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Characterization and expression of the *knox1*gene in relation to leaf shape in some species of Araliaceae in Egypt

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Abstract: The Araliaceae family has 47 genera and more than 1,350 species. In several genera, leaf forms of Araliaceae species have a tremendous array, e.g., simple, palmately lobed, palmately compound or up to three times pinnate, in several genera. Here, the expression of the *Knotted1*-Like homeobox *(KNOX1)* transcription factor gene is measured in relation to leaf shape in 12 plant species from the Araliaceae family in Egypt. Results obtained from real-time quantitative PCR (RT-qPCR) showed variable expression levels among the plant samples under study. High expression levels of *KNOX1* genes were seen in compound pinnate-leaved plants (*Polyscias fruticosa*, *P. guilfoylei*, and *P. scutellaria*), whereas relatively low expression levels were seen in lobed- leafed plants (*Hedera helix* and *Tetrapanx papyrifer*). The RT-qPCR data indicate that the expression level of *KNOX1* in the leaves of species studied coincides with the degree of leaf lamina serration and complexity. Genomic fragments of *KNOX1* were also amplified from the 12 plant samples of Araliaceae under study. The PCR product amplified from plant sample 2 (*Hedera helix*) was eluted, purified, and sequenced. The nucleotide sequence of the *KNOX1* gene fragment was aligned with some reference genes in GenBank and showed a high degree of sequence similarity, indicating a common structure and function of the *KNOX1* gene in higher plants.

Key words: Araliaceae, *KNOX1* gene, RT-qPCR, leaf morphology.

1. Introduction

The Araliaceae family has 47 genera and more than 1,350 species, found mostly in tropical regions. The largest genera (*Schefflera*, *Polyscias*, *Dendropanax*, *Oreopanax*, and *Osmoxylon*) are best represented in tropical or subtropical zones, although several smaller genera (*Panax*, *Hedera*, *Brassaiopsis*, *Macropanax*, *Gamblea*, and *Oplopanax*) grow in the northern temperate and subtropical zones [1].

Leaf form in several genera of the Araliaceae family is tremendously diverse, e.g., simple, palmately lobed, palmately compound, or up to three times pinnate. Gross morphology, petiolar, and nodal leaf anatomy have been used in taxonomic treatments of the family [2, 3, 4, 5]. In angiosperms, the evolution of compound leaf forms has been subject to morphological, genetic, and phylogenetic analysis. Based on these studies, [6] suggested that compound leaf forms arose in dicotyledons on 29 different occasions and the derivation of simple leaves from compound leaves occurred at least six times. Leaf shape and architectural criteria were also used to aid the delimitation of genera and species by [7] and [8]. The taxonomic implications of petiole vascularization of simple, lobed, and compound leaves in certain taxa of Araliaceae have also been studied by [9], who found that the simple-leaved Meryta denhamii should be separated from the lobed Hedera helix and Tetrapanax papyrifer and the compound Polyscias spp., and Schefflera ssp.

Homeodomain (HD) transcription factors play an important part in leaf development [10]. Several classes of HD proteins play a role in developmental signal integration and coordinated growth in complex organisms, the TALE (Three Amino Acid Loop Extension) superclass being one of them. The TALE superclass is defined by three extra amino acids between helix 1 and helix 2 of the HD protein [11,12]. The TALE superclass, according to [12]), is made up of two protein subgroups: *Knotted1*-Like homeobox (*KNOX*) and *BEL1*-Like homeobox (*BLH*). The two phylogenetic classes of KNOX genes are further differentiated, *KNOX1* and *KNOX2*. *KNOX1* transcripts are found in certain domains of shoot apical meristems (SAMs), whereas *KNOX2* genes are found in almost all plant tissues [13, 14]. Plant *KNOX1* proteins have been extensively studied as critical participants in developmental processes in monocotyledons and dicotyledons. [15, 16]. The evolutionary diversification of leaf morphology is aided by *KNOX1*. Distinct degrees of leaf lamina serration and complexity correspond to different *KNOX1* expression patterns in leaves **[6].** In certain simple-leafed plant species, overexpression of *KNOX* genes causes the production of strongly lobed leaves and marginal outgrowth [15]. [17] study Plant development elementary changes determine leaf shape complexity.

The present work aims to study the expression of *Knotted1-Like* homeobox *(KNOX1)* transcription factors in relation to leaf shape in some species of Araliaceae in Egypt. The *KNOX1* gene was PCR amplified, sequenced, and aligned to other related genes in GenBank.**2.**

Materials and methods

2.1. Plant materials

Plant samples representing 12 species and six genera of Araliaceae were collected from the Botanical Public Gardens in Egypt. Sample identification was confirmed by comparison with herbarium specimens in the herbaria of Ain Shams and Cairo University. Leaf morphology was described from the fresh specimens according to [18].

2.2. PCR amplification of *KNOX1* genes:

Total genomic DNA was extracted from fresh plant 12 using the GeneJET Genomic DNA Purification Kit (K0721, Thermo Fisher) according to the manufacturer's protocol. Gene-specific PCR was conducted using forward and reverse primers designed using the Primer-Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the *KNOX1* gene from *Elaeis guineensis* as a design template (XM_010929028.2). Table 1 shows the primers.

Table 1: Sequence features of specific *KNOX1* gene primers.

Primer	Sequence	Tm	GC content
Forward	5′ATTCAGTGATGGAAGCGGCA -3′	60. 7°C	50%
Reverse	5′TTTGCTTCTGGTCGAGTCCC -3′	59. 2°C	55%

The following conditions were used for PCR: denaturation at 95°C for 30 seconds, followed by annealing at 37°C for 1 minute, and extension at 72°C for 2 minutes, for a total of 40 cycles. As a post extension phase, there was a 15-minute delay at 95°C at the start of the first cycle and a 10-minute delay at 72°C at the end of the last cycle. Amplicons were moved in a 1.5 percent agarose gel and imaged using a gel documentation system utilizing a MultiSUB Mini Horizontal Electrophoresis System with Power Pro 300 Power Supply (Cleaver Scientific, UK) (OmniDOC, Cleaver Scientific, UK). Totallab analytic software was used to do the data analysis (www.totallab.com, Ver.1.0.1).

2.3. Sequencing and alignment of *KNOX1* genes:

The E.Z.N.A.®Gel Extraction Kit-Amplified PCR Fragments was used to purify amplified PCR fragments (D2500-01, Omega BIO-TEK, USA). Micron-Corp in Korea sequenced the purified amplicons using an ABI PRISM® 3100 Genetic Analyzer. To confirm their identity, the amplified *KNOX1* sequences were aligned using BLAST on the NCBI website (http://www.ncbi.nlm.nih.gov/website). The Pairwise Distance technique was used to calculate genetic distances and multialignments across species using Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/). The nucleotide sequences were also compared to the GenBank sequences with the highest identity, as determined by BLAST.

3.2. RNA extraction and real-time quantitative PCR (RT-qPCR) of *KNOX1* expression

Total RNA was purified from leaf samples using TRIzols Reagent (15596026, Life Technologies, USA). Total RNA yield and quality were assessed by spectrophotometry at 260 and 260/280 nm ratios, respectively.

The Maximas SYBR Green/Fluorescein qPCR Master Mix by Rotor-Gene Q (Qiagen, USA) was utilised as a housekeeping gene in a two-step cycle RT-qPCR procedure to detect KNOX1 gene expression. For each plant sample, three biological duplicates were done.

3. Results:

3.1. Leaf morphology of plant samples:

Plant samples representing 12 species and six genera of Araliaceae were studied. Leaf shape was described from the fresh specimens (Table 2). All plants of sample 1 (*Hedera canariensis*), sample 4 (*Oreopanax guatemalensis*), and sample 3 had a simple leaf blade. Juvenile plants of sample 2 (*Hedera helix*) had a simple leaf blade; however, adult plants had lobed palmate leaves. Some plants of sample 12 (*Tetrapanax papyrifer*) had a simple leaf blade, while some plants of the same sample were lobed palmate. All plants of samples 5, 6, and 7 (*Polyscias fruticosa*, *P. guilfoylei*, and *P. scutellaria*, respectively) had compound pinnate leaves. Similarly, all plants of samples 8, 9, 10, and 11 (*Schefflera actinophylla*, *S. arboricola*, *S. elegantissima* and *S. pueckleri*) had compound palmate leaves (Table 2).

3.3. KNOX1 gene sequence:

KNOX1 gene fragments were amplified from the 12 plant species of Araliaceae under study using genomic DNA as template and specific *KNOX1* forward and reverse primers constructed from *Elaeis guineensis* (XM_010929028.2) (Table 1). All plant samples had a PCR product of the expected size (approximately 850 bp), except plant samples 1, 4, and 12 (Figure 1).

The PCR product amplified from plant sample 2 (*Hedera helix*) was eluted, purified and sequenced. We were able to sequence 750 bp of the amplified fragment. The nucleotide sequence of the *KNOX1* gene fragment was aligned with some reference genes in GenBank (Table 3). The amplified *KNOX1* gene sequence had a high degree of similarity with the reference genes in GenBank (Table 3). The highest degree of similarity (94.30%) was with the *Eloies guineensis*-predicted mRNA (XM_010929028.2), whereas the lowest degree of similarity was with *Dactylorhiza fuchsia* (JQ229970.1).

Table 2: Leaf shapes of the 12 plant samples of Araliaceae under study.

Number	Plant sample	Leaf shape
1	**Hedera canariensis**	**Simple**
2	H. helix	Simple - Lobed palmate
3	Meryta denhamii	Simple / Lobed pinnate
4	Oreopanax guatemalensis	Simple
5	Polyscias fruticosa	Compound pinnate
6	P. guilfoylei	Compound pinnate
7	P. scutellaria	Compound pinnate
8	Schefflera actinophylla	Compound palmate
9	S. arboricola	Compound palmate
10	S. elegantissima	Compound palmate
11	S. pueckleri	Compound palmate
12	Tetrapanax papyrifer	Simple / Lobed palmate

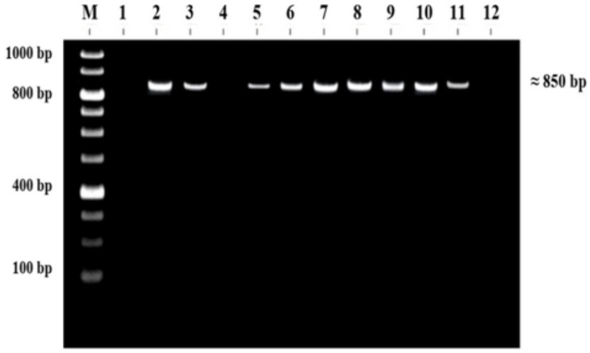
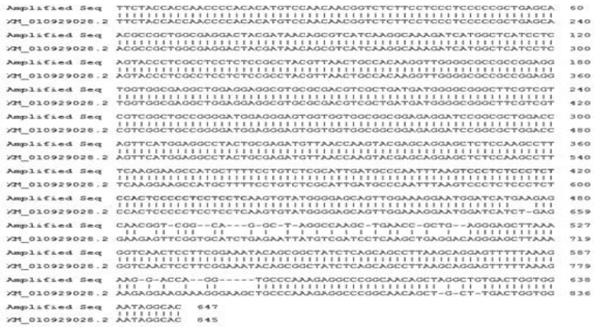


Figure 1: PCR products of amplified *KNOX1* gene fragments (approximately 850 bp) from the 12 species of Araliaceae under study.



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Amplified Seq   TTCTACCACCAACCCCACACATGTCCAACAACGGTCTCTTCCTCCCTCCCCCGCTGAGCA  60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
XM_010929028.2  TTCTACCACCAACCCCACACATGTCCAACAACGGTCTCTTCCTCCCTCCCCCGCTGAGCA  240
Amplified Seq   ACGCCGCTGGCGAGGACTACGATAACAGCGTCATCAAGGCAAAGATCATGGCTCATCCTC  120
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
XM_010929028.2  ACGCCGCTGGCGAGGACTACGATAACAGCGTCATCAAGGCAAAGATCATGGCTCATCCTC  300
Amplified Seq   AGTACCCTCGCCTCCTCTCCGCCTACGTTAACTGCCACAAGGTTGGGGCGCCGCCGGAGG  180
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
XM_010929028.2  AGTACCCTCGCCTCCTCTCCGCCTACGTTAACTGCCACAAGGTTGGGGCGCCGCCGGAGG  360
Amplified Seq   TGGTGGCGAGGCTGGAGGAGGCGTGCGCGACGTCGCTGATGATGGGGCGGGCTTCGTCGT  240
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
XM_010929028.2  TGGTGGCGAGGCTGGAGGAGGCGTGCGCGACGTCGCTGATGATGGGGCGGGCTTCGTCGT  420
Amplified Seq   CGTCGGCTGCCGGGGATGGAGGGAGTGGTGGTGGCGGCGGAGAGGATCCGGCGCTGGACC  300
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
XM_010929028.2  CGTCGGCTGCCGGGGATGGAGGGAGTGGTGGTGGCGGCGGAGAGGATCCGGCGCTGGACC  480
Amplified Seq   AGTTCATGGAGGCCTACTGCGAGATGTTAACCAAGTACGAGCAGGAGCTCTCCAAGCCTT  360
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
XM_010929028.2  AGTTCATGGAGGCCTACTGCGAGATGTTAACCAAGTACGAGCAGGAGCTCTCCAAGCCTT  540
Amplified Seq   TCAAGGAAGCCATGCTTTTCCTGTCTCGCATTGATGCCCAATTTAAGTCCCTCTCCCTCT  420
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
XM_010929028.2  TCAAGGAAGCCATGCTTTTCCTGTCTCGCATTGATGCCCAATTTAAGTCCCTCTCCCTCT  600
Amplified Seq   CCACTCCCCCTCCTCCTCAAGTGTATGGGGAGCAGTTGGAAAGGAATGGATCATGAAGAG  480
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||   |||
XM_010929028.2  CCACTCCCCCTCCTCCTCAAGTGTATGGGGAGCAGTTGGAAAGGAATGGATCATCT-GAG  659
Amplified Seq   CAACGGT-CGG--CA---G-GC-T-AGGCCAAGC-TGAACC-GCTG--AGGGAGCTTAAA  527
                 ||  || |||  ||   | |  | | | | |   | || | |  |  ||||||||||||
XM_010929028.2  GAAGAGTTCGGTGCATCTGAGAATTATGTCGATCCTCAAGCTGAGGACAGGGAGCTTAAA  719
Amplified Seq   GGTCAACTCCTTCGGAAATACAGCGGCTATCTCAGCAGCCTTAAGCAGGAGTTTTTAAAG  587
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
XM_010929028.2  GGTCAACTCCTTCGGAAATACAGCGGCTATCTCAGCAGCCTTAAGCAGGAGTTTTTAAAG  779
Amplified Seq   AAG-G-ACCA--GG-----TGCCCAAAGAGGCCCGGCAACAGCTAGGCTGTGACTGGTGG  638
                ||| | |  |  ||      ||||||||||||||||||||||||| | || ||||||||||
XM_010929028.2  AAGAGGAAGAAAGGGAAGCTGCCCAAAGAGGCCCGGCAACAGCT-G-CT-TGACTGGTGG  836
Amplified Seq   AATAGGCAC  647
                |||||||||
XM_010929028.2  AATAGGCAC  845
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Fig 2: Sequence alignment of the *Knotted-1-like* (*KNOX1*) gene amplified from plant sample 2 (*Hedera helix*) with *Eloies guineensis*-predicted mRNA (XM_010929028.2) in GenBank indicating 94% similarity.

Table 3: Sequence similarity of the *Knotted-1-like* (*KNOX1*) gene amplified from plant sample 2 (*Hedera helix*) with some reference genes in GenBank.

(*KNOX1*) gene	**Similarity%**
Dactylorhiza fuchsia (JQ229970.1)	71.53 %
Cocos nucifera (GU937114.1)	74.40 %
Eloies guineensis-predicted mRNA (XM_010929028.2)	94.30%
Eloies guineensis (DQ890420.1)	90.80 %
Phoenix dactylifera – predicted mRNA (XM_017844142.3)	84.40 %
Phoenix dactylifera (XM_039116003.1)	84.40 %
Phoenix dactylifera (XM_039116002.1)	74.40 %
Phoenix dactylifera (XM_039131360.1)	85.45%
Eloies guineensis (XM_029266954.1)	80.31%

3.4. Expression of *KNOX1*

RT-qPCR was conducted to detect *KNOX1* gene expression. Table 4 and Figure 3 present the expression folds of the KNOX1 gene.

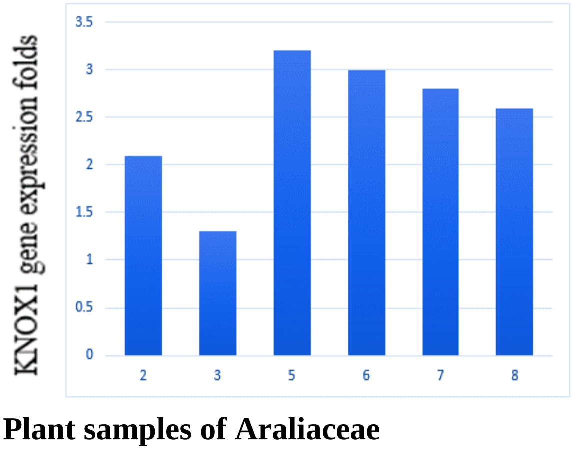

Fig 3: *KNOX1* gene expression levels in some selected plant samples of Araliaceae as indicated in Table 2.

Table 4: Expression levels of *KNOX1* in some selected plant samples of Araliaceae as indicated in Table 2.

Samples	**Gene being Tested Experimental (TE)**	**Gene being Tested Control (TC)**	**Housekeeping Gene Experimental (HE)**	**Housekeeping Gene Control (HC)**	**ΔCt values for the experimental (ΔCTE)**	**ΔCt values for the control (ΔCTC)**	**Delta Ct Value (ΔΔCt)**	**2^-ΔΔCt** (expression fold change**)** -fold Expression level in the experimental condition the expression as in the control condition
2	33	23	32.1	21	0.9	2	-1.1	2.1
3	33.6	23	32	21	1.6	2	-0.4	1.3
5	32.4	23	32.1	21	0.3	2	-1.7	3.2
6	32.7	23	32.3	21	0.4	2	-1.6	3.0
7	32.8	23	32.3	21	0.5	2	-1.5	2.8
8	32.9	23	32.3	21	0.6	2	-1.4	2.6

ΔCt values (Experimental) = Ct (Tested Exp) – Ct (Housekeeping Exp)

ΔCt values (Control) = Ct (Tested Control) – Ct (Housekeeping Control)

ΔΔCt values = ΔCt values (Experimental) - ΔCt values (Control)

Relative gene expression fold change = 2^-ΔΔCt

Plant sample 5 (*Polyscias fruticosa*) showed the highest expression fold change (3.2), followed by plant sample 6 (*Polyscias guilfoylei*) with a high expression fold change of 3.0. Plant sample 7 (*Polyscias scutellaria*) and plant sample 8 (*Schefflera actinophylla*) showed relatively high expression levels (2.8 and 2.6 folds, respectively). By contrast, plant sample 2 (*Hedera helix*) had a relatively low expression level (2.1), whereas plant sample 3 (*Meryta denhamii*) showed the lowest expression level (1.3).

4. Discussion

The placement of Araliaceae among the major lineages of the order Apiales has made significant progress [19, 20, 21, 22, 23] and understanding relationships within and among Araliaceae genera that are closely related [24, 25, 26, 27, 28]. In angiosperms the evolution of compound-leaf forms is broadly studied using morphological, genetic, and phylogenetic analysis. Leaf shape and architectural criteria have been used to aid in the delimitation of genera and species by [7, 8].

In the present study, the relationship between the expression of *KNOX1* transcriptional factor genes and the shape of leaves of 12 plant samples of the family Araliaceae was investigated. Plant samples representing 12 species and six genera of Araliaceae were collected, and the leaf shape of plant samples was described from fresh specimens. Our results indicated great variations in leaf shape among plant samples under study (simple, palmately lobed, palmately compound, or up to three times pinnate). These results are in agreement with previous studies on the Araliaceae [2, 3, 4, 5].

In the present study, expression levels of the *KNOX1* gene were detected for some selected plant species of Araliaceae. RT-qPCR showed variable expression levels among the plant samples under study. Our results indicated a clear relationship between *KNOX1* gene expression levels and leaf shape. High expression levels of *KNOX1* genes were found in compound pinnate-leafed plants (*Polyscias fruticosa, P. guilfoylei,* and *P. scutellaria*), whereas relatively low expression levels were indicated in lobed-leaved plants (*Hedera helix* and *Tetrapanax papyrifer*). These data were consistent with previous literature showing that expression levels of *KNOX1* genes in plant leaves coincide with the degree of leaf lamina serration and complexity [6]. Overexpression of *KNOX* genes resulted in the production of highly lobed leaves and marginal outgrowth in a variety of simple-leafed plant species, according to [15].

Cytokinins (CK) are master regulators of plant development that stimulate cell division and induce SAM formation in plant tissue culture. Several plant species with overexpressed *KNOX1* genes produce a lot of cytokinins [29, 30, 31, 32]

KNOX1 gene fragments were amplified from the 12 plant samples of Araliaceae under study. Lobed- and compound- leafed plant samples had genomic PCR product of the expected size (approximately 850 bp). Surprisingly, simple leafed plant samples (*Hedera canariensis, Oreopanax guatemalensis*) showed negative results. These results confirmed the vital role of the *KNOX1* gene in leaf development. Also plant samples of *Tetrapanax papyrifer* showed negative PCR results which can be correlated with the simple leaf blade exhibited by most individuals of this species, although some plants of the same sample were lobed palmate.

PCR product amplified from plant sample 2 (*Hedera helix*) was eluted, purified, and sequenced. The nucleotide sequence of the *KNOX1* gene fragment was aligned with some reference genes in GenBank, which indicated a high degree of sequence similarity. Our results indicate a common structure and function of the *KNOX1* gene in higher plants [6, 15, 16].

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