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Original article

Identification and Gene Expression Analysis of TIR1 in Easy and Hard to Root Olive (*Olea europaea* L.) Cultivars

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Abstract

Many plant species can be efficiently propagated vegetatively through the use of cuttings. Although simple to implement, the success rate of this method is restricted by the plants ability to produce adventitious roots. Auxin hormones are commonly used to induce adventitious roots in propagation by cuttings. The auxin metabolism influences plant growth and is significant because it can also affect the rooting performance of the IBA hormone commonly used during steel production. Olive trees (*Olea europaea* L.) are among the species that are commonly propagated using cuttings. However, the rooting success of olive cultivars grown for agriculture varies. For these reasons we analyzed the gene structure and expression rates of Olive *TIR1*, which is the receptor of auxins. We chose Gemlik (easy to root) and Domat (Hard to root) cultivars for the gene expression analysis. Our analysis revealed that *OeTIR1* is coded by *Oeu047472.1* and it contains 3 exons and codes for 594 amino acid protein and a coding sequence of 1785 nucleotides. Gene expression results showed different expression patterns between cultivars. Results indicate that in Gemlik cultivar expression of *OeTIR1* does not show any upregulation after IBA application. Domat however showed an increase in expression only after 7 days.

Keywords: Auxin Receptor, Auxin metabolism, Gene Expression, Propagation with Cuttings.

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INTRODUCTION

Many cultivated plant species are successfully propagated vegetatively by cuttings. Although it is easy to perform, the success rate of this technique is limited by the ability of cuttings to form adventitious roots. Olive (*Olea europaea* L.) is one of the tree species that is routinely produced with cuttings. On the other hand, the rooting success of cultivated olive varieties is different. Some varieties with economic importance, such as Domat from Turkey, are propagated by grafting due to their low rooting rates. Investigation of the molecular mechanisms underlying this issue has significant potential. Application of indole butyric acid (IBA) is a common approach in olive propagation. After the application of indole butyric acid (IBA) to olive cuttings, the adventitious rooting process takes place in 3 stages; Molecular and biochemical changes do not cause histological changes during the induction phase covering the first 4 days, histological changes such as root meristems occur in initiation phase between the 4th and 14th days, and finally, there is the expression phase where root development begins (Pacurar et al., 2014). While auxins such as IAA have a stimulating effect on rooting in these processes, the high amount of auxin in the initial stages can have a suppressing effect (Porfirio et al., 2016). These results suggest that the speed and timing of auxin levels are important for the rooting success of the cuttings.

Apart from its synthesis, external applications as IBA or conversion to different auxin forms (IBA to IAA), the receptors that perceive auxin levels in the tissues also take role in the regulation of the adventitious rooting process. Auxin is perceived by TIR1 and Aux/IAA protein complex. When bound, auxin causes degradation of Aux/IAAs and releases the repression of ARF-based transcription (Korasick et al., 2015; Salehin et al., 2015; Wang & Estelle, 2014). In tree species the overexpression of *Populus TIR1* homolog increased adventitious rooting, while knock out lines showed delayed adventitious root formation (Shu et al., 2019). Apart from Populus, Olive is also advantageous for adventitious rooting research for there is cultivars showing different levels of rooting capacity and has sequenced genome data. Olive propagation also could benefit from increased adventitious rooting as propagation by cuttings are the preferred method. With this study we report the Olive *TIR1* homologs and show their expression patterns in easy and hard to root cultivars under auxin applications. The results revealed a difference in gene expression between the two cultivars, which can be targeted to increase rooting ability of Olive cutting.

MATERIALS and METHODS

Obtaining of Sequences of OeTIR1 and Selection of Primers: The Sylvestris Olive genome was used to obtain the genomic and transcript sequences of the olive TIR1 (Unver et al., 2017). A local blast database was created in Geneious R8 (Kearse et al., 2012) software by downloading the entire genome, transcriptome and proteome data of the related cultivar from the NCBI database (Phytozome genome ID: 451). Then, the protein sequences of the TIR1 (AT3G62980) were downloaded from the TAIR

(arabidopsis.org) database and used in the Blastp analysis against the local olive protein database in Geneious software. Sequences obtained as a result of blast analysis were aligned in Geneious software, and phylogenetic trees were created with UPGMA algorithm. Olive TIR1 homolog sequences were selected depending on their similarity to the reference sequences depending on both blast and phylogenetic results and their conserved domain structures. Quantitative real-time PCR primers were selected over exon junction regions with the help of the NCBI primer blast tool.

Plant material: Micro-stems of Gemlik and Domat cultivars were obtained from trees from the Çanakkale Onsekiz Mart University Dardanos Olive collection (Türkiye). Short cuttings containing four nodes were cut and cleared of leaves except the shoot tip. Then, the lower parts of the cuttings, approximately 1 cm from the cuts, were immersed in 4000 ppm IBA solution for 5 seconds. In the control groups, the cuttings were immersed in IBA-free solvent solution (%40 Ethanol) for 5 seconds. Afterwards, the cuttings were taken into pots containing perlite and placed in plant growth cabinets at high humidity (>90%) and 25 ℃. At least 10 cuttings were prepared for each 3 biological replicates. The IBA-applied parts of the cuttings were cut from approximately 1 cm length on the 0 and 24th hour, 7th, and 15th days, immediately frozen in liquid nitrogen, and stored at -80°C until RNA isolation.

Gene Expression Experiments: RNA isolations from tissues were carried out in liquid nitrogen using a pestle and pestle. Extraction was performed using TRIzol (Fisherscientific – USA) and PureLink RNA Mini Kit (Life Technologies – USA) according to the manufacturer's protocol. After extraction, RNA quality was checked with 1% agarose gel electrophoresis and their concentrations were measured with Qubit 2.0 (Invitrogen, Life Technologies – USA). For all samples, 1µg RNA was treated with DNAse I (Fisher Scientific - USA) for 30 minutes at 37°C before cDNA synthesis. Next, cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Fisher Scientific – USA) following the manufacturer's protocol. The qPCR reactions were set up by mixing 5µl of SYBR Green PCR Master Mix (Applied Biosystems, USA), 0.3µl (200nM) of each primer, and 1µl of cDNA, adjusting the total reaction volume to 10µl with nuclease-free water. Step One Plus (Applied Biosystems, USA) system was used for RT-PCR. The first denaturation step was carried out at 95°C for 10 minutes followed by 15 s denaturation at 95°C, 60 s bonding at 60°C, and 1 minute elongation at 72°C for 40 cycles. At the end of the reaction, Melt Curve Analysis was performed between 60-95°C. The qPCR efficiencies were calculated with the standard curve method using serial dilutions of the pooled cDNA of the samples used in the project. In gene expression analyzes, only primer pairs with an efficiency value of 90-110%, an R2 value higher than 0.98 and a single peak in melt curve analyzes were used. *OUB2* and *ACT7a* genes were selected as reference genes in the study (Hürkan et al., 2018; Noceda et al., 2020). Gene expression studies were performed with SYBR GREEN method and 3 biological / 3 technical replicates. Data were analyzed according to (Pfaffl, 2019) and sorted and formatted in R platform. The graphs were prepared with the boxplot function of ggplot in R.

RESULTS and DISCUSSION

Auxin reception and signaling is important for many developmental aspects of plant development. Because of its importance in adventitious rooting and potential effects on propagation by cutting, we characterized the structure and expression patterns of auxin receptor *TIR1* in Olive.

Identification of Olive *TIR1*: Analysis of the Olive genome and the blast analysis to identify the Olive *TIR1* (*OeTIR1*) showed the sequence that showed most similarity was *Oeu047472.1* with 0 E-value, %78.8 pairwise similarity and %97.81 coverage. It is 581 amino acid long, has a theoretical pI of 6.32 and molecular weight of 65,49 KDa. *Oeu047472.1* has a 1746 nucleotide long transcript and 3 exons (Figure 1). The genomic region covering all exons was 2998 base pairs. *AtTIR1* also has 3 exons, 594 a.a. protein and a coding sequence of 1785 nucleotides.

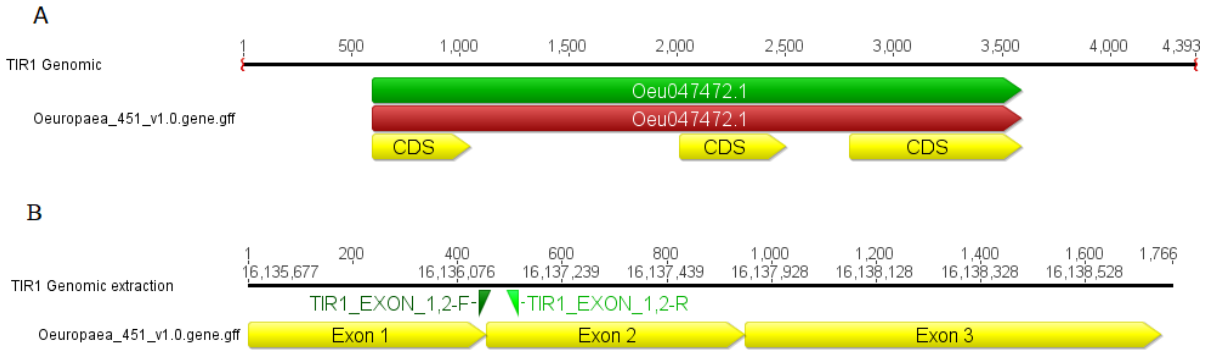

Figure 1. A) Genomic structure of *OeTIR1*. B) The primer positions for gene expression studies.

To compare OeTIR1 sequences, protein homologs of AtTIR1 from several plant species were retrieved from the phytozome homologs database. All sequences were aligned and used for creating phylogeny trees (Figure 2). The tree generally showed a grouping of different plant families like Brassicaceae, Solanaceae and Rosaceae. The *OeTIR1* was grouped with *Mimulus guttatus*, *Solanum tuberosum* and *Solanum lycopersicum* sequences. Alignments and distance calculations showed high level of similarities between homologs from different families with pairwise identities ranging from %76 to %99.

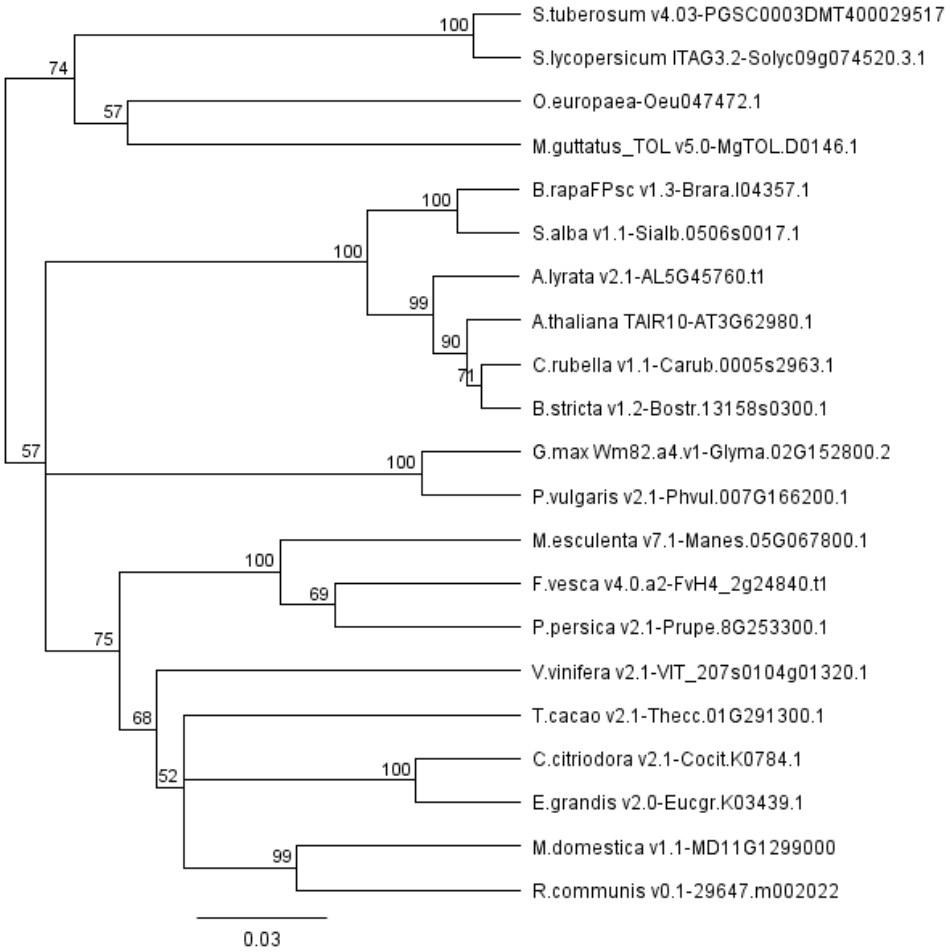

Figure 2. Phylogenetic tree of *TIR1* homologs. Descriptions contain the species and the accession numbers (https://phytozome-next.jgi.doe.gov) of each sequence. Numbers on the nodes represents bootstrap support values.

Alignments and distance calculations showed high level of similarities between homologs from different families with pairwise identities ranging from %76 to %99. We also compared the presence and locations of functional conserved domains of *AtTIR1* and *OeTIR1* (Figure 3). Both sequences contained several leucine rich repeats characteristic to these proteins. They also contained a Transport inhibitor response 1 protein domain (pfam18791, PSSMID 436740) which is found in Auxin receptor *TIR1*. The F Box domain on the N terminal region is an N-terminal F-box domain which is also found in other auxin signaling f-box proteins apart from *TIR1*. Both also have AMN1 domains which were discovered in *Saccharomyces* but their functions in plants are not yet clear.

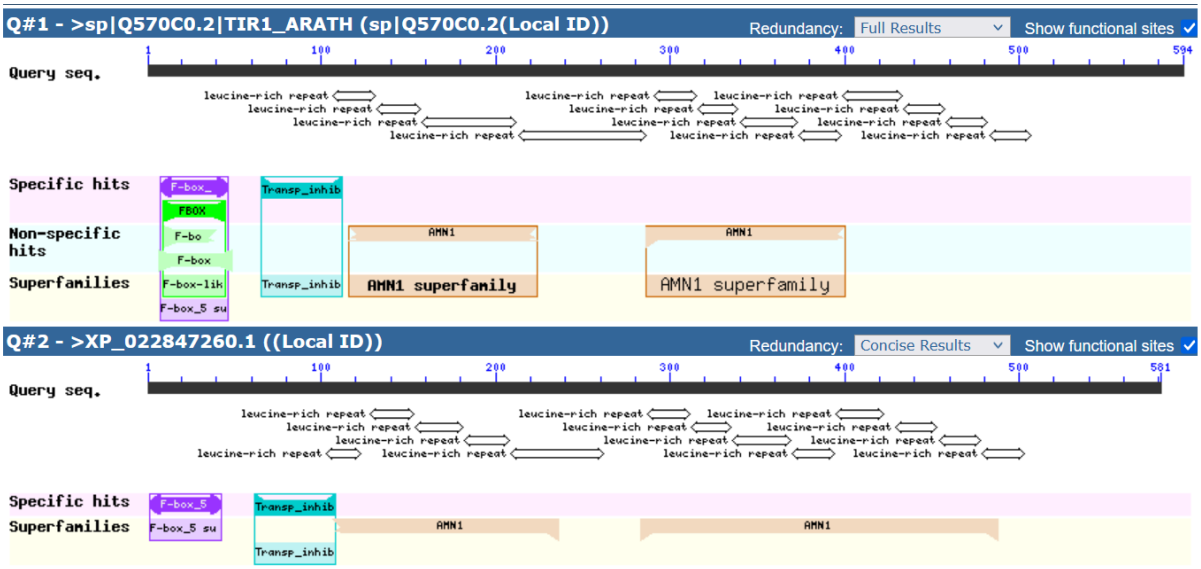

Figure 3. Conserved functional domains of Olive and *Arabidopsis TIR1*. Q1 *Arabidopsis thaliana*, Q2 *Olea europaea*.

Expression of *OeTIR1* under IBA Application: Gene expression studies were set up to mimic the common Olive propagation method by cuttings. For this purpose, micro-cuttings were treated with 4000ppm IBA solution and basal parts of cuttings were harvested after 24 hours, 7 and 15 days. Expression of *OeTIR1* showed different expression pattern between cultivars and sampling time (Figure 4).

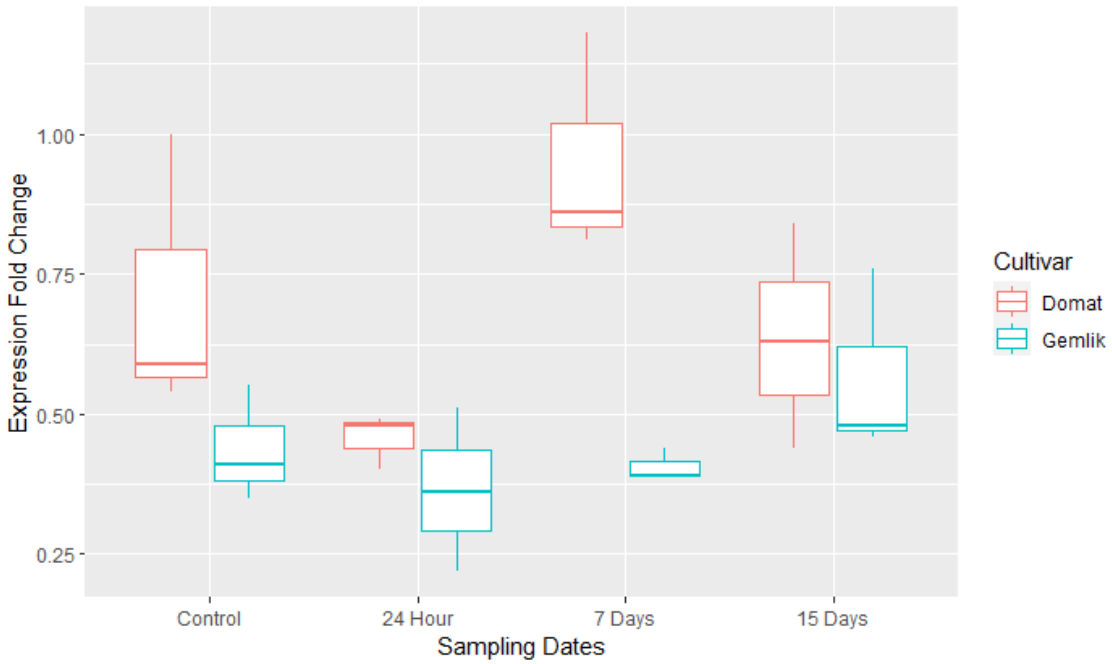

Figure 4. Expression patterns of *OeTIR1* after IBA application

Interestingly highest expression difference was observed at samples taken after 7 days. Hard to root Domat cultivar showed higher levels of expression compared to easy to root Gemlik in day 7 samples. Contrarily, 24 hour and day 15 samples did not show a significant expression difference between cultivars. Results indicate that in Gemlik cultivar expression of *OeTIR1* does not show any upregulation after IBA application. Domat however showed an increase in expression only after 7 days.

Conclusion

Auxin reception and signaling is important for many developmental aspects of plant development. Because of its importance in adventitious rooting and potential effects on propagation by cutting, we characterized the structure and expression patterns of auxin receptor *TIR1* in Olive. Our analysis revealed the structure of Olive TIR1, which shows high resemblance to functionally proven Arabidopsis *TIR1* (*AT3G62980*). Gene expression results showed different expression patterns between cultivars. Results indicate that in Gemlik cultivar expression of *OeTIR1* does not show any upregulation after IBA application. Domat however showed an increase in expression only after 7 days. This expression patterns could indicate a difference in molecular control of adventitious rooting between hard to root and easy to root cultivars. Similar expression differences was observed in other genes that have roles in auxin metabolism or abiotic stress caused by wounding from cutting procedures (Arnholdt-Schmitt et al., 2006; Hedayati et al., 2015; Velada et al., 2018, 2020). These articles focus on the polar auxin transporter PIN proteins and stress related alternative oxidase (AOX) genes. Our results showed the first information about the Auxin receptor *OeTIR1* in Olive. Further functional research of *OeTIR1* across cultivars of olive could create new potential markers for rooting capability of olives.

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