigated for genetic improvements. Our analysis reveals that members of the WARKY, TPPS and TPS genes family have an important role in the plant's response to abiotic stress and are viable candidates for further characterization.

Keywords: *Moringa oleifera, Genome, Morphological, DNA Extraction, WARKY, MoTPS & MoTPP genes, Medicinal plant.*

1. Introduction

Moringa oleifera, a member of the Moringaceae family, has been prized for its nutritional and therapeutic benefits (Alkuwayti, El-Sharif, Yap, & Khattab, 2020). *Moringa oleifera* is a plant native to India that thrives throughout the tropics and subtropics of the world. Other names for it include "drumstick tree" and "horseradish tree." There are around 13 distinct tree species

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that make up the tropical tree known as *Moringa oleifera*. This plant is frequently grown in tropical and subtropical climates (Zhang, et al., 2017). 0n the other hand, *M. oleifera* is the most well-known crop which is alsogrown in Northern India, parts of Northern Europe, the Red Sea region of Asia, and Africa, including Madagascar (Trigo , Castello, Ortola, Garcia-Mares, & Desamparados Soriano, 2021). It has now become established and naturalized throughout thetropics and subtropics (Gupta & Ahmed , 2020).

M. oleifera is best suited to the warm and arid climatesof India's peninsular, central, and northwestern regions as well as Pakistan (Shahzad , Jaskani, Awan , Shahjahan, & Abbas , 2018). Only two species of family Moringaceae are found in Pakistan: *M. concanensis* and *M. oleifera*. The former species is uncommon and may be restricted to a single location (Tharparker).*M. oleifera*, also known as "Sohanjna" is widely planted and cultivated in Khyber Pakhtunkhwa, Pakistan (Anwar & Bhanger, 2003). *M. oleifera* is fast growing perennial deciduous tree with a height of and 12 meters (Jacques, Arnaud, & Jacques, 2020). The *M. oleifera* tree is a multi-purpose, fast-growing and drought-tolerant tree (Daba, 2016). It has traditionally been used as diuretic, stimulant, antispasmodic and expectorant. Its various elements have been screened for digestive disorders, heart complains, antioxidants, fever, inflammation, anti-hypertensive, analgesic, diuretic anti-inflammatory, and anti-tumor activity in pharmacological studies (Gowda, et al., 2020).

M. oleifera is well known as a non-cultivated plant for its disease and stress resistance. Responses are always stress dependent in environment swift, adaptability allows the organism to survive in bad conditions, or surrounding (Ciarmiello, Woodrow, Fuggi, Pontecoryo, & Carillo, 2011). Salinity, high temperature, drought, and other abiotic stresses affect agricultural yield around the world. Abiotic stress is said to negatively affect plant growth, physiological and biochemical characteristics, vitality, and crop output in a variety of agricultural crops. Many crops agricultural output is claimed to be harmed by salt stress, which is themost severe environmental concern. Salinization of a soil is progressively rising, particularly in arid, semiarid, and extensively irrigated areas with salt-containing water (Mushtaq , Faizan, & Gulzar, 2020).

Stress tolerance is the outcome of plants' progressive evolution of intricate biochemical processes that respond to environmental stress. Complex regulatory networks can result in osmoticmaterial accumulation, stomata closure, and decreased photosynthesis, among other molecular, biochemical, and physiological alterations

(Pandy, et al., 2016). Plants have ability to feel abiotic threats and adjust their metabolism, growth and development in response. Abiotic stress changeadaptation of cellular functions to environmental changes which may regulate gene regulation. Many genes and biochemical-molecular pathways are involved in the complicated plant reaction towards different stress conditions.

Improving crop production requires the understanding of molecular mechanism of tolerance of higher plants. It was suspected that hundreds of genes are involved. Among these modifications, the buildup of trehalose is an important mechanism for plants to escape damage when they are under stressed. A non-reducing glucose disaccharide that rises in reaction to stress from the environment is trehalose (Wang, Ouyang, & Wang, 2019).These WRKY protein family is one of the largest families of transcription factors. They play crucial roles in plant growth and development, signaling, and stress responses. However, there is little information about the WRKY family of drumsticks (*Moringa oleifera*, Lam.). Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) chain reaction were used to assess the levels of expression of MOWRKY genes in response to five different abiotic stresses (Zhang, et al., 2019).

2. Materials and Methods

2.1. Morphological Traits

Morphological traits of the selected *Moringa oleifera* were examined before proceeding for the molecular analyses. Different morphological parameter in *Moringa oleifera* were studied including plant height (cm), number of leaves on main stem, number of branches, leaf length, leaf breath, petiole size, internode number, plants fresh weight(g) plants dry weight (g), fresh herb yield per plant (g) and dry herb yield per plant (g). The experiment was replicated three times. The morphometric parameters were recorded for selected plant species in order to study for variation of same age group plants.

First of all three replicates of each control and treatment were being placed at the selected place for the trial within Department of Biological Sciences, UVAS. Seeds which were collected from the (NARC), were sown in the pots filled with mud and compost mixture that was taken from the botanical garden. Seeds of both control and treatment plants were sown separated by one pot for each. Controlled replicates were served with filtered water taken from the fisheries department water plant and the treated replicates were served with filtered water mixed with 200mM NaCl for the duration of the day after one day.

2.2. DNA Extraction

Genomic DNA was extracted by using CTAB method with minor modifications (Doyle & Doyle , 1990) from fresh leaves samples of *Moringa oleifera*.

2.3. Gel Electrophoresis

The DNA integrity was checked by gel electrophoresis. A stock of 50X TAE Buffer a 1% agarose gel.

2.4. RNAs Treatment

RNA contamination from DNA was eliminated using the Ambion RNA-free TM Kit.

2.5. Quantification of Genomic DNA

The concentration of total DNA samples was easily quantified (V.3.3.0).

2.6. Primer Designing and PCR Amplifications of Candidate Abiotic Stress Related Genes

Primers of TPS and TPP gene for PCR analysis was designed using Primer3 software according to the sequence of the reference genes and potential candidate genes.

Table.2.1. Primer Designing by Primer3 Software

Gene name	Primer	Temperature
WARKY 46	GGCACGTACCCACTTGAGAA	60℃
	ACAGCCACGAAGACAGACAG	
BdTPS6	GAGAGCAGGACAATATTGCTTG	60℃
	CAAAACTTCTGCACTAGGTGTC	
BdTPP	CTTCGAACATGATGCCGTTTTC	60℃
	AATTCTCCTGGCCAAAGACTAA	

2.7. PCR Amplification of Selected Candidate Salt Responsive Genes

The amplification through Polymerase Chain reaction for all the selected genes were optimized and *Moringa oleifera* in thermal cycler using standard PCR protocol.

2.8. Gel elution and Sequencing of PCR Amplicons

Gel extracted by gel extraction kit. Quality and quantity of eluted samples were checked again on 1% agarose gel.

Table.2.2: PCR Reagents under in this work.

Reagent	Quantity
Template DNA	300-700 ng
M13 Primers (10 pmol)	1 µl
Big dye sequencing mix	1 µl

5Xsequencing buffer	1 µl
Sterile dH_2O	up to 10 µl

2.9. Bioinformatics Analysis of the Candidate Salt Responsive Genes

Sequence data was processed using base-calling software (Chromas) and then assembled using CAP3 software.

2.10. Sequence Alignment and Phylogenetic Analysis:

Multiple sequence alignment (MSA) was conducted using the known protein sequences of the chosen salt stress related genes from *Moringa oleifera* for analyzing the evolutionary perspective, the sequence result was used to form a phylogenetic tree using neighbor-joining methods utilizing publicly available online software (MEGA) (Lin, et al., 2018). Multiple sequence alignment was carried out using the CLUSTALW with EMBnet (http://www.ch.embnet.org/software/ClustalW.html).

2.11. Gene Structure, Protein Properties, and Conserved Motif Analysis

Motifs in the abiotic stress responsive protein sequences was predicted using the online program (http://meme-suite.org). The Compute pI/MW tool (https://web.expasy.org/compute_pi/) was used to predict the isoelectric point (pI) and molecular weight (MW) of the respective proteins.

3. Results

3.1. Growth and Morphological Characters

Before proceeding with molecular analysis, the morphology of *Moringa oleifera* was examined. Different morphological parameters such as plant height, number of leaves on the main stem, length and breadth of the leaves, size of the petiole, number of internodes, weight of the plant both in fresh and dry forms. Table is showing the characteristics of *M.olifera* plant as pretreatment with 20mM Nacl.

Table 3.1: Pre-treatment of 200mM NaCl

Plant Specie	Height of plant (cm)	No. of Leaves	No. of Branches	Leaf Length (cm)	Leaf Breadth (cm)	Petiole Size (cm)	No of internodes
Control *Moringa oleifera* C1	4.4cm	15cm	3cm	1.1cm	0.8cm	1cm	2

Moringa oleifera C2	6.2cm	11cm	3cm	1.2cm	0.9cm	1.7cm	1
Moringa oleifera C3	**7cm**	**14cm**	**3cm**	**1.3cm**	**1.1cm**	**1.9cm**	2
Treatment							
Moringa oleifera T1	**0.4cm**	**17cm**	**3cm**	**1.1cm**	**0.6cm**	**1.7cm**	1
Moringa oleifera T2	1.6cm	9cm	2cm	1.8cm	0.7cm	23cm	4
Moringa oleifera T3	1.7cm	15cm	3cm	1.7cm	0.8cm	1.6cm	2

Table 3.2: Pre-treatment with 200mM NaCl

Plant Specie	Height of plant (cm)	No. of Leaves	No. of Branches	Leaf Length (cm)	Leaf Breadth (cm)	Petiole Size (cm)	No. of internodes	Fresh weight of plant (g)	Dry weight of plant (g)
Control									
Moringa oleifera C1	24.2 cm	10 cm	7 cm	2.1 cm	1.5 cm	3.8 cm	4	2.55 g	0.15 g
Moringa oleifera C2	24.9 cm	25 cm	9 cm	2.3 cm	1.4 cm	4.2 cm	4	2.59 g	0.17 g
Moringa oleifera C3	22 cm	28 cm	8 cm	2 cm	1.5 cm	4.4	5	2.10 g	0.19 g
Treatment									
Moringa oleifera T1	9.4 cm	11cm	7 cm	1.4 cm	1 cm	2.4 cm	3	1.30 g	0.06 g

Moringa oleifera T2	15 cm	14 cm	4 cm	1.8 cm	1.5 cm	3.3 cm	3	4.20 g	0.24 g
Moringa oleifera T3	11.6 cm	9 cm	4 cm	1.6 cm	0.7 cm	2.2 cm	4	3.20 g	0.21 g

3.2. RNAs Treatment

To remove RNA contamination from DNA, by using the1µlof RNAase (Sigma, Germany) the genomic DNA was treated with RNAase for 40 min. at 37ºC for each sample separately. On a 1% agarose gel, plasmid DNA was examined for 30 minutes at 80 volts. Using "GrabIT 2.5" software, a picture of the gel was obtained using a UV light Trans illuminator

3.3. Quantification of DNA

DNA quantification was done by using genetic Bio Analyzer (3500). The Bio Analyzer plots showed quantity of total DNA. Both 260/280 and 260/230 ratios are 2.0, which confirmed the DNA purity with good concentration.

Table 3.3: Quantification of DNA through Bio Analyzer

Sample Identity	Quantity of DNA ng/ µl	Ratio A260/280
Moringa oleifera C1	67.6	1.38
Moringa oleifera C2	138.8	1.20
Moringa oleifera C3	103.0	1.87
Moringa oleifera T1	163.2	1.71
Moringa oleifera T2	123.1	1.56
Moringa oleifera T3	120.1	1.74

3.4. PCR Amplification of selected candidate salt-responsive genes

The amplification through polymerase chain reactions for all the selected genes was optimized and done in a thermal cycler using standard PCR protocol. With a total of 03 arbitrary primers (represented as WRKY- F, TPS-F, and TPP-F) in combination with 03 anchored primers represented as (WRKY-R, TPS-R and TPP-R), a total of twelve differentially expressed gene fragments were isolated from leaf samples of control andtreated *Moringa oleifera.* The PCR amplicons of control and treated samples were resolved side by side on 1% gene transcripts were confirmed

as final transcripts on reamplification trials. For each primer pair, PCR was repeated using genomic DNA from at least two independent preparations.

3.5. Gel Elution and Sequencing of PCR amplicons

Using the Basic Local Alignment Search Tool, the DNA, EST, and protein sequences from various databases were compared with the nucleotide sequence or the deduced amino acid sequence of each gene (BLAST). The selected genes, WRKY-46, TPS-6, and TPP, were further confirmed to be present in the *M. oleifera* under inquiry by the BLAST results, and all of the selected genes shared the highest degree of similarity among taxa.

Table 3.4*: Moringa EST* against *CLCuV* stress and their homologies

EST name	GS-Primer	Size in (bp)	With Land Plants
WRKY-*C*	WRKY-F&R	480	WRKY-46(*Moringaoleifera)* JX555963.1
WRKY-T	WRKY-F&R	480	WARKY-46 (*Moringa oleifera)* JX555963.1
TPS-C	TPS-F&R	500	Trehalose-6- phosphate synthase *(Brachipodium dectyrone)* MT665004.1
TPS-T	TPS-F&R	500	Trehalose-6- phosphate synthase *(Brachipodium dectyrone)* MT665004.1
TPP-C	TPP-F&R	500	Trehalose-6- phosphate *(Arabidopsisthaliana)* EU108704.1
TPP-T	TPP-F&R	500	Trehalose-6- phosphate *(Arabidopsisthaliana)* EU108704.1

3.6. Multiple Sequence Alignment and Phylogenetic Analysis

Multiple sequence alignment (MSA) was conducted by utilizing the known protein sequences of the chosen salt stress related gene from *Moringa oleifera*. Through BLAST, a sequence similaritysearch was implemented to identify top 10

proteins from different plant species to which the deduced amino acid sequence of *M. oleifera* was more strongly allied.

4. Discussion

Plants under salt stress undergo a variety of morphological and physiological changes. Growth inhibition, rapid aging and senescence, and death following extended exposure are common signs of salt stress damage. The primary damage that causes secondary symptoms was growth inhibition. Saline soil causes physiological and metabolic changes in plants that have an impact on their growth, development, yield, and quality. Salinity, seed germination, survival rate, morphological traits, development, yield, and its components all have a negative impact on plants (Jouyban, 2012). The miracle tree *Moringa oleifera* was likewise affected by salt stress. Same outcomes were found in (Nouman, et al., 2012) Salt stress has a detrimental impact on *Moringa oleifera's* ability to thrive under salt stress.

As more concentration can stunt the growth of leaves and branches as lines in the findings by (Sivritepe , Sivritepe , & Eris, 2003) High NaCl content causes the leaves and branches to shrink, which stunts growth. Salt stress cause an effect in the fresh and dry weight of the plant as lined in (Shafi, et al., 2010). The wheat plant was subjected to salinity stress, and he came to the conclusion that salt causes the plant's fresh and dry weight to decrease. The impact of salt stress on genes involved in the metabolic pathway is particularly intriguing. The involvement of many genes in salt stress was recognized as similar in the findings of (Zhang, et al., 2019). WARKY-35, recognized by the WARKY family of genes, regulates salt stress response and plant growth.

The trehalose gene was found in the *Moringa oleifera* and give a great impact on salt stress tolerance. It's noteworthy to note that other genes from the trehalose family have been revealed to play a severe part in the stress response to salt stress as lined in the findings of Wang (2019) examined the TPS gene family in *M. oleifera*, and it offers guidance for future investigations into the precise roles of TPS genes in this species. Furthermore, assessments of their expression profiles by qRT-PCR validation of different Moringa tissues in drought, salinity, and extremely hot conditions. Stresses provide information about MoTPSs available.

These findings contrast with earlier research on Populus, Arabidopsis, and rice, as lined in the findings of (Lin, et al., 2018) which discovered at least seven ancient TPS genes in the common ancestor of the monocot and dicot. The TPS gene

family may have contracted during the divergence from angiosperms to dicots and monocots, and it is also plausible that different plants have lost members of the family. Reference genes are necessary for better amplification and identification when molecularly identifying all the genes. The best reference gene so far has been GAPDH as similarly found in the findings of Sinha, et al., (2015) sugar cane tissue and genotype as a whole was examined for expression analyses during salt stress, GAPDH proved to be the most reliable housekeeping gene.

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