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AOX Special Issue

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Physiological responses and gene diversity indicate olive alternative oxidase (AOX) as a potential source for markers of efficient adventitious root induction

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ABSTRACT

Olive (Olea europae L.) trees are mainly propagated by adventitious rooting of semi-hardwood cuttings. However, efficient commercial propagation of valuable olive tree cultivars or landraces by semi-hardwood cuttings require better rooting capacity. We hypothesize that root induction is a plant cell reaction linked to oxidative stress and that activity of stress-induced alternative oxidase is importantly involved in adventitious rooting. To identify AOX as a source for potential functional marker candidate sequences that may assist tree breeding, genetic variability has to be demonstrated that can affect gene regulation.

The paper presents an applied research approach demonstrating first multi-disciplinary indications of an important relationship between AOX activity and differential adventitious rooting in semi-hardwood cuttings. Root induction in the easy-to-root Portuguese cultivar ‘Cobrançosa’ could be significantly reduced by treatment with SHAM an inhibitor of AOX activity. On the contrary, treatment with H₂O₂ or pyruvate, both known to induce AOX activity, increased the degree of rooting.
Recently, identification of several OeAOX gene sequences from olive (O. europaea) has been reported from our group. Here we present for the first time partial sequences of OeAOX2. To search for polymorphisms inside of OeAOX genes, partial OeAOX2 sequences from the cultivars ‘Galega vulgar’, ‘Cobrançosa’ and ‘Picual’, were cloned, including exon, intron and 3’-UTRs sequences. Data revealed polymorphic sites in several regions of OeAox2. The 3’-UTR was the most important polymorphic source showing 5.7% of variability. The identified polymorphisms can be explored in future studies for linkage to olive rooting phenotypes in view of marker-assisted plant selection.

ABBREVIATIONS

AOX- alternative oxidase; AS- alternative splicing; COX- cytochrome oxidase; IBA- Indol-3-butyric acid; InDel- Insertion/ deletion; KNC- potassium cyanide; NMD- nonsense-mediated RNA decay; ORF- open reading frame; ROS- reactive oxygen species; SHAM- salicyl-hydroxamic acid; SNP- single nucleotide polymorphism
INTRODUCTION

Olive propagation through efficient rooting of semi-hardwood shoot cuttings is of commercial interest. However, application of this methodology is limited to easy-to-root genotypes. Several valuable olive cultivars and breeding lines from diverse countries of the Mediterranean climate regions suffer from low rooting efficiencies at a rate around 20% or below. However, high variability in rooting phenotypes observed among olive cultivars and landraces point to the possibility for tree improvement in available olive material. Rooting efficiency further strongly depend on the physiological state of the tree or tree part, the environment and seasonal and annual changes. Root induction at shoot cuttings originates from reprogramming of basal shoot cells. Shoot cutting and subsequent treatments with auxins means a stress to the involved cells. Directed growth responses on stress are discussed as being a plant acclimation strategy to diminish stress exposure (Potter et al. 2007). Treatment with exogenous auxin increases the number of meristematic root primordia. Meristem maintenance and development is known to be regulated by a balance of auxin and cytokinin (Loio et al. 2008). Both growth regulators are able to interact with reactive oxygen species, such as \( \text{H}_2\text{O}_2 \). \( \text{H}_2\text{O}_2 \) seems to play a crucial role in environmental stress perception and signaling related to the central role of mitochondria in homeostasis and cell fate determination under stress (Sung et al. 2005, Amirsadeghi et al. 2006). In guard cells auxin seems to limit \( \text{H}_2\text{O}_2 \) levels through preventing its generation, but unlike cytokinin did not reduce exogenous hydrogen peroxide levels (Song Xi-Gui et al. 2006). Several reports are now available that point to a critical role of ROS signaling in root induction and development (Nag et al. 2001, Dunand et al. 2007). Seed germination of rice seedlings could be promoted by applying exogenous \( \text{H}_2\text{O}_2 \) (Sasaki et al. 2005). Treatment of olive shoot cuttings with \( \text{H}_2\text{O}_2 \) could increase early-rooting and the number of roots across genotypes and environments. However, clear effects of olive cultivars and years could be observed during treatment. Quantitative and qualitative differences in rooting between genotypes and environmental conditions during mother tree growth could not be equalized by \( \text{H}_2\text{O}_2 \) application. No interaction of years or cultivars with the treatment was obtained (Rugini et al. 1997, Sebastiani and Tognetti 2004). The strong influence of genetic factors on olive rooting efficiencies encourages searching for appropriate molecular markers for tree breeding.
Genes of interest for functional marker development can be identified by high-throughput differential gene analyses or in hypothesis-driven research approaches (Arnholdt-Schmitt 2005). Gene candidate approaches for marker-assisted selection are actually rated as the most promising strategies in molecular plant breeding (Collins et al. 2008). To verify a gene as functional marker candidate three prerequisites must be fulfilled. The gene must be (1) importantly involved in the final trait, (2) the gene must show polymorphic sequences to mark genotypic differences and, finally, (3) the polymorphic marker must be stably linked to the target phenotype. The present paper deals with the first two aspects. The importance of a gene can be assessed by manipulating gene activities and physiological responses related to the target trait, through functional genomics, including transgenic strategies, linkage mapping and/or association studies. Once a gene is identified as candidate by a hypothesis-driven or an analytical approach, it is not necessarily needed to understand causal biochemical or molecular details of the gene function and complex regulation to develop a gene sequence as marker. Identification of a close relationship between the gene and/or the polymorphic sequence and the target trait is sufficient.

The present paper focuses on the development of alternative oxidase (AOX) as a functional marker for adventitious rooting of olive shoot cuttings (Arnholdt-Schmitt et al. 2006a, Arnholdt-Schmitt et al. 2006b, Santos Macedo et al. 2006a,b). AOX is nuclear encoded, but active in the alternative respiration pathway in mitochondria. It is positioned up-stream to important energy and carbon turnover regulation. AOX to COX rate is an important measure for carbon efficiency rates related to environmental factors, growth and development (Hansen et al. 2009 in this issue). AOX is known to be involved in plant reactions upon all types of abiotic and biotic stress. ROS seems to play a critical role for this link (Amirsadeghi et al. 2006). Several articles report about a complex interaction between AOX activity and H$_2$O$_2$ (e.g. Vanlerberghe and McIntosh 1996, Popov et al. 1997, Gray et al. 2004, Umbach et al. 2005, Amirsadeghi et al. 2006). Consequently, inhibitors of AOX activity, such as SHAM were found to interact with H$_2$O$_2$ cell levels. In SHAM-treated Arabidopsis thaliana roots, peroxidase was inhibited and root length was strongly reduced. H$_2$O$_2$ disappeared from the root hair zone, but was accumulated in cell walls in the meristemic region (Dunand et al. 2007). H$_2$O$_2$ treatment of rice seedlings promoted germination and seedling growth and AOX1a was up-regulated by this treatment at an early stage (Sasaki et al. 2005). An up-regulation of AOX transcription upon H$_2$O$_2$-treatment was observed by Polidoros et al.
(2005) in maize and maintenance of high levels of AOX activity was suggested to reinforce establishment also of maize seedling germination and growth under stressful environmental conditions (Camacho et al. 2004). Giraud et al. (2008) characterized an aox1a mutant of *A. thaliana* with reduced growth rates in early stages of seedling growth. Whereas *AOX1* subfamily genes are known to be related to diverse types of stress reactions, *AOX2* member genes are thought to have a closer relation to tissue specificity and development. However, this correlation is not fixed, since a relationship to stress-induction was also indicated for *AOX2* in *A. thaliana* and *Vigna unguiculata* (see the review Polidoros et al. 2009 in this issue). Alternative respiration can be promoted through blocking the cyanine-sensitive respiration pathway via cytochrome oxidase. KCN treatments of dormant grapevine cuttings induced an increase of bud break and rooting (Mizutani et al. 1994), which can point to the importance of AOX. AOX expression can be up-regulated in plant metabolism by organic acids, such as pyruvate (Juszczuk and Rychter 2003, Oliver et al. 2008). Therefore, it was used in our study besides H$_2$O$_2$ to test its effect as an external stimulator for olive root induction.

The importance of *AOX* genes in affecting physiological responses under determined conditions must be verified at species level since orthologous genes can have different functions in different species. Furthermore, the importance of a gene can be influenced by the genetic background of a defined genotype. Thus, a functional marker for rooting efficiency from *AOX* genes need to be developed at species level and with preference in genetic material that contains already the overall characteristics of a commercially important variety for breeding. A later introgression of additional genetic characteristics for plant or tree design will be possible under testing the stability of the identified functional marker. The present paper reports about AOX inhibitor and stimulator studies with the easy-to-root cultivar ‘Cobrançosa’ and screening of a partial sequence of *OeAOX2* from cultivars ‘Cobrançosa’ and ‘Picual’, and the non-easy-to root cultivar or landrace ‘Galega vulgar’. Here we publish for the first time initial experimental results that strengthen the hypothesis that alternative oxidase (AOX) gene sequences can serve as a source for functional markers for efficient adventitious rooting of olive shoot cuttings.
MATERIAL AND METHODS

I - Rooting assays: treatment of shoot cuttings with IBA, SHAM, H₂O₂ and pyruvate

1. Plant material
Olive semi-hardwood shoot cuttings were obtained from trees of the easy-to-root Portuguese olive cultivar ‘Cobrançosa’. The ‘Cobrançosa’ orchard has a polyclonal origin being property of the Viveiros Plansel Lda., a nursery company located in Montemor-o-Novo, Alentejo, in the southern region of Portugal. The orchard was established on a range of 1 m row and 3 m between rows, being the plants trained as shrubs. Annually the orchard is submitted to a vigorous pruning with the objective to maximize the production of vegetative material for tree propagation. Shoots cuttings of about 14 cm length were collected from the medium portion of one year old branches in a 10-years old mother plant field.

2. Root assay conditions
The rooting trials were conducted in benches in a greenhouse cooled by ‘aqua cooling’ and an automatic shading system. The benches were equipped with an intermittent sprinkling system and basal heating. Greenhouse temperature was maintained at 24°C and the substrate temperature at 27 to 28 ºC. Shoots were cut to about 14 cm in length and only the top four leaves were maintained. After the treatments, the shoot cuttings were placed into the benches with ‘perlite’ as rooting substrate.

3. Shoot cutting treatments
Shoot cuttings were treated with IBA (Sigma, Canada), which is used in commercial olive propagation as a root promoting auxin, the inhibitor of AOX activity SHAM (Aldrich, Germany) and two stimulators of AOX activity, H₂O₂ (J.T. Baker, Holland) and pyruvate (Fluka, Switzerland). All compounds were applied as water solutions through immersion of the cutting bases (4 to 6 cm). Due to their insolubility in water, IBA was previously soluted in a few drops of NaOH 1N and SHAM in 95 % ethanol.
4. Experimental design and data analysis
The experimental conditions applied to SHAM trials correspond to a fully randomized scheme within a three-factorial design: 3 experimental periods x 4 treatments x 3 replications. Independent experiments were performed during March/April in 2007, October/November in 2007 and during May/June in 2008. Treatment concentrations and contact times are presented in Table 1. Each replication with 20 semi-hardwood cuttings was used as an experimental unit for statistical data analysis. The trials with hydrogen peroxide and pyruvate were also conducted applying a completely randomized scheme within a simple factorial assay with 6 treatments and 3 replications (Table 1). For both trials, each replication with 10 semi-hardwood cuttings was used as experimental unit for statistical data analysis. In all trials rooting rates were evaluated after 60 days and the collected data submitted to variance analysis using Statistica V.7.0 as software and the results are presented as 95 % confidence interval charts.

II - Molecular studies - OeAOX2 expression in root-inducible tissues of shoot cuttings

1. Plant Material
Shoot cuttings randomly collected from ‘Cobrançosa’ trees were used for OeAOX2 gene expression studies. The samples consist of olive tissue rings, each with about 1cm high, taken from the basal portion of ten shoot cuttings as a bulked sample. The samples were collected in the field (T0) and 12 h and 14 days after IBA treatment. 10 days after IBA application, root primordial induction can typically be observed by histology (Peixe et al. 2007a).

2. RNA extraction and cDNA synthesis
Total RNA was extracted from the bulked samples of 10 shoot cuttings and purified using the RNeasy plant mini Kit (Qiagen, Germany). Single strand cDNA was synthesized by help of the enzyme RevertAid™ HMinus M-MuLV Reverse Transcriptase (Fermentas, Canada) using the oligo(dT) primer 5’-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTTV (V=A,C or G)-3’ (Roche, Germany). The primer (2 µM) was incubated with 5 µg of total RNA for 5 min.
at 70 ºC. After this procedure the mixture was placed on ice for 5 min, and then added
the following reagents: 1x enzyme buffer RevertAid\textsuperscript{Tm} HMinus M-MuLV Reverse
Transcriptase (Fermentas, Canada), 1 mg/ml of BSA (Ambion, USA), 1 mM each
dNTP (Fermentas, Canada), 0,05 µg/µl of Actinomycin D (Roche), 2 mM of DTT
(Fermentas, Canada), 0,8 U/µl of RNase inhibitor (Fermentas, Canada), and 6 U/µl of
the enzyme RevertAid\textsuperscript{Tm} HMinus M-MuLV Reverse Transcriptase (Fermentas, Canada).
This reaction mixture was incubated for 90 min at 42 ºC.

3. Gene expression
RT-PCR was performed in final volumes of 25 µl with pure Taq Ready-to-go PCR
Beads (GE Healthcare, England) using 1 µl of 1:10 cDNA dilution and 0.2 µM of each
primer OeAOX2_1111Fw: 5’-CCACTAGTTGTTGGTGGTGTCGTG-3’ and
OeAOX2_1246Rev: 5’-AGTAAGTGGCATGTTTCTGTAG-3’ (both from Eurofins
MWG Operon, Germany). PCR was carried out for 35 cycles in the 2720 Termalcycler
(Applied Biosystems, Singapore). Each cycle consisted in 30 sec at 94 ºC for
denaturation, 30 sec at 55 ºC for primers annealing and 30 sec at 72 ºC for DNA
synthesis. An initial step at 94 ºC for 5 min and a final step at 72 ºC for 10 min were
included.
PCR products were analyzed by electrophoresis in 1.4 % (w/v) agarose after staining in
a ethidium bromide (EtBr) solution (2 ng/ml) using the Gene Flash Bio Imaging system
(Syngene, UK).

III - Gene diversity and 3’-UTR sequence and length variability

1. Plant material
Genomic DNA from young leaves of individual olive trees from three cultivars were
used for analysis of OeAOX2 gene diversity: ‘Galega vulgar’ (clone 1053),
‘Cobrançosa’ and ‘Picual’. The trees were grown in fields in Montemor-o-Novo
(‘Galega vulgar’ and ‘Cobrançosa’) and Elvas (‘Picual’), Alentejo, Portugal.
For studies on 3’-UTR leaves of five micro shoots of ‘Galega vulgar’ clone 1053 were
macerated together and used for RNA extraction. The establishment of micro shoots
cultures were performed as previously described by Peixe et al. (2007b).
2. DNA extraction
Genomic DNA (gDNA) was extracted using the DNEasy kit (Qiagen, Germany) according to the manufacture protocol. DNA integrity was assessed by electrophoresis in 1% agarose and DNA quantification was performed in agarose gels using defined amounts of lambda DNA as a standard. Nucleic acids were visualized through EtBr staining as described before.

3. RNA extraction and cDNA synthesis
All the procedure was made as described under II-2.

4. AOX2-specific fragment isolation from three cultivars
AOX2-specific primers were designed in the region of exon 3 (OAOX2_33Fw: 5’-ATTGCATCTCAGGTCTCTTCGC-3’) and 3’-UTR (OAOX2_647Rev: 5’-CAGGCATAAGTAAAGTGCGAC-3’) (both from Eurofins MWG Operon, Germany) (see Fig. 1). For the PCR mix 0.02 U of a Phusion™ High-Fidelity DNA Polymerase (Finnzymes Oy, Finland) were used with 1x manufacturer supplied Phusion HF buffer containing 1.5 mM MgCl₂, 0.2 mM of each dNTPs (Fermentas, Canada) and 0.2 μM of each primer. The PCR was carried out with 0.2 ng/µl of gDNA as template running an initial step of 30 sec at 98 ºC followed by 35 cycles, each consisting of 10 sec at 98 ºC, 15 sec at 58 ºC and 60 sec at 72 ºC.

PCR fragments were analysed in 1.4% agarose gels and visualized as described. Selected PCR fragments were purified using the GFX PCR DNA and Gel Purification kit (GE Healthcare, UK). The addition of adenines to the 3’end of amplicons required for the cloning procedure was made by adding to a mix 0.1 U/µl of Taq polymerase (Promega, USA), 1x manufacturer supplied (NH₄)₂SO₄ buffer, 2.5 mM MgCl₂ and 0.2 mM dATP (Fermentas, Canada). The final mix was incubated during 30 min at 72 ºC in a 2720 Termalcycler (Applied Biosystems, Singapore). The amplicons were cloned into a pGEM®-T Easy System I vector (Promega, USA) and subsequently used for the transformation of E. coli competent cells JM109 (Promega, USA). Plasmid DNA of selected white clones was extracted using the alkaline lyses protocol (Sambrook et al.1989) and was characterized by the restriction enzyme EcoRI. Recombinant clones were used for commercial sequencing.
5. 3’-UTR characterization in cDNAs of ‘Galega vulgar’

3’-UTR sequence and length variability was analyzed in transcripts of micro shoots (clone 1053). Amplicons were produced by the forward primer OAOX2_33Fw combined with the reverse primer Vial9: 5’-GACCACCGTGATCGATGTCGAC-3’ (Roche, Germany).

The PCR reaction was performed with pure Taq Ready-to-go PCR Beads (GE Healthcare, England). Each reaction contained 10 ng sample DNA and ran at a concentration of 0.2 µl of each primer.

The PCR was carried out with an initial step of 5 min at 94 ºC followed by 35 cycles, each consisting in 30 sec at 94 ºC, 90 sec at 68 ºC and 10 min at 72 ºC. PCR fragments were analysed in 1.4 % agarose gels, purified, cloned and selected as described above. Nine recombinants bacterial clones from ‘Galega vulgar’ were used to study 3’-UTR variability.

6. Sequence analysis and sequence alignment

Selected clones were sequenced by commercial services through the company MACROGEN using the specific primers T7 and SP6 for the cloning vector (Promega). Sequence homologies in the NCBI data base (National Center for Biotechnology Information, Bethesda, MD) were studied by using the Blast algorithm (Altschul et al 1990) (http://www.ncbi.nlm.nih.gov) (BLASTX and BLASTN).

Genomic DNA and cDNA sequences were manipulated and compared using the programs EditSeq and Bioedit (Hall 1999). For sequence alignment the Clustal W algorithm of Megalign (Lasergene, GATC Biotech, Konstanz) was applied.

7. Computational prediction and validation of miRNA precursors and mature miRNAs in intron sequences

Putative miRNA precursors were searched in eight intron sequences of the OeAOX2 gene from ‘Galega vulgar’, ‘Cobrançosa’ and ‘Picual’ by using the software miR-abela, which is publicly available at (http://www.mirz.unibas.ch/cgi/pred_miRNA_genes.cgi).

For validation of potential pre-miRNAs the software MiPred was applied, which is publicly available at (http://www.bioinf.seu.edu.cn/miRNA/) (Jiang et al. 2007). Prediction of the secondary structure of pre-miRNA was run on the web-based software MFOLD 3.1, which is available at http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi) (Mathews et
al. 1999, Zuker 2003). The criteria applied for screening the candidates of potential pre-miRNA were described by Xie et al. (2007). To screen the candidates of potential miRNAs the validated pre-miRNAs were run with the software miRBase::Sequences, which is publicly available at (http://microrna.sanger.ac.uk/sequences/search.shtml).

8. Computational prediction of target miRNA binding sites

To predict the existence of putative sites at the 3’-UTR for miRNA annealing the software miRanda v3.0 was applied by using the largest sequence from all samples (Gal_3).

RESULTS

AOX inhibitor and activator effects on olive rooting

SHA M treatment of shoot cuttings. Figure 2 shows the rooting rates of shoot cuttings treated with SHAM for three independent experiments with ‘Cobrançosa’. The data sets show clearly the same tendency across all three experiments. The positive control, which corresponds to the treatment with IBA, gave in all cases highest rooting percentages, around 60 % in average. SHAM treatment either alone or in combination with IBA significantly reduced the level of root induction to the values observed for the negative control. Rooting rates in the presence of SHAM reached maximal 28 %. A slight but non-significant higher percentage of rooting was observed in two of the three experiments when SHAM was applied together with IBA.

H\textsubscript{2}O\textsubscript{2} treatment of shoot cuttings. The effect of H\textsubscript{2}O\textsubscript{2} was time-dependent (Fig. 3). The 14 hs treatment demonstrated a stimulating effect on rooting rates which achieved the same rooting rates as the IBA variant and, thus, being appropriate to substitute IBA treatments actually a common practice in commercial olive propagation. A trend for increased rooting rates was recognizable from 3 hs onwards, but it reached a degree of statistical significance only at 14 hs.
Pyruvate treatment of shoot cuttings. Pyruvate was applied at two different concentrations and contact times. A short pulse of 20 sec (Fig. 4, treatment codes 11 and 12) was not sufficient to have any effect on rooting. This was observed independently from a concentration of 0.1 or 0.01 mM. However, when the time was prolonged to 3 hs a stimulating effect was obtained that raised root rates to the level of the auxin-treated variant (Fig. 4, treatment code 10 in relation to 12). A similar stimulating effect through a longer contact time could be achieved by a 10 times lesser concentration (Fig. 4, treatment codes 9 and 11).

OeAox2 expression in shoot cuttings

Figure 1 shows the scheme of the partial OeAOX2. The genomic sequence consists of a partial ORF of 475 bp, a complete intron of 499 bp and a partial 3’-UTR of 159 bp starting at the stop codon. The exon contains three of the four regions known as more conserved regions of the alternative oxidase (NERMHL, LEEEA and RADE__H region, Berthold et al. 2000), which include 5 di-iron binding sites (Fig. 5). Three helical regions that are assumed to be involved in the formation of a hydroxo-bridged binuclear iron center and a possible membrane-binding domain are included in that region (Siedow et al. 1995, Andersson and Nordlund 1999, Berthold et al. 2000).

Figure 6 demonstrates the expression pattern of OeAOX2 during the root induction treatment in a bulked sample of shoot cuttings. Shortly after shoot cutting (10 min) still before the treatment with IBA, a high transcript level was observed that was later clearly reduced, as can be seen 12 h after IBA treatment. At the time, when histological evidence for root induction can typically be found (Peixe et al. 2007a), an increase in OeAOX2 transcript level was obtained.

Gene diversity studies

The partial sequence of OeAOX2 was isolated from three cultivars (‘Galega vulgar’, ‘Cobrançosa’ and ‘Picual’) and DNA polymorphisms were identified. Table 2 presents the DNA polymorphisms that were identified among all available sequences.
Open reading frame (ORF)

From 10 ORF sequences identified from ‘Galega vulgar’, 6 presented variations between each other (Table 2). 12 SNPs were detected among the sequences of this cultivar in the conserved region of exon 3 and one SNP in the exon 4 region. From these 13 SNPs, 7 were responsible for non-synonymous translations: 23C/A→K/H; 114A/G→Q/R; 122T/C→W/R; 134G/C→V/L; 141T/C→V/A; 399A/G→D/G; 429T/C→F/S (Fig. 5). In sequence 4 a substitution of Phe (F) by Ser (S) due to the SNP at position 429 occurred within one of the structural elements in the fourth helical region proposed to influence AOX regulation (Siedow et al. 1995, Andersson and Nordlund 1999, Crichton et al. 2005). In sequence 5 the Asp (D) / Gly (G) substitution due to a SNP in position 399 occurred two positions upstream the di-iron binding site of RADE_-H box, which is included in the fourth helical region, previously assumed to be involved in the formation of a hydroxo-bridged binuclear iron center (Siedow et al. 1995, Andersson and Nordlund 1999). A multiple alignment made with the peptide sequences of plant AOXs available at the NCBI data basis showed at this position an Asp in all sequences (data not showed). In sequence 8 a Gln (Q) / Arg (R) substitution by a SNP in position 114 was identified eight positions upstream of the di-iron binding NERMHL box, included in the second helical region (Siedow et al. 1995, Andersson and Nordlund 1999).

Variation at the possible membrane binding domain were observed in the sequences 2 and 8 due to a Val (A) / Leu (L) (SNP at the position 134) and a Val (V) / Ala (A) (SNP at the position 141) substitutions, respectively.

From 7 gDNA sequences identified as OeAOX2 from ‘Cobrançosa’, 4 presented variations (Table 2). Among these sequences 6 SNPs were identified. All SNPs are repetitive, since they were already observed in ‘Galega vulgar’. Additionally, an insertion / deletion (InDel) of two nucleotides (nts) were identified at positions 248 and 249. From these 8 sources of variation, 6 were responsible for synonymous translations and 2 SNPs were responsible for non-synonymous translations (114A/G→Q/R; 134G/C→V/L). In sequence 3 a Gln (Q) / Arg (R) substitution due by the SNP at position 114 (A/G) was observed at the second helical region (Siedow et al. 1995, Andersson and Nordlund 1999). Sequence 3 presented a mutation due to Val (A) / Leu (L) substitution (SNP at the position 134) which is included in the possible membrane binding domain (Andersson and Nordlund 1999, Berthold et al. 2000). The InDel
converts the codon ACT to a premature stop codon (TGA) at nucleotide 255 of the coding region, named *nonsense* mutation. This mutation was detected in 5 of the 7 sequences from this cultivar. Two of them were identical to sequence 2 presented in Table 2. The stop codon is located 160 bp upstream of the 3’-most exon-exon junction and five positions upstream the di-iron binding site of the LEEEA box (Fig. 5). Only 2 partial *OeAOX2* sequences from ‘Cobrançosa’ can be translated to a peptide (only sequence 3 is presented in Table 2) (see Cob_3 in Fig. 5).

Four gDNA sequences were cloned from ‘Picual’ and 3 of them varied between each other (Table 2). However, the ORF sequences are identical, and all presented the same *nonsense* mutation that was described before in ‘Cobrançosa’ (Fig. 5). All SNPs identified in ‘Picual’ were repetitive, i.e. the same variation have been identified already in at least one of the other two cultivars (Table 2).

In summary, the ORF of *OeAOX2* displays a rich source of polymorphic sites of which most are found to be repetitive in allelic sequences and between the cultivars. However, ‘Galega vulgar’ is the only one, which showed unique SNPs that could only be discovered in this cultivar (positions 23, 25, 122, 141, 196, 370, 399 and 429). No *nonsense* mutation was observed in the 10 sequences of this cultivar as described for both other cultivars. However, it must be considered that 9 of the sequences were deduced from actively transcribed sequences (cDNA) (Table 4).

**Intron**

All intron sequences from the three cultivars show the typical nuclear DNA exon-intron junctions 5’-GT/AG-3’ (Saisho et al. 1997) (Table 2). The intron region is more U-rich than the flanking exons (41% vs. 28% U on average). Similar results were previously reported in *A. thaliana* showing 41% U vs. 26% U on average for introns (Goodall and Filipowicz 1989, Ko et al. 1998, Deutsch and Long, 1999). It was suggested that U-richness can be important for intron recognition (Ko et al. 1998).

The intron of ‘Cobrançosa’ presented 7 SNPs and 1 InDel among the four sequences (Table 2). In the intron of ‘Picual’, 5 SNPs and 1 InDel have been identified. All SNPs identified in the intron are repetitive between cultivars, which means they have not been single events that occurred only in one cultivar, but could be observed in at least two cultivars. In all variable positions no more than two different nucleotides can be found. This was also observed in the exon.
In all introns from the three cultivars a putative miRNA precursor could be predicted (Table 3). The predicted region contains a substitution of G by T in 2 sequences of ‘Cobrançosa’. After computational validation using the software MiPred, only these sequences 1 and 3 presented a potential miRNA precursor with a higher probability of 65.9% (Table 3).

Micro RNA precursors possess a characteristic secondary structure, with a terminal loop and a long stem (Bartel 2004) by which the miRNA is positioned (Reinhart et al. 2002). The secondary structure of the predicted pre-miRNA is shown at the figure 7.

The sequence between 13 and 32 nt (5’-GAAAAUAGGAAUUUGUUGA-3’) was identified with homology to a miRNA of *A. thaliana* ath-miR417 (78-GAAGGUAGGAAUUUGUUCGA-98) with a score of 91.7. The ath-miR417 had been validated by Northern blot hybridization (Wang et al. 2004). Additionally, the sequence between 13 and 29 nt was extracted with homology to a miRNA of *O. sativa* osa-miR417 (44-GAAUGUAGGAAUUUGUUC-64) with a score of 82.5 (see Fig. 8).

**3’ Untranslated Region (3’-UTR)**

Among the 3’-UTRs of ‘Galega vulgar’ sequences 5 SNPs and two single nt InDels were identified (Table 2). In the cultivar ‘Cobrançosa’ this region demonstrated 5 SNPs and two single nt InDel. In ‘Picual’ the 3’-UTRs of sequence 1 and 3 are identical, however, between these and sequence 2 4 SNPs and a single nt InDel were detected. Whereas most SNPs were repetitive between the cultivars, ‘Galega vulgar’ showed a unique SNP in position 996 and a deletion in position 1063. Cultivar ‘Cobrançosa’ demonstrated a unique deletion in position 1058.

Comparing the three regions within the partial genomic gene sequence as sources of polymorphisms among all studied sequences of the three cultivars, the ORFs presented 16 sites of variation which means a variability of 3.4%, the introns 10 sites equal to 2% and the 3’UTRs 9 sites which means 5.7% of variability. Calculations were based on the known sequence of each region (475 bp of ORF, 499 bp of intron and 159 bp of 3’-UTR).
3’-UTR sequence and length variability in transcripts of *OeAOX2*

Transcription of the *OeAOX2* resulted in transcripts with variable length due to alternative polyadenylation (AP). AP affects a large number of higher eukaryote mRNAs, producing mature transcripts with 3’-ends of variable length. Table 4 shows the length of the 3’-untranslated region and the number of clones detected for each 3’-UTR size in ‘Galega vulgar’.

Alternative polyadenylation is an important mechanism in generating a diversity of mature transcripts. Conventional genetic mutagenesis studies revealed that plant poly(A) signals are composed of three major groups: far upstream elements (FUE), near upstream elements (NUE; an AAUAAA like element characteristic in animals), and cleavage elements (CE) (Loke et al. 2005). The composition of plant consensus signals, such as CEs, which is an expansion of the CS (cleavage site), including the YA dinucleotide (CA or UA) in the CS and two U-rich regions, one before and another after the CS, both spanning about 5 to 10 nt (Loke et al. 2005). NUE is an A-rich region and spans about 6 to 10 nt located between 13 and 30 nt upstream of the CS (referred to as locations -13 to -30; Hunt 1994, Li and Hunt 1995). FUE, the control or enhancing element, is a combination of rather ambiguous UG motifs and/or the sequence UUGUAA (Hunt 1994) and spans a region of 25 to 125 nt upstream of the NUE (Loke et al. 2005). The alternative sites for the poly(A) signal are indicated in figure 9.

In search for these nuclear mRNA poly(A) signals different regions of FUE and NUE elements were detected (Fig. 9). All identified FUE regions in olive *OeAOX2* include different patterns found previously in *A. thaliana* (Loke et al. 2005) as the top 50 FUE patterns: the first region in *OeAOX2* named FUE.1 (Fig. 9) include the pattern UUGUUG found as 25th most frequent pattern in *A. thaliana*; the FUE.2 GUUUUGUU include the patterns UUUGUU (1st), UUUUGU (2nd) and GUUUUG (22th); the FUE.3 region GUUGUGU are related to the 21th and 23th patterns identified in *A. thaliana* (UGUUGU and UUGUGU, respectively); the FUE.4 region, UAUUUUGUAAGUUUGUUCUUU, is the largest region and belong to a group of seven patterns previously identified in *A. thaliana*: 2nd, 7th (UUUUGU), 9th (UUCUUU), 14th (UUUGUA), 16th (UUUUUU), 18th (UAUUUU) and 47th UUGUAA. The pattern 9th in *A. thaliana* had been identified as the 6th most frequent in *O. sativa* (Shen et al. 2008). This last region include also the pattern UUGUAA referred by Hunt (1994) as typical for the FUE element.
NUE elements are A-rich and three were identified in \textit{OeAOX2}. NUE.1 is common to the second NUE pattern (UUAAUU) identified in \textit{O. sativa} (Shen et al. 2008); NUE.2 corresponds to the pattern AAUAAA, highly conserved in vertebrate cells, and NUE.3 refers to AAAUAU. Gal_2 demonstrates a deletion in NUE.2.

The four FUE regions identified are complete and can be suggested to be functional for the NUEs downstream of each one. Gal_1 contains the shortest 3’-UTRs and ends at the beginning of NUE.2 in the other sequences. No CS element was discovered. For all other sequences, Gal_2 to Gal_7, a CS element could be identified, which included the di-nucleotide YA corresponding to TA or CA. The di-nucleotide is in different positions in the 3’-UTRs: for Gal_6 in position 113, Gal_5 in 151, in Gal_7 in 259, Gal_2 in 297, Gal_3 in 298, and for Gal_4 in position 301. In all sequences a T-rich region was observed upstream the CS element that is characteristic for the cleavage element.

Table 5 shows predicted target sites for miRNAs in the 3’-UTR of Gal_3. Many miRNA families are evolutionary conserved across all major lineages of plants, including mosses, gymnosperms, monocots and dicots (Axtell and Bartel 2005). This conservation makes it possible to identify homologous miRNAs known from other species (Floyd and Bowman 2004, Axtell and Bartel 2005). A previous study has shown that most known plant miRNAs bind to their mRNA targets with perfect or nearly perfect sequence complementarity and degrade the target mRNA. The plant miRNAs targets sites are mostly located in the protein-coding sequence acting in a way similar to RNA interference (Wang et al. 2004), but can be found also in the 3’-untranslated region (Rhoades et al. 2002). Five putative target binding sites for miRNAs could be predicted within regions of the 3’-UTR in Gal_3 sequence (Table 5). The only information available about this binding sites is related to the ppt-miR1212 localized in the 3’-UTR of \textit{A.thaliana} B-box zinc finger coding-protein sequence (Talmor-Neiman et al. 2006).

**DISCUSSION**

The presented research focuses on application. It is the aim of these studies to validate 1) the involvement of \textit{AOX} genes in root induction of olive shoot cuttings and 2) to reveal whether \textit{AOX} genes can be a source of polymorphic sequences that may be
developed in a later step as functional markers for the selection of individual trees with a more efficient root induction. Thus, it is not the primary goal of this research to contribute to fundamental knowledge in AOX research, i.e. to understand why and how AOX is involved in the root induction process.

We applied the inhibitor of AOX activity, SHAM, and two compounds, hydrogen peroxide and pyruvate that are known to stimulate AOX activity (Feng et al. 2008, Oliver et al. 2008). SHAM is known to inhibit the alternative pathway and it was not the aim of this study to quantify electron partitioning between both respiration-pathways (Lambers et al. 2005). The results confirmed our expectation. SHAM strongly reduced root induction in a reproducible manner and both stimulators tended to increase rooting. Additionally, the results seem to confirm that root induction is an example of a stress-related cell reprogramming event as hypothesized (Arnholdt-Schmitt et al. 2006a and b). OeAOX2 was highly expressed 10 min after shoot cutting before the IBA treatment, decrease rapidly and was then increased at the time, when root primordial starts to be visible at histological level (Peixe et al. 2007a). Further, application of hydrogen peroxide as an important component of oxidative stress signaling succeeded to increase rooting. The current state of knowledge on an interaction of ROS and AOX suggests that AOX plays an important role in preventing ROS production, but also in the perception and coordination of oxidative stress signaling that influence mitochondrial dysfunction, the mitochondrial retrograde signaling pathways and cell recovery or cell death strategies (Fiorani et al. 2005, Umbach et al. 2005, Amirsadeghi et al. 2006, Clifton et al. 2006, Giraud et al. 2008, Van Aken et al. 2009 in this issue). The experiments presented indicate an involvement of both AOX and H$_2$O$_2$ in olive rooting under the applied conditions used for commercial application. The results are sufficient encouragement for our applied research strategy to go ahead and search for polymorphisms in AOX genes that can be related to differential gene regulation and subsequently for the rooting process. This strategy is bearing in mind that it would be already sufficient for marker-assisted breeding to find a close association or correlation between a polymorphic sequence related to AOX and the efficiency of the rooting process.

The presented studies on polymorphic AOX sequences are at this stage a first approximation and restricted to the identified OeAOX2 gene in search for variability.
The partial sequence from three cultivars contains part of the ORF, one intron and the 3′-UTR regions. Recent knowledge on AOX gene regulation suggests that differential regulation cannot be sufficiently explained by defined motifs in the promoter region (see review Polidoros et al. 2009 in this issue). Variation in within-gene sequences at genome level and 3′-UTR micro-heterogeneity are currently considered as important factors that might cause diseases and differential regulation in genes (Goto et al. 2001, Lambert et al. 2003, Novelli et al. 2007). The principle aim of this study was to highlight the existence or non-existence of polymorphic sequences within the selected OeAOX2 gene as an example.

Several SNPs and InDels revealed OeAOX2 as a rich source for polymorphic sequences. Allelic and genotypic differences were identified from all gene regions. Highest variability was discovered for the 3′-UTR region, followed by the ORF and the intron. The relatively low variability in the intron is surprising, since introns are typically known to exhibit a higher variability than protein-coding sequences (Gibbs 2003). Since most parts of the ORF consist of the highly conserved region related to exon 3 from A. thalianas (Saisho et al. 1997), this was expected to be especially true. Interestingly, the majority of the SNPs identified in a sequence of one cultivar were common to allelic sequences and sequences from other genotypes. However, eight SNPs could be identified in the ORF of ‘Galega vulgar’, which could be observed only in this cultivar. Another unique SNP was found in the 3′-UTR of the same cultivar. This is of special interest for our approach, since this cultivar is a bad rooting cultivar and we are looking for differences that can be explored in future studies for any relationship to the rooting ability. Abe et al. (2002) reported the existence of a SNP in the OsAOX1a leading to a non-synonymous translations, that was completely linked to the presence of the QTL for low temperature tolerance. Five of the eight SNPs in the ORF of ‘Galega vulgar’ were responsible for non-synonymous translations. The substitution of Asp (D) by Gly (G) in position 399 in a sequence of ‘Galega vulgar’ (Table 4) indicates a position near the iron-binding site di-iron binding site of RADE_ _H box where Asp is found to be highly conserved among diverse species. The effect of substitutions in neighbour residues of the di-iron binding sites had already been demonstrated. Nakamura et al. (2005) reported in T. vivax 70 % reduction of the enzyme activity by artificial site-direct mutations in the conserved Glu to Ala positioned one and two positions downstream the LEEA box di-iron binding site proposed by Berthold et al. (2000). Albury et al. (2002)
performed the same site-direct mutation two residues downstream the di-iron binding site by changing the conserved residue to Asn and achieved total inactivation of the enzyme in *S. guttatum*.

The *OeAOX2* sequence of ‘Cobrançosa’ and ‘Picual’ carried an InDel mutation of two nucleotides (at positions 248 and 249) in exon 3 (considering the most conserved structure of *AOX* genes consisting in 4 exons) converting an ACT to a premature stop codon (TGA) in the 85 amino acid of peptide. The predominant consequence of nonsense mutations is not the synthesis of truncated proteins, but the recognition of nonsense transcripts and their efficient degradation by a phenomenon called nonsense-mediated RNA decay (NMD) (Conti and Izaurralde 2005). This mechanism seems to guarantee that only full-length proteins are produced (Byers 2002). Frischmeyer and Dietz (1999) considered NMD as an extremely heterogeneous process that might be transcript-, cell type- or genotype specific. The nonsense decay pathway participates in the control of gene expression by regulating the stability of physiological transcripts (Lew et al. 1998, Culbertson 1999). Nonsense mutations are related to a decrease of correspondent gene transcript accumulation (Nawarath et al. 2002, Aung et al. 2006). Horri and Watanabe (2007) reported that transcripts with stop codons located distant from the mRNA 3’termini or >50 nts upstream of the 3’-most exon-exon junctio are recognized as substrates for NMD. In both cultivars of olive the premature stop codon is located at 160 nts upstream the 3’-most exon-exon junctio. Nonsense mutations were related with one-third of inherited human genetic disorders and many forms of cancer (Frischmeyer and Dietz 1999). Several authors described that the phenotypic severity of selected diseases caused by nonsense mutations can be predicted by the extent of reduction in the level of mRNA from the mutant allele (Dietz et al. 1993, Hall and Thein 1994). In plants there are several examples showing that the nonsense mutation in specific genes are related to phenotype variations (Olsson et al. 2004, Aung et al. 2006, Sattler et al. 2009).

Introns are known to participate in nonsense decay (Frischmeyer and Dietz 1999) as a mechanism of gene expression control. This confirms the important role of plant introns in the control of gene expression (Rose 2002, Giani et al. 2003, Fiume et al. 2004) allowed introns to gain new credit in the scientific community (Rodriguez-Trelles et al. 2006, Roy and Gilbert 2006).
Pre-mRNA can influence splicing decisions and induce either exon skipping or intron retention (Aoufouchi et al. 1996, Valentine 1998). There are a number of cis elements located in exons and introns known as exonic or intronic splicing elements or silencers, due to their stimulating or repressing effects, respectively (Liu et al. 1998, Ladd and Cooper 2002). Single base changes that affect splicing can have dramatic effects on gene function and consequently in the phenotype, usually because the splice mutation results in a shift in the amino acid reading frame. SNPs located at introns and exons were related with alternative splicing (Kawase et al. 2007, Seli et al. 2008) with a strongest correlation were those closest to the intron-exon boundaries of the splicing events (Hull et al. 2007). Thus the two SNPs leading to non-synonymous translation at positions nearby the exon-intron (SNP at position 399) and intron-exon boundaries (SNP at position 429) that were only identified in ‘Galega vulgar’ can be of interest for future studies. The effects of polymorphisms on splicing may represent an important mechanism by which SNPs influence differential gene function.

Defective splicing can be also related with nonsense mutations (Aoufouchi et al. 1996), which can be due by SNPs (Isshiki et al. 2001, Nawrath et al. 2002, Aung et al. 2006, Sattler et al. 2009) or InDel events (Olsson et al. 2004).

The capacity of introns to regulate gene expression is related to intronic regulatory elements, such as miRNAs which inhibit translation of target genes by binding to their mRNAs. Recently discovered, the miRNAs have emerged as important players in plant stress responses, playing vital roles in plant resistance to abiotic as well as biotic stresses (Chiou et al. 2006). The control of plant developmental processes has been related with miRNA, including regulation of root growth (Wang et al. 2005), leaf development (Mallory et al. 2004), flower development and fertility (Achard et al. 2004). In two sequences of ‘Cobrançosa’ a putative miRNA precursor sequence (pre-miRNA) was located in a region characterized by a SNP at position 754 (T/G). This is an example how a polymorphism can influence prediction of regulatory sequences. A similar example was reported for intron 3 of the carrot AOX2a gene (Cardoso et al. in this issue). A pre-miRNA sequence was identified in a region of frequent large InDel events. The results need to be validated in future experiments.

Variation in the 3’-UTR region is not restricted to nucleotide polymorphisms but also encompasses length polymorphisms. Examining the 3’-UTR structure in ‘Galega vulgar’ a heterogeneity in 3’-UTR size was revealed due to both local
microheterogeneity and alternative polyadenylation. Microheterogeneity, probably caused by polymerase slippage, could be considered in the case of the length variation from 297 to 301 nucleotides in the 3'-UTRs of 5 clones in sequences Gal_2, Gal_3 and Gal_4 (Table 4). Alternative polyadenylation could be considered the variance in 3'-UTR size among the group of these three and the rest of the other sequences. All required polyadenylation signals could be identified (Fig. 9). In the shortest 3'-UTR sequence (Gal_1) a typical cleavage site is missing that was found in all other 3'-UTRs. The presence of 5 classes of alternative polyadenylation in OeAOX2 in a single cultivar raises the possibility for differential responses (Polidoros et al. 2009 in this issue). 3'-UTRs play an important role in post-transcriptional regulation known to be mediated by miRNAs in animals (Stark et al. 2005). In plants miRNA sites exist anywhere along the target mRNA (Zhang et al. 2006). However, several examples exist also in plants where the miRNA target is located in the 3'-UTR (Rhoades et al. 2002). In maize AOX1a 3'-UTR a putative miR163 target motif was identified (Polidoros et al 2009 in this issue). The maize AOX1a is transcribed with different 3'-UTR length and two major classes, a shorter and a longer (Polidoros et al. 2005). The miR163 target motif is present only in the longer class. Although the functional significance of this motif in maize AOX1a is obscure, its differential presence in the maize AOX 3'-UTR can be suggested how modulation of the 3'-UTR length can have significant effects of the regulation of AOX genes. A search for miRNA sites in olive 3'-UTR revealed 5 putative miRNA targets that had overall pairing energy ΔG<-20 Kcal/mol. Three of these targets were present in all seven variants but the other two were absent in the shorter three variants. The functional significance of these sites remains to be examined. However, discovery of AOX 3'-UTR microheterogeneity in two phylogenetically distinct plant species strengthens the possibility that this phenomenon is widespread in plant AOX genes.

Polyadenylation requires two major components: the cis-elements or polyadenylation signals of the pre-mRNA, and the trans-acting factors that carry out the cleavage and addition of the poly(A) tail at the 3'-end (Loke et al. 2005). Analyses of OeAOX2 transcripts with different 3'-UTR length has been restricted to a single olive cultivar and the observed variation could be correlated with the presence of the respective polyadenylation signals in the correct position (Fig. 9). It is currently not known if the structure of the OeAOX2 3'-UTR is conserved in other cultivars but it is conceivable that sequence polymorphisms that affect the polyadenylation signals could result in differences of transcript 3'-UTR lengths among different cultivars. This may provide an
additional source of genetic variation that can be exploited in the development of a marker-assisted strategy for breeding purposes.

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FIGURES LEGENDS

Figure 1. Structure of the partial OeAOX2 gene from Olea europaea cultivar ‘Galega vulgar’.

Figure 2. Rooting rates observed in the three SHAM trials (LSD=95%). (1: Control; 2: 17 mM IBA during 20 sec; 3: 10 mM SHAM during 20 sec; 4: 10 mM SHAM + 17 mM IBA during 20 sec).

Figure 3. Rooting rates observed in the H₂O₂ trial (LSD=95%). (1: Control; 2: 17 mM IBA during 20 sec; 5: 10 mM H₂O₂ during 1 h; 6: 10 mM H₂O₂ during 3 hs; 7: 10 mM H₂O₂ during 6 hs; 8: 10 mM H₂O₂ during 14 hs).

Figure 4. Rooting rates observed in the Pyruvate trial (LSD=95%). (1: Control; 2: 17 mM IBA during 20 sec; 9: 0.01 mM pyruvate during 3 hs; 10: 0.1 mM pyruvate during 3 hs; 11: 0.01 mM pyruvate during 20 sec; 12: 0.1 mM pyruvate during 20 sec).

Figure 5. Alignment of the translated amino acidic sequences obtained from the three genotypes of Olea europaea L. The underlined sequence corresponds to the peptide sequence of exon 4, all the other peptide sequences correspond to exon 3 in reference to A. thaliana. Amino acid residues differing are shown on a black background, deletions are shown by minus signs. Sites of translation stop are shown by points and are related with a nonsense mutation. In black boxes are three regions defined by Berthold et al. (2000) as highly conserved in AOX. The di-iron site positions are indicated by filled triangles. Asterisks indicate residues found to confer resistance to the AOX inhibitor salicylhydroxamic acid (Berthold 1998). In grey boxes are two structural elements proposed to influence AOX regulatory behavior (region 3 and 4 proposed by Crichton et al. 2005), the residues potentially involved in regulation of AOX activity are indicated by filled circles. Helical regions that are assumed to be involved in the formation of a hydroxo-bridged binuclear iron center (Andersson and Nordlund 1999, Berthold et al. 2000) are shown by two-headed arrows above the amino acid sequences. Possible membrane-binding domains center (Andersson and Nordlund 1999, Berthold et al. 2000) are shown with line above the amino acid sequences.
Figure 6. Differential expression of *OeAOX2* in *Olea europaea* cultivar ‘Cobrançosa’ at shoot cutting and during root induction in a bulked sample of 10 shoot cuttings (immersion during 20 sec in a solution with 17 mM of IBA). RT-PCR was individually controlled by amplification of *actin* mRNA.

Figure 7. Secondary structure of miRNA precursor predicted in the *OeAOX2* intron sequence of *Olea europaea* cultivar ‘Cobrançosa’.

Figure 8. Identification of a putative miRNA in *Olea europaea*. Alignment of the predicted pre-miRNA of *OeAOX2* with ath-miR417 and osa-miR417.

Figure 9. Alignment of the 3’-UTR sequences identified in seven different *OeAOX2* transcripts of *Olea europaea* cultivar ‘Galega vulgar’.
Table 1. Treatments of SHAM, H₂O₂ and Pyruvate applied to semi-hardwood olive shoot cuttings (for details see in Material and Methods).

<table>
<thead>
<tr>
<th>Code</th>
<th>Treatment</th>
<th>Concentration [mM]</th>
<th>Contact Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Negative control</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>IBA (positive control)</td>
<td>17</td>
<td>20 s</td>
</tr>
<tr>
<td>3</td>
<td>SHAM</td>
<td>10</td>
<td>20 s</td>
</tr>
<tr>
<td>4</td>
<td>IBA + SHAM</td>
<td>17 + 10</td>
<td>20 s</td>
</tr>
<tr>
<td>5</td>
<td>H₂O₂</td>
<td>10</td>
<td>1 h</td>
</tr>
<tr>
<td>6</td>
<td>H₂O₂</td>
<td>10</td>
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<td>H₂O₂</td>
<td>10</td>
<td>6 h</td>
</tr>
<tr>
<td>8</td>
<td>H₂O₂</td>
<td>10</td>
<td>14 h</td>
</tr>
<tr>
<td>9</td>
<td>Pyruvate</td>
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<td>3 h</td>
</tr>
<tr>
<td>10</td>
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<td>0.1</td>
<td>3 h</td>
</tr>
<tr>
<td>11</td>
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<td>0.01</td>
<td>20 s</td>
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<tr>
<td>12</td>
<td>Pyruvate</td>
<td>0.1</td>
<td>20 s</td>
</tr>
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</table>
Table 2. Polymorphic sites identified in the three regions of the gene *OeAOX2* (ORF, intron and 3’-UTR) among three cultivars. The fragment of the ORF corresponds to the sequence limited by the annealing position of the forward primer (17 nucleotides downstream of the first nucleotide identified in the cultivar ‘Galega vulgar’) and the stop codon. The intron is complete and consists of 499 bp. In the 3’-UTR region 159 bp downstream of the stop codon were analyzed. (Cv. corresponds to the cultivars; *M* corresponds to the material used for the gene isolation; *bc* corresponds to the number of sequences identified after *OeAOX2* cloning and sequencing).

<table>
<thead>
<tr>
<th>Cv. Origin</th>
<th>M</th>
<th>Nucleotide position in the partial ORF (475 bp until the stop codon)</th>
<th>Nucleotide position in the intron (total size of 499 bp)</th>
<th>Nucleotide position in the 3’-UTR (159 bp after the stop codon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galega vulgar gDNA</td>
<td>1</td>
<td>A G A T C G T C T C T C T C T C A T</td>
<td>T C C A - - - T G T</td>
<td>C C</td>
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<tr>
<td></td>
<td>2</td>
<td>A G A T T G C C A C T C T C T C A T</td>
<td>T C T A A - - T G T</td>
<td>T C T C A A</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>A G A T T G T C T C T C T C A T</td>
<td>T C T A A - - T G T</td>
<td>T C T C A A</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>A G A T T G T C T C T C T T C A C</td>
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<td>T C T C A A</td>
</tr>
<tr>
<td></td>
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<td>T C C G A A A A C A C</td>
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<tr>
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<td>6</td>
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<tr>
<td></td>
<td>7</td>
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</tr>
<tr>
<td></td>
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<td>T C C C C C C C C C</td>
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<tr>
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<td>T C C A - - - T G T</td>
<td>T C C C C C C C C</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>T C C A - - - T G T</td>
<td>T C T C A A</td>
</tr>
<tr>
<td></td>
<td>3</td>
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<tr>
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<td>5</td>
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<td>T T T C G G T G T A T</td>
<td>T C C C C C C C C</td>
</tr>
<tr>
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<td>A G A T T G T C T C T C T C A T</td>
<td>T C C A - - - T G T</td>
<td>T C T C A A</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>A G A T T G T C T C T C T C A T</td>
<td>T C C A - - - T G T</td>
<td>T C T C A A</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>A G A T T G T C T C T C T C A T</td>
<td>T C C A - - - T G T</td>
<td>T C T C A A</td>
</tr>
</tbody>
</table>
Table 3. Computational prediction of intronic miRNA precursors in *OeAOX2* of three *Olea europaea* cultivars: ‘Galega vulgar’ (Gal), ‘Cobrançosa’ (Cob) and ‘Picual’ (Pic).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Putative pre-miRNA sequence</th>
<th>bp</th>
<th>MFE</th>
<th>Prob.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal8</td>
<td>AUAGAUUUUGUCAUUUUUUGUCCCCCUAUAUCCACCUUUGCCGCUUAUUAUUUGCAAGAUAGAUUGCCAGAGCUAU</td>
<td>82</td>
<td>-13.22</td>
<td>no</td>
</tr>
<tr>
<td>Cob2</td>
<td>UUUUUUUUGUUGUCGAAAUAAGAAUUUGUUUGAUUUAAUAUAUAGCAAUUCUGUUUAGAUAAUAUAAGAAA</td>
<td>73</td>
<td>-16.30</td>
<td>65.9%</td>
</tr>
<tr>
<td>Cob3</td>
<td>AUAGAUUUUGUCAUUUUUUGUCCCCCUAUAUCCACCUUUGCCGCUUAUUAUUUGCAAGAUAGAUUGCCAGAGCUAU</td>
<td>81</td>
<td>-11.72</td>
<td>no</td>
</tr>
<tr>
<td>Cob5</td>
<td>AUAGAUUUUGUCAUUUUUUGUCCCCCUAUAUCCACCUUUGCCGCUUAUUAUUUGCAAGAUAGAUUGCCAGAGCUAU</td>
<td>82</td>
<td>-13.12</td>
<td>no</td>
</tr>
<tr>
<td>Cob9</td>
<td>UUUUUUUUGUUGUCGAAAUAAGAAUUUGUUUGAUUUAAUAUAUAGCAAUUCUGUUUAGAUAAUAUAAGAAA</td>
<td>73</td>
<td>-16.30</td>
<td>65.9%</td>
</tr>
<tr>
<td>Pic1</td>
<td>AUAGAUUUUGUCAUUUUUUGUCCCCCUAUAUCCACCUUUGCCGCUUAUUAUUUGCAAGAUAGAUUGCCAGAGCUAU</td>
<td>81</td>
<td>-11.72</td>
<td>no</td>
</tr>
<tr>
<td>Pic2</td>
<td>AUAGAUUUUGUCAUUUUUUGUCCCCCUAUAUCCACCUUUGCCGCUUAUUAUUUGCAAGAUAGAUUGCCAGAGCUAU</td>
<td>81</td>
<td>-11.72</td>
<td>no</td>
</tr>
<tr>
<td>Pic3</td>
<td>AUAGAUUUUGUCAUUUUUUGUCCCCCUAUAUCCACCUUUGCCGCUUAUUAUUUGCAAGAUAGAUUGCCAGAGCUAU</td>
<td>81</td>
<td>-11.72</td>
<td>no</td>
</tr>
</tbody>
</table>
Table 4. Alternative polyadenylation of the *OeAOX2* transcripts ('Galega vulgar') detected by 3'-RACE experiments. The length of the 3'-UTR in bases, the number and percentage of clones detected for each 3'-UTR size and the nomenclature of the sequence are shown.

<table>
<thead>
<tr>
<th>3'-UTR size (bp)</th>
<th>Number of clones</th>
<th>Sequence identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>76</td>
<td>1</td>
<td>Gal_1</td>
</tr>
<tr>
<td>113</td>
<td>1</td>
<td>Gal_6</td>
</tr>
<tr>
<td>151</td>
<td>1</td>
<td>Gal_5</td>
</tr>
<tr>
<td>259</td>
<td>1</td>
<td>Gal_7</td>
</tr>
<tr>
<td>297</td>
<td>1</td>
<td>Gal_2</td>
</tr>
<tr>
<td>298</td>
<td>2</td>
<td>Gal_3</td>
</tr>
<tr>
<td>301</td>
<td>2</td>
<td>Gal_4</td>
</tr>
</tbody>
</table>
Table 5. miRNA target sites predicted at the 3'-UTR region of the sequence Gal_3 (software miRanda v3.0).

<table>
<thead>
<tr>
<th>miRNA identification</th>
<th>nt position</th>
<th>Alignment</th>
<th>Energy (kCal/Mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ath-miR401</strong></td>
<td>2-23</td>
<td>Query: 3' acAGCCAGC-UGUGGUGCAAagc 5'</td>
<td></td>
</tr>
<tr>
<td>(Sunkar and Zhu 2004)</td>
<td></td>
<td>Ref: 5' tgTTGGTCGTGTGCTAGTTcat 3'</td>
<td>-22.10</td>
</tr>
<tr>
<td></td>
<td>35-51</td>
<td>Query: 3' acAGCCAGCUGUGGUGCAAAGc 5'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ref: 5' aaTTGGTCC----CCGGTTTTg 3'</td>
<td>-20.10</td>
</tr>
<tr>
<td><strong>ppt-miR1212</strong></td>
<td>27-47</td>
<td>Query: 3' gcGUAAGAUACGACAGGGUgc 5'</td>
<td></td>
</tr>
<tr>
<td>(Talmor-Nieman et al. 2006)</td>
<td></td>
<td>Ref: 5' cgCGTCCGCTAGCTCCGg 3'</td>
<td>-21.36</td>
</tr>
<tr>
<td><strong>smo-miR1110</strong></td>
<td>153-171</td>
<td>Query: 3' agGAACUGGUGUAGCGGAUCg 5'</td>
<td></td>
</tr>
<tr>
<td>(Axtell et al. 2007)</td>
<td></td>
<td>Ref: 5' tgCTGAT---CTGCGCTTAGt 3'</td>
<td>-22.56</td>
</tr>
<tr>
<td><strong>tae-miR1125</strong></td>
<td>158-177</td>
<td>Query: 3' ggcGGCGUCAACCAGAGCAACCAa 5'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ref: 5' gatCTGCGCTTAGT----TTGGTc 3'</td>
<td>-23.97</td>
</tr>
</tbody>
</table>
For Peer Review

OAOX2_33Fw → Exon → Intron → Exon → 3’-UTR → OAOX2_647Rev

- partial sequence 430 bp
- 499 bp
- 57 bp

VIAL 9
• **ath-miR417 : 1-21**  
  • score: 91.7, evalue: 28  

  UserSeq  
  1  UUUUUUGUUGUCGAAAAUAG-GAAUUUGUUUGAUUUCAAUUAAAUAAAGUCAUUCUGUUUAGAUAUAAGAAA  73  
  ath-miR417  
  1  GAAGGUAGUGAAUUUGUUCGA                                           21

• **osa-miR417 : 1-18**  
  • score: 82.5, evalue: 91  

  UserSeq  
  1  UUUUUUGUUGUCGAAAAUAG-GAAUUUGUUUGAUUUCAAUUAAAUAAAGUCAUUCUGUUUAGAUAUAAGAAA  59  
  osa-miR417  
  1  GAAGGUAGUGAAUUUGUUCGA                                           21