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Molecular identification of resistant genotype of *Swietenia mahagony* in Egypt using cpDNA trnL-F transgenic spacer region

Eglal, M. Said, Hanafy, Manal M. and Hesham A. El-Aryeb

Horticultural Research Institute, Agricultural Research Center, Giza, **Egypt**.

***Correspondence: eglal2017@hotmail.com** **Accepted:** 19 Sep. 2017 **Published online:** 30 Sep. 2017

The genotype of *Swietenia mahogany* plant, grown at Nobaria, Horticulture Research Station, Egypt, showed good tolerance to unfavorable environmental conditions (salinity and calcareous soil) was observed. In this paper, physiological, biochemical and molecular studies were evaluated in order to understand adaptability and tolerance mechanisms of this new genotype. The chloroplast trnL-F region was amplified from extracted total genomic DNA using polymerase chain reaction (PCR) and sequenced. The results showed that the investigated regions of chloroplast genome are variable in the two tested genotypes. These regions served as useful molecular markers in phylogenetic studies or for population studies *of Swatinea mahogany* species.

Keywords: *Swietenia mahagony*, trnL-F, cpDNA, genotype

INTRODUCTION

Genetic diversity is the basis for adaptability and is essential for long term stability of populations and tree breeding for production, whether in plantations or by natural regeneration. It provides the potential for species to resist pests and diseases, and adapt to different environments. Although very strong environmental variation may produce adaptive differences over short distances, despite continued high levels of gene flow (Broadhurst et al. 2008).

Phenotypes of individual plants are determined by genotypes that underlie quantitative traits, environmental conditions, and the interactions between genotype and environment (Wang et al. 2010). The ability of plants to sense environmental changes and produce plastic responses is determined by a portion of the genetic variation, and that plastic phenotypic responses can both provide a buffer against rapid environmental changes and assist rapid adaptation (Nicotra et al. 2010). For any given plant species, different genotypes may respond similarly or differently to the same environmental changes, or show no response at all, resulting in the differential responses in phenotype (Aspinwall et al. 2015).

Evaluation of chloroplast DNA (cpDNA) diversity in plants is important for characterization of cytoplasm and also for population genetics/phylogeographic analyses. Studies on cpDNA diversity are also important for population genetics and phylogeographic analyses of rare, endemic, and endangered species (Baldwin et al. 1995). The trnL-F intergenic spacer of cpDNA is non-coding characters, and this region is more variable than the coding regions. Some studies on non-coding region of cpDNA showed higher variations and more often mutation than that of coding regions. Therefore, by amplification and direct sequencing of these non-coding regions, the resolution of cpDNA can be increased both for evolutionary studies, and for identifying intraspecific genetic markers (Saiki et al.1988).

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Swietenia mahogany belongs to family Meliaceae. Small-leaved mahogany was introduced in India from the West Indies in 1795, and since then has been planted throughout the tropics on a small scale in timber plantations and as an ornamental, occasionally also in tropical Africa. Across their wide geographic distributions, mahogany species appear to be adapted to a wide range of different environments and soils (Navarro et al. 2003).

A pattern resistant genotype of *Swietenia mahogany*, showed good tolerance to unfavorable environmental conditions (salinity and calcareous soil) was observed grown at Nobaria, Horticulture Research Station, Egypt, planted almost 15 years ago. In this paper, physiological, biochemical and molecular studies using cpDNA trnL-F region were evaluated in order to understand adaptability and tolerance mechanisms of this resistant genotype.

MATERIALS AND METHODS

This study was carried out at Biotechnology Research Laboratory, Horticulture Research Institute, ARC, Egypt during the period of 2016-2017, on two different genotypes of *Swietenia mahagoni*. One genotype grown at the orchard of Horticultures Institute Research, Giza governorate, and the second genotype grown at Nubaria, Horticulture Research Station, North Tahreer region, Beheira province. Selected trees were about 15 years old.

Soil characteristics

Representative soil samples were collected from each of the studied orchards at depth of 30 to 60 cm and analyzed for Physico-chemical characteristics according to wilde *et al.* (1979) as shown in Table (3).

Photosynthetic Pigments

Photosynthetic pigments (chlorophyll a, b and carotenoids) were measured in *Swietenia mahagony* leaf by the formula of Lichtenthaler (1987).

Estimation of proline content

Free proline content in the plant tissues was determined following the method of (Bates et al. 1973).

Determination of antioxidant properties

The antioxidant activity of plant methanol extracts was determined based on the radical scavenging ability in reacting with a stable DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical according to Blois (2002).

DNA Isolation

Total DNA was isolated from leaves tissue of the two genotypes as described by Dellaporta *et al.* (1983).

DNA amplification

The chloroplast trnL-F regions were amplified from extracted total genomic DNA using the polymerase chain reaction (PCR) method. The intron of the chloroplast trnL (UAA) gene and the trnL-trnF regions were amplified with primer combinations of (C+D), and (E+F), respectively as recommended by Taberlet et al. 1991 (Table 1 and Fig 1).

DNA Sequencing and Phylogenetic Analysis

PCR products were subjected to gel electrophoresis and cleaned up using a PCR clean-up kit (Promega, USA). Purified PCR products were directly sequenced from one directions, using ABI 3730xl automated DNA sequencer (Applied Biosystems) at GATC Company, USA. The obtained sequence was compared to the sequences in GenBank using the BLAST algorithm to search for close evolutionary relatives.

Gen Bank accession numbers

The representative sequence of the new genotype of *Swietenia mahagoni* was deposited in GenBank of National Centre for Biotechnology Information.

Statistical analysis

Statistical analysis was carried out according to Snedecor and Cochran (1976) using analysis of variance and significant difference was determined using L.S.D. values at P= 0.05 , and the distance matrix was represented in a phonogram using UPGMA clustering method (Sneath and Sokal, 1973).

Table 1: Sequences of the trnL-F primers used in this study.

Primer	Sequence 5'-3'
C	CGAAATCGGTAGACGCTACG
D	GGGGATAGAGGGACTTGAAC
E	GGTTCAAGTCCCTCTATCCC
F	ATTTGAACTGGTGACACGAG

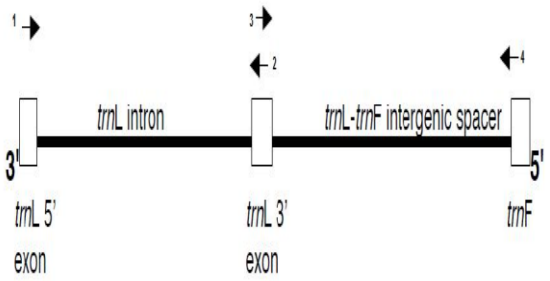

Figure 1: Approximate location of trnL-F primers used in this study

RESULTS AND DISCUSSION

Photosynthetic Pigments

Chlorophyll (Chl. *a* and *b*), total chlorophyll, carotenoids and total pigments content of leaves were differences between the two investigated genotypes of *Swietinea mahagony*, table (2). Carotenoids and total pigments decreased significantly in tolerance genotype 2, whereas total chlorophyll content revealed insignificant differences between the two genotypes. The decrease in chlorophyll content can be attributed to the sensitivity of this pigment to increasing environmental stresses, especially to drought and salinity (Guerfel et al. 2009). Sharma and Hall (1991), highlighted that saline stress induces degradation of β-carotene, which causes a decrease in the content of carotenoids that are integrated constituents of thylakoid membranes and act in absorption and light transfer to chlorophyll; besides, they protect chlorophyll from photo oxidation, thus degradation in carotenoid synthesis may imply degradation of chlorophylls (Lima et al. 2004).

Proline

The proline content tended to increase in tolerant genotype 2 compared to genotype 1, table (2), although the results have not differed significantly ($p < 0.05$), that proline accumulation was very small in *Swietinea mahagony* species, which indicates that *Swietinea mahagony* has limited capability to synthesize proline as a compatible compound that could increase tolerance or mitigate salt stress effects on this species (Angélica et al. 2011).

Antioxidant effect (DPPH scavenging effect) %

DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging method is an easy and rapid method to evaluate free radical scavenging abilities of various samples (Errabii et al. 2006). To prevent and respond to oxidative stress, an anti-oxidative defense system is expressed in the chloroplast, consisting of proteins and scavenging molecules (Froehlich et al. 2003). Data presented in table 2, shows the scavenging effects of extracts on DPPH' radicals, the highest value of (92.73%) in genotype 2 was observed, comparison to genotype 1 which reached to (83.44%). Generally with the advent of any stress condition, reactive oxygen species (ROS) are produced. These ROS cause oxidative damage to multiple cellular components like proteins, DNA, RNA and lipids Jacob et al. (1996). All types of abiotic stresses (salinity, chilling, freezing and drought stress) induce oxidative stress in plant cells (De Klerk and Pumisutapon, 2008).

Soil analysis

Data in Table 3 shows that soil texture of Nubaria station is mainly calcareous sandy loam. The values of the EC are 11.7ds/m which is classified very high saline soil while, $CaCO_3$ values are 30.7, and the pH values are 7.99. The high contents of calcium carbonates causes fixation of phosphorus, low availability of certain micronutrients (B, Fe, Zn, Ni, and Cu), and weak top soil structure Anter et al. (1973).

Table 2: Change in photosynthetic pigments (mg g/FW), antioxidant % and proline content (mg g/FW), in leaves of two different genotypes of *Swietinea mahagony* trees.

Genotype	Chlorophyll *a*	Chlorophyll *b*	Total Chlorophyll	Carotenoids	Total pigments	Antioxidant %	Proline
1	0.6269[a]	0.3085[a]	0.9354[a]	6.7884[a]	7.7238[a]	83.4489[b]	0.1546[a]
2	0.2809[b]	0.4646[a]	0.7455[a]	3.3672[b]	4.1137[b]	92.7337[a]	0.2427[a]

Means having the same letters in a column were not significantly different at $p<0.05$

Table 3: Physico-chemical characteristics of the investigated orchard loamy sand soil

Samples	PH	EC ds/m	SP	Soluble aninos (mg/l)				Soluble cations (mg/l)				$CaCO_3$ %
				CO_3	HCO_3	Cl	SO_4	K^+	Na^+	Mg^{++}	Ca^{++}	
1	7.96	7.8	42	-	1.2	1.2	4.3	0.5	43.5	11.6	19.4	3.1
2	7.99	11.7	55	-	1.5	105	3.5	0.13	56.3	13.7	24.7	30.7
	Particle size distribution (%)											
	Sand				Silt			Clay				
1	85.5				9.5			5				
2	72.5				18.6			8.9				

1 and 2 = soil samples of orchard of Horticultures Research Institute, Giza governorate and Nubaria Horticulture Research Station, Beheira province respectively. EC = Electrical conductivity, PH = Acidity algorithm, ds/m = descisiemen/meters, SP= saturation percentage

Amplification of DNA coding for trnL- F region

The genomic DNA of two genotype of *Swietinea mahagony* was subjected for isolation of the DNA coding for trnL- F (Fig 1). Due to a sequencing problem in the total trnL- trnF region, two primers combinations were used separately. A (C+D) primers amplified the intron of the trnL, while (E+F) primers amplified the partial trnL gene and trnL-trnF regions (Fig 1). PCR amplification of the trnL- trnF region was successfully obtained for (Genotype 2) only, meanwhile amplification was not successful for the second genotype (Genotype 1). The samples had PCR product of about 600bp. and 433bp. with the (C+D) and (E+F) primer sets, respectively (Fig. 2). The PCR product for both primers; (C+D) and (E+F) were directly sequenced from one direction after gel purification

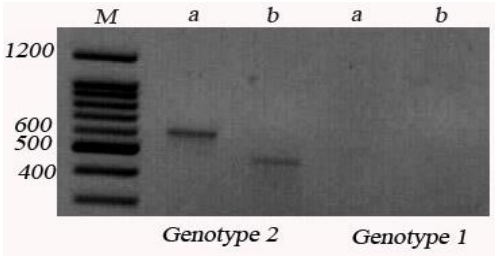

Figure.2. Agrose gel showing amplified trnL intron (a), and trnL-F intergenic spacer (b) for two *Swatinea mahogany* genotypes. Lane M: 100 bp. Ladder

The results showed that the investigated regions of chloroplast genome are variable in the two tested genotypes. These regions served as useful molecular markers in phylogenetic studies or for population studies *of Swatinea mahogany* species

Thus an intraspecific chloroplast DNA polymorphism could result from normal intraspecific variation, which has not been studied for non –coding region, or from intraspecific chloroplast DNA transfer (Taberlet et al. 1991).

The sequences obtained was as follows: Sequence of trnL intron, 600bp.

1 GGAACGGAGT TTAGTCATGA TATGGAACCT ACTAAGTGAT AACTTTACAC ATTCAGAGAA
61 ACCCTGGAAT CAAAAATGGG CAATCCTAGA GCCAAATCCT GTTTTACAAG AACAAACAAG
121 GGTTCAGAAA GCGAAAAAGG GGATAGGTGC AGAGACTCAA TGGAAGCTGT TCTAAGAAAT
181 GGGGTTGACT GCCTTTTTTT TTTTTGGTAA AAAAAGGAAA AAAAATCCTT CTATCAAATA
241 TCAAAACTCC ATAAAGGATG AAGGATAAGC GTATATACAC TATGTATACA CAATGAAAAA
301 CTATCTCAAA AATGACAACC GAATCCGTAT TTTTTTTTAG GAAAAAAAAA AAAATTGTTA
361 TGAATCAATT CCAAGTTGAA AAAAAAATCA AATATTCATT GATCAAATCA TTCACCCCAA
421 AGTCGGACCA ATCTTTTCTT TTGAAAAACT GATTAACCGG ACAAAAATAA AGATAGAGCC
481 CCATTCTACA TGTCAATATCA ATACCGGCAA CAATGAAATT TAAAGTAAAA GGAAAATCCG
541 CCAACTTTAA AAATCGGGAG GGTTCAAGTC CCTCCTATCC CCAACTCTTC GAACTATCCC

Sequence of trnL-F region, 433bp.

1 GGTCGCGAAT AATCACCATT GTGCTAAATT TCTCCTACCC TCTTTTTGTG TTAGTGGTTC
61 AAAATTCGTT AGGTTTCTCA TTCATCCTAC TCTTTTCCAG ATACAAATGT ATCTGAGCAG
121 AATTTTTTTC TCTTATCACA AGTCGTGTTA TATATATGAT AGACGTACAA ATTAACACCC
181 TTGAGCAAGG AATCCCCAGT TGAATGATGC ACAATTCATA TTATTGCTCA TACTGAAACT
241 TACAAAGTCT TCCTTTTGAA AATTCAAGAA ATGAAATTCC CCGTGCAAGA CTTTTAATAC
301 TTTTTTTTGT CTTTTTTAAT TGACATAGAC CCAAGTCATC TAGTAAAATC AGGATGGTGT
361 GTTGGGAATG GTCGGGATAG CTCAGCTGGT AGAGCAGAGG ACTGAAAATC CTCGTGTCAC
421 CAGTAAAAA AAA

The chloroplast DNA (cpDNA) *trnT-F* region in land plants consists of the transfer RNA genes trnT UGU, *trnL* UAA, and *trnF* GAA arranged in tandem and separated by non-coding spacer regions.

The amplification of the trnL- trnF region in the resistant genotype 2 only, demonstrated that this region may be served as useful molecular markers for stress resistance genotypes.

Sequence analysis and alignment

The sequence of the trnL and trnL-F intergenic spacer of resistant genotype (Genotype 2) was aligned to determine phylogenetic assessment. The trnL region showed 94% identity with *Swietenia mahagoni*, *Khaya grandifoliola* and *Khaya nyasica*, whereas the trnL-trnF region showed 99% identity with *Swietenia mahagoni*, *Khaya nyasica*, *Khaya grandifoliola*, *Dacryodes buettneri*, *Dacryodes buettneri* and *Cedrela odorata*.

As a consequence, the *trnL-F* region, comprising the *trnL* intron and *trnL-F* spacer, has become one of the most widely used accumulation of an increasingly large number of sequences of the *trn* (*T*-)*LF* region from a wide range of plants has allowed further study of structures, functions, and evolution in different orders of flowering plants (Bakker *et al.*, 2000). Sequences from the *trnL-F* region (excluding the *trnT-L* region and *trnL* 5' exon) have recently been used, in combination with those from further chloroplast markers *rbcL* and *matK*, as a source of characters for phylogenetic reconstruction in the tropical flowering plant family Annonaceae Juss. These phylogenies have been used to answer questions about morphological character evolution (Sauquet, 2003), classification, biogeography (Pirie et al. 2006). These markers appeared to contain complementary phylogenetic signals, as is expected from different sequences sampled from the plastid genome (Chase and Cox, 1998), and were thus applied in combined analyses.

CONCLUSION

A pattern resistant genotype of *Swietenia mahogany*, showed good tolerance to unfavourable environmental conditions (salinity and calcareous soil) was observed grown at Nobaria, Horticulture Research Station, Egypt, planted almost 15 years ago. In this paper, physiological, biochemical and molecular studies using cpDNA trnL-F region were evaluated in order to understand adaptability and tolerance mechanisms of this resistant genotype

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CONFLICT OF INTEREST

The present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study.

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