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Original Research Paper

Identification and expression profile of a thioredoxin h in olive (*Olea europaea* L.)

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Abstract: The cellular response to stress also includes the accumulation of reactive oxygen species (ROS). When in excess, these second messengers can cause significant detrimental biochemical changes, such as stress-induced lipid peroxidation. In plants, a powerful endogenous antioxidant system is based on the enzymatic modification of redox-sensitive cysteines, carried out for example, by thioredoxin proteins. In this work, we report the cloning and characterization of a thioredoxin gene from olive (*Olea europaea* L.), which was named *Oe-Trx h I*. We also performed an expression analysis in drupes following biotic stress, as a first step towards the definition of its possible defensive role against the olive fruit fly [*Bactrocera oleae* (Rossi)]. The data indicate that the cloned thioredoxin h is a potential member of the biotic stress response in drupes and suggest that *Oe-Trx h I* may be a component active towards an increased resistance against oxidative stress. This work opens the way to further studies to assess the protective role of this gene against ROS accumulation and lipid peroxidation in drupes following larval feeding.

Keywords: biotic stress; cloning; drupes; gene expression; *Olea europaea*; stress response

1. Introduction

The sustainable increase of yield requires an improved understanding of the plant reaction to the various limiting conditions that crops face during their lifetime (Tester et al., 2010). Plant stress response is a complex physiological process that also involves second messengers (Takahashi et al., 2019). These intracellular signaling molecules integrate different stress cues and related downstream responses. For instance, under unfavorable environmental conditions, plant cells can generate a high level of reactive oxygen species (ROS). These molecules affect processes ranging from stress tolerance to programmed cell death, from stomatal behavior to DNA damage and repair (Baxter et al., 2014), ultimately resulting in yield penalties (Czarnocka et al., 2018). ROS toxicity also affects various cellular compounds. In particular, unsaturated fatty acids, such as those abundantly present in plant cell membranes, are readily oxidized by ROS (Su et al., 2019). ROS homeostasis is mediated by both non-enzymatic and enzymatic systems (Nadarajah 2020; Su et al., 2019). Among the latter, thioredoxins (TRXs) are key players in modulating ROS scavenging in cells (Dos Santos et al., 2006). TRXs are small proteins (around 14 kDa) present in virtually all organisms, from bacteria and algae to mammals and higher plants. TRXs are characterized by two vicinal cysteines (C) in the -C-X-X-C- motif (where X is any amino acid), central for their biological activity (Gelhaye et al., 2005). The primary function of these proteins is the reversible reduction of oxidized cysteine residues and the cleavage of disulfide bonds. TRXs are reduced by Thioredoxin Reductases in a NADPH-dependent system (NTRs). In addition, photosynthetic organisms also possess a chloroplastic system characterized by Ferredoxin-dependent Thioredoxin Reductases (FTRs) (Geigenberger et al., 2017; Gelhaye et al., 2005).

Higher plants have a larger number of TRXs than mammals, yeasts, and bacteria (Meyer et al., 2005). Plant thioredoxins are typically coded by a multigene family. The number of members strongly varies among species, suggesting a functional specificity for the different isoforms (Fernández-Trijueque et al., 2019; Cavalcante et al., 2019). Plant TRXs are classified in six groups (f, h, m, o, x and y) because of previous biochemical evidence, phylogenetic relationship and subcellular localization (Meyer et al., 2002). Members of the m, x and y more closely resemble prokaryotic TRXs, while f, h and o are typical of eukaryotes. Chloroplastic (f and m) and mitochondrial thioredoxins (x) are nuclear encoded proteins with a transit peptide. The h group takes the name because initially considered characteristic of heterotrophic plant tissues (Johnson et al., 1987). This group is the most variable in higher plants (Reichheld et al., 2002; Gelhaye et al., 2004; Geigenberger et al., 2017), with some members also targeted to the mitochondria or the secretory pathway (Laloi et al., 2001). One of the first well characterized function of TRXs h was their promoting role in cereal grain germination, essentially through disulphide bond reduction of insoluble storage proteins (Hägglund et al., 2016; Kobrehel et al., 1992). It is now clarified that these proteins are involved in protection against oxidative stress in cells, mediating intracellular signal transduction through ROS-dependent oxidation of cysteine residues (Dos Santos et al., 2006; Gelhaye et al., 2004). Moreover, the cellular role of TRXs is probably multifaceted, mainly because a number of studies indicated that TRX h can interact with different proteins (Gelhaye et al., 2004; Montrichard et al., 2009), not only those acting in the redox-dependent signaling cascades. Despite their central role in ROS signaling, TRXs have been mainly studied in herbaceous crops and model systems (Schürmann et al., 2000).

In this study, we present the new cDNA sequence of a thioredoxin h cloned from *Olea europaea* (L.) and an expression analysis in drupes, with a focus on biotic stress [i.e., *Bactrocera oleae* (Rossi)], as a first step to define the functional role of this gene. *Bactrocera oleae*-dependent ROS-induced lipid peroxidation ultimately increases olive oil acidity, diminishing the organoleptic characteristics, the economic value, and the health-promoting properties of the commercial product (Gómez-Caravaca et al., 2008; Tamendjari et al., 2009). Therefore, the characterization of key members of the enzymatic antioxidant system provides information useful to understand and manage the olive protective mechanisms against oxidative stress in drupes.

2. Materials and Methods

2.1. cDNA cloning

The initial partial cDNA was identified in a Suppression Subtractive Hybridization (SSH) library previously described (Corrado et al., 2012). The recovery of full-length coding sequence was performed by 5′ and 3′ rapid amplification of cDNA ends, using the "5′ RACE System for Rapid Amplification of cDNA Ends" (Thermofisher, Milan, Italy) and the "3′ RACE System for Rapid Amplification of cDNA Ends" (Thermofisher) kits, following already reported procedures (Corrado et al., 2012).

2.2. PCR amplification

DNA isolation from olive leaves was carried out as described (Corrado et al., 2011). PCR reactions were assembled in a final volume of 25 µl with the following reagents: 25 ng genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µl of each primer (100 µM), 5 U of Taq DNA polymerase (Promega, Milan, Italy) and its 1X Buffer. The primers employed were (sequences are in 5' to 3' order): TRX-CLO-FW: GTA CCT TCG ACA AAG AGA GAG T; TRX-CLO-RV: GAA ACC TCA AGC ACT AGC AG; TRX-RT-FW: AGA GGG ACA GGT TAT CGG CTG; TRX-RT-RV: CCA CAC CAC GAA GCT GTG AA. Reactions were performed in a Mastercycler Gradient S thermocycler (Eppendorf, Milan, Italy) using a previously reported thermal profile (Corrado et al., 2012).

2.3. DNA sequencing and analysis

Plasmid inserts and PCR products were purified with the "Purelink Quick Plasmid Miniprep Kit" and "Purelink PCR Purification Kit" (Invitrogen, Milan, Italy), respectively, following the manufacturer's instruction. Sequencing was carried out at GenoPOM laboratory (Portici, NA, Italy), according to the Sanger's method. Raw data were analysed with Sequence Scanner 1.0 (Applied Biosystems, Milan, Italy). Similarity search was performed using BLAST (Altschul et al., 1990) at the National Center for Biotechnology Information (NCBI). Multiple alignment was carried out with Clustal X 2.0 (Larkin et al., 2007) and visualized with Jalview (Waterhouse et al., 2005). Intracellular targeting was predicted with TargetP-2.0, Psort and iPSORT. BLAST scan of the olive genome was performed in Phytozome (Waterhouse et al., 2005). The computation of the theoretical isoelectric point (pI) and molecular weight was performed at web.expasy.org/compute_pi/.

2.4. Gene expression profile

For the mechanical wounding experiment, drupes of approximately 1.5 cm of the cultivar 'Leccino' were diagonally punctured (ten times) with a sterile steel needle 110 days after flowering, without damaging the stone. Olives were picked at time 0 (unwounded control), and 24- and 48-hours following treatment. At each harvest, drupes were manually destoned, and the tissue was frozen in liquid nitrogen and stored at -80 °C until RNA isolation. We analyzed two different pools of five drupes per plant coming from different branches, with the experiment carried out using two biological replicates (i.e. two different olive trees). For the analysis of the response to the biological wounding caused by *Bactrocera oleae*, we analyzed fruits without obvious symptoms of pathogen attack, deformation, or damage. Olives at different attack stages (punctured or with a single II or III instar larva) were examined under a light microscope and sliced to remove the active larva. Fruit tissue was immediately frozen in liquid nitrogen and stored at -80 °C until use. For the real-time quantification of gene expression, RNA isolation from drupes, first-strand cDNA synthesis and reverse transcription PCRs were carried out as previously reported (Grasso et al., 2017). The *Elongation Factor 1-alpha* gene was used as reference gene and the unwounded or the undamaged olives as calibrator condition (Grasso et al., 2017). Relative gene expression was calculated according to the DeltaDelta Ct method. Reactions were performed in triplicate on two biological replicates per experimental conditions.

3. Results

3.1. Cloning and classification of the Oe-Trx h I

A cDNA fragment with a sequence similar to a thioredoxin gene was obtained from a SSH library screening (Corrado et al., 2012). Its sequence was employed to capture the mRNA ends by 5′ and 3′ RACE-PCRs. The assembly of the rescued 5' and 3' ends yielded a 604 bp cDNA, which was named *Oe-Trx h I* (Genbank accession JQ711530). This sequence contained a complete ORF (from bp 50 to 421) putatively coding for a 123 aa polypeptide with a theoretical pI and a molecular weight of 5.91 and 13.55 kDa, respectively (Figure 1).

Target prediction indicated the lack of putative signaling or targeting peptides, with the most likely localization (65%) being the cytoplasm. Similarity search (blastp) in the UniProtKB/Swiss-Prot database indicated that the *Oe-Trx h I* protein had the highest sequence identity (77%) with the Thioredoxin H-type 1 from *Nicotiana tabacum*. Search in the *Olea europaea* sp. *europaea* var. *sylvestris* genome localized the locus coding for the *Oe Trx h I* in the chromosome 14 (strand forward; position of the ATG: 5818269). On the basis of the transcript alignment, the olive gene is predicted to have two introns, of 1235 bp and 127 bp. To verify the gene structure, we compared the amplification pattern of the olive genomic DNA (gDNA) and cDNA using different primer combinations.

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-49                                                  AGAG
-45  AGTTGAAATTCTTGAAATTTGGGTTTTTTTGTGAGAGGAAAAAAA

  1  ATGGCCGCAGAAGAGGGACAGGTTATCGGCTGCCACTCCGTTGAT
      M  A  A  E  E  G  Q  V  I  G  C  H  S  V  D

 46  CAGTGGAAGGAGAACTTCAACAAGGGCATCGAGACCAAGAAATTG
      Q  W  K  E  N  F  N  K  G  I  E  T  K  K  L

 91  GTGGTGATCGATTTCACAGCTTCATGGTGTGGGCCCTGCCGAGTT
      V  V  I  D  F  T  A  S  W  C  G  P  C  R  V

136  ATTGCCCCAATTTTGGCTGAGATTGCCAAGAAGACGCCACATGTT
      I  A  P  I  L  A  E  I  A  K  K  T  P  H  V

181  ATATTCTTGAAGGTGGATGTGGATGAACTAAAGGATGTTGCTAAA
      I  F  L  K  V  D  V  D  E  L  K  D  V  A  K

226  GAATTCAACGTGGAGGCCATGCCAACGTTCGTGTTTCTCAAGGAT
      E  F  N  V  E  A  M  P  T  F  V  F  L  K  D

271  GGGAAAGAAGTGGATAGGCTCGTCGGTGCAAGGAAGGAAAATTTG
      G  K  E  V  D  R  L  V  G  A  R  K  E  N  L

316  CAGGATACAATCAACAAGCATGCTACTGCTACTGTTACTGCTACT
      Q  D  T  I  N  K  H  A  T  A  T  V  T  A  T

361  GCTACTGCTTGAGGTTTCTTTGTTAAGAAACATTTGGGCTTGTAA
      A  T  A  *

406  TAATCTATAGTTTGTTAAGACTTATGACTGTTTTATTATGACCGT
451  TTTAACTTTAGTGTGATGGTCTATCATTGTGAATGTAACGAAGTC
496  TTATGATTTTCTCCTACTGCAGGGGAAATCTTAGTATGTTACCGT
541  GTGTTTTATCGTGAG
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Figure 1. Nucleotide sequence of *Oe-Trx h I* with its deduced amino acid sequence. The start and stop codons are in bold face. The putative poly(A) additional signal is underlined.

The PCR analysis confirmed the different lenght fragments in cDNA e gDNA and the presence of a large intron at the 5' region (Figure 2). Moreover, the exon-intron junction regions were Sanger sequenced using the same primers (not shown). While the second intron could be fully sequenced, the length of the first intron, along with the presence of homopolymeric sequences, allowed to obtain around 50% of its predicted full-length sequence.

The identity of the predicted *Oe-Trx h I* protein with the putative translational product of the *O. europaea* reference genome was 88%. Multiple alignment of the *Oe-Trx h I* amino acid sequence to other well characterised plant TRX revealed the presence of several conserved residues, most notably the canonical WCGPC site (Figure 3). The *Oe-Trx h I* presents the conserved protein domain of the TRX family Group I of the Thioredoxin_like Superfamily (cl 00388; i.e., Protein Disulfide Oxidoreductases with a canonical TRX fold). Moreover, *Oe-Trx h I* contains the conserved N-terminus tryptophan residue (W17), a frequent signature of the thioredoxin h sequences (Lemaire et al., 2000).

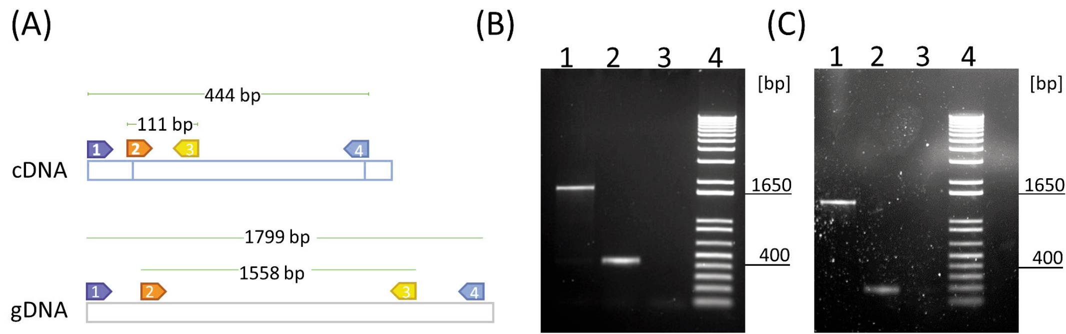

Figure 2. (A) Schematic representation of the expected amplification products of the cDNA and genomic DNA (gDNA) of the *Oe-TRX h I* (not to scale). For each template, indicated on the left, the colored arrows indicate the primers' location used in this study (primer 1: TRX-CLO-FW; primer 2: TRX-RT-FW; primer 3: TRX-RT-RV; primer 4: TRX-CLO-RV). (B) PCR amplification with the primer 1 and 4; 1: gDNA; 2: cDNA; 3: negative control; 4: 1 kb plus DNA ladder (Thermofisher). (C) PCR amplification with the primer 1 and 3; gDNA; 2: cDNA; 3: negative control; 4: 1 kb plus DNA ladder (Thermofisher). Representative marker sizes (in bp) are reported on the right-hand side.

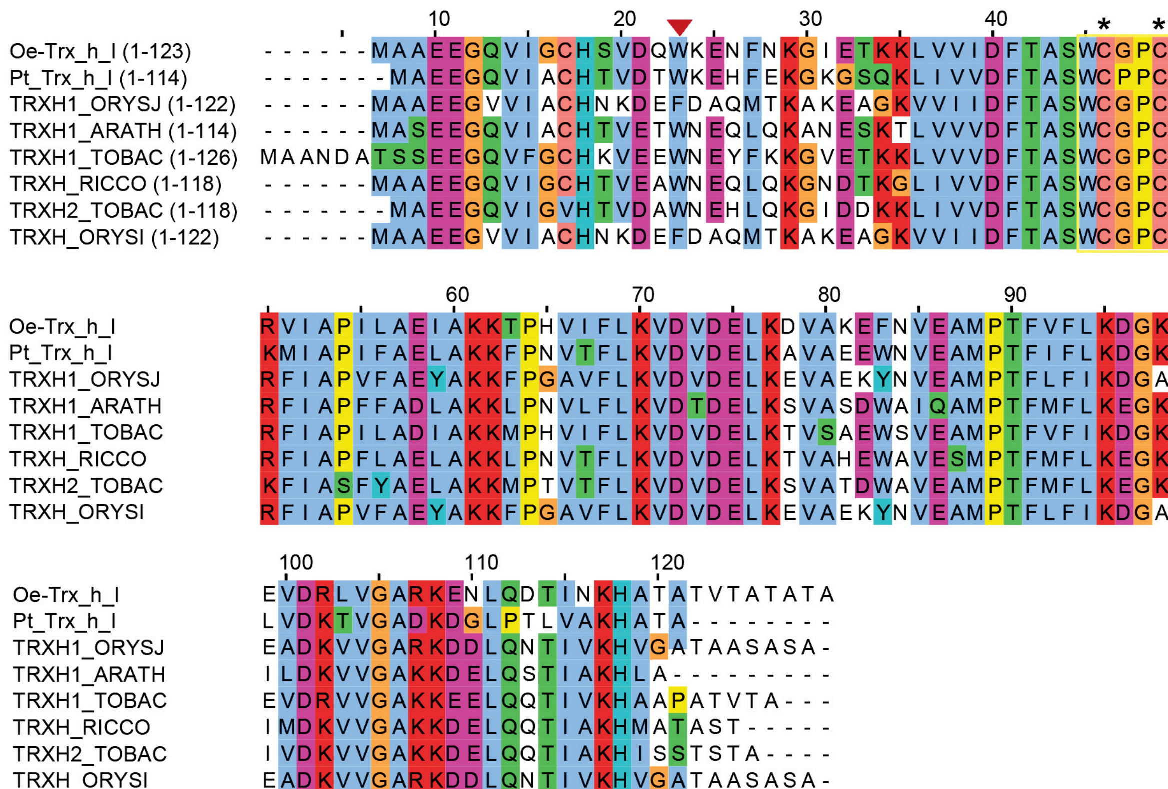

Figure 3. Multiple alignment of the amino acid sequences of representative plant thioredoxin h, chosen among those with a structure-link or manually annotated and reviewed (in Swiss prot). Conserved residues are colored according to the following scheme: hydrophobic in blue; positively charged in red; negatively charged in magenta; polar in green, cysteines in pink, glycines in organge, prolines in yellow, aromatic in cyan and uncorserved/gaps in white. The canoncial amino acid motif of thioredoxins h is framed by a yellow box. The asterisks indicate the catalytic cysteins. A red triangle indicates the conserved N-terminal tryptophan (W) residue. The aligned thioredoxin proteins (plant species, accession) are: Oe-Trx_h_I (*Olea europaea*, this study); Pt_Trx_h_I (*Populus thricocarpa*, Q07090); TRXH_ORYSJ (*Oriza sativa*, A2YIW7); TRXH1_ARATH (*Arabidopsis thaliana*, P29448); TRXH1_TOBAC (*Nicotiana tabacum*, P29449); TRXH_RICCO (*Ricinus communis*, Q43636); TRXH2_TOBAC (*N. tabacum*, Q07090), TRXH_ORYSI (*O. sativa*, Q0D840).

3.2. Expression analysis of the Oe-Trx h I

We studied the expression of the *Oe-Trx h I* in drupes in response to biological (female puncture and larval feeding of *B. oleae*) and physical (mechanical wounding) stress. The relative quantification of gene expression, carried out by real-time PCR, indicated that the *Oe-Trx h I* was significantly overexpressed in olives presenting a feeding tunnel with an active larva, while differences were not observed for punctured olives (Figure 4A). The expression of the *Oe-Trx h I* gene also increased following mechanical damage, with the transcript reaching a significant higher relative expression two days after injury (Figure 4B).

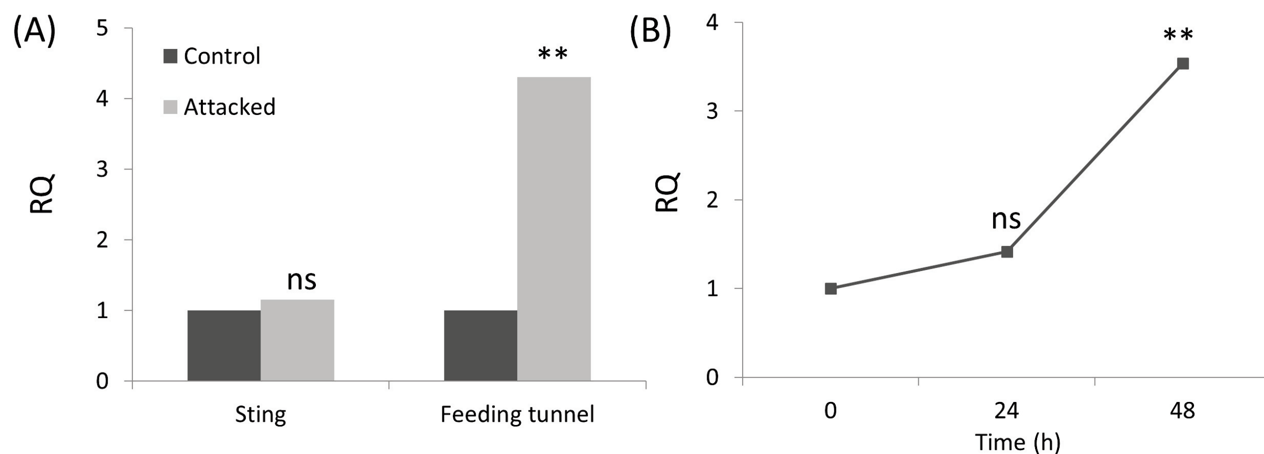

Figure 4. Real-time PCR analysis of the relative expression levels of the Oe-Trx h I gene in relation to biotic and mechanical stress. Asterisks indicate significant difference compared to control ($p<0.01$); ns: not significant. RQ: Relative quantities. (A) Relative expression level of *Oe-Trx h I* in olives with oviposition punctures (sting) or a larval feeding tunnel. For each biotic stress (sting and feeding tunnel), the RQ of the attacked drupes (ligh grey bar) is expressed relatevely to the control/uninfested drupe (dark grey bar). (B) A time-course of *Oe-Trx h I* expression in drupes following mechanical damage, at 0 (undamaged control), 24 and 48 h following treatment.

The data indicated that the *Oe-Trx h I* is inducible in drupes. Moreover, the differential pattern of expression within the same tissue suggests that the gene has a specific function more closely associated to a long-term damage.

4. Discussion

This work presents the nucleotide sequences of a thioredoxin h from *O. europaea* and a first characterization of its response to biotic stress in drupes. This knowledge is essential for screening sequence variants present within the Italian olive germplasm (Lombardo et al., 2019), with the long-term aim of identifying possible functional polymorphisms for molecular breeding and selection (Bettaieb et al., 2020; Corrado et al., 2017). For instance, a trait-marker association analysis, carried out on an Italian germplasm collection, indicated that some structural variants of the *oleate desaturases 2 (FAD2-2)* gene may contribute to the variation of the oleic and linoleic acid content in drupes (Salimonti et al., 2020). Using different primer combinations and Sanger sequencing, it was verified that that the *Oe-Trx h I* gene contains two intronic sequences. Frequently, thioredoxins m have only one intron, while thioredoxins f have two introns (both located within the sequence encoding the mature protein) and encode a transit peptide for the subcellular localization of the protein (Sahrawy et al., 1996).The position and

length of the non-coding sequences of the *Oe-Trx h I* parallel those of the cytosolic thioredoxins h of other higher plants (for instance, the *Glycine max* XP_003527904, the *Ricinus communis* XP_002510456, the *Populus tricocharpa* XP_002310830, the *A. thaliana* NP 1906721, and the *O. sativa* NP 001059069). All these genes present a three exons structure, with the first being the smallest. While the size of the first intron is typically longer than 1000 bp (with the notable exception of Arabidopsis), the second intron is typically very small (around one hundred bps) and it is located in the same position as the second intron of the thioredoxin f (Sahrawy et al., 1996). At the protein level, similarity search and multiple alignment indicated that the putative translation product has a strong amino-acid identity with well characterized plant thioredoxins h. Specifically, Oe-Trx h I possess the distinctive features of the this class, such as the WCGPC signature motif, essential for a correct protein structure and catalytic activity, and the lack of a N-terminal signal peptide.

Considering that *Bactrocera oleae* is the most devastating biotic threat of olive in Mediterranean areas, we studied the response of the *Oe-Trx h I* to the fruit fly in drupes. We also performed a wounding assay because of the impossibility of studying the olive-fruit fly interaction in controlled conditions. The *Oe-Trx h I* gene is activated by biotic stress in drupes. In plants, not all the thioredoxins h are stress inducible, with some being developmentally regulated. For instance, in tobacco, only *NtTRX3* (and not *NtTRX1* and *NtTRX2*) was induced by viruses, reaching the maximum expression 48 hours following inoculation (Sun et al., 2010)**.** The gene expression analysis implied that *Oe-Trx h I* is related to a slower response to stress, compared for instance, to other olive genes involved in direct defense against pests (Corrado et al., 2012). The production of compounds involved in direct defense in plants depends on a network that also includes ROS (Suzuki et al., 2012; Torres 2010). Specifically, the molecular response to the olive fruit fly includes genes and proteins involved in the regulation of the redox status (such as the metallothionein-like proteins, GSTs, catalases and thioredoxins), indicating that ROS production is a relevant element of the inducible olive defense (Corrado et al., 2016).

5. Conclusions

The data indicated that the cloned thioredoxin h is a potential member of the stress signaling cascade in olive fruits following mechanical damage and fruit fly feeding. The bioinformatics analysis and the gene expression study suggest that *Oe-Trx h I* may be a component active towards an increased resistance against oxidative stress, rather than be involved in the direct activation of stress response in drupes. Thioredoxin h genes show different expression patterns in different organs and in response to various stress (Reichheld et al., 2002; Cazalis et al., 2006) and further studies will have to characterize the features of the gene presented in this work, as well as its biochemical role in the oxidative stress response. In particular, it will be interesting to verify whether this protein can counteract ROS accumulation and lipid peroxidation following larval feeding (Malheiro et al., 2015), a question with strong implications for the quality of the olive oil (Rallo et al., 2018).

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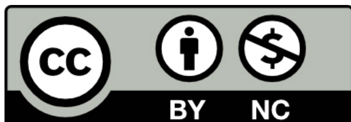

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