Physiologia Plantarum
An International Journal for Plant Biology

Physiologia Plantarum 137: 532–552. 2009

Physiologic responses and gene diversity indicate olive alternative oxidase as a potential source for markers involved in efficient adventitious root induction

Elisete Santos Macedo[a], Hélia G. Cardoso[a], Alejandro Hernández[a], Augusto A. Peixe[a,b], Alexios Polidoros[c], Alexandre Ferreira[a], António Cordeiro[d] and Birgit Arnholdt-Schmitt[a,*]

[a]ICAAM, University of Évora, 7002-554 Évora, Portugal
[b]Department of Plant Production, ICAAM, University of Évora, 7002-554 Évora, Portugal
[c]Institute of Agrobiotechnology, Center for Research and Technology Hellas, Thermi, Greece
[d]INRB/ L-INIA, URGEMP. Apartado 6, 7350-951 Elvas, Portugal

Correspondence
*Corresponding author,
e-mail: eu_chair@uevora.pt

Received 20 May 2009; revised 23 September 2009

doi:10.1111/j.1399-3054.2009.01302.x

Olive (*Olea europaea* 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 can often be restricted by a low rooting capacity. We hypothesize that root induction is a plant cell reaction linked to oxidative stress and that activity of stress-induced alternative oxidase (AOX) is importantly involved in adventitious rooting. To identify AOX as a source for potential functional marker sequences that may assist tree breeding, genetic variability has to be demonstrated that can affect gene regulation. The paper presents an applied, multidisciplinary research approach demonstrating first 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 salicyl-hydroxamic acid, an inhibitor of AOX activity. On the contrary, treatment with H_2O_2 or pyruvate, both known to induce AOX activity, increased the degree of rooting. Recently, identification of several *O. europaea* (Oe) *AOX* gene sequences 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 from genomic DNA and cDNA, including exon, intron and 3′-untranslated regions (3′-UTRs) sequences. The data revealed polymorphic sites in several regions of *OeAOX2*. The 3′-UTR was the most important source for polymorphisms showing 5.7% of variability. Variability in the exon region accounted 3.4 and 2% in the intron. Further, analysis performed at the cDNA from microshoots of 'Galega vulgar' revealed transcript length variation for the 3′-UTR of *OeAOX2* ranging between 76 and 301 bp. The identified polymorphisms and 3′-UTR length variation can be explored in future studies for effects on gene regulation and a potential linkage to olive rooting phenotypes in view of marker-assisted plant selection.

Abbreviations – AOX, alternative oxidase; AP, alternative polyadenylation; CE, cleavage elements; COX, cytochrome oxidase; CS, cleavage site; EtBr, ethidium bromide; FUE, far upstream elements; gDNA, genomic DNA; H_2O_2, hydrogen peroxide; IBA, indol-3-butyric acid; InDel, insertion/deletion; NMD, nonsense-mediated RNA decay; nts, nucleotides; NUE, near upstream elements; ORF, open reading frame; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; SHAM, salicyl-hydroxamic acid; SNP, single nucleotide polymorphism; 3′-UTR, 3′-untranslated region.

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. Furthermore, rooting efficiency strongly depends on the physiologic 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 constitute a stress to the involved cells. Directed growth responses on stress are supposed to be a plant acclimation strategy to diminish stress exposure (Potters et al. 2007). Plant reactions upon stress involve hormone-transmitted metabolic changes, molecular transduction pathway activation, protein degradation and protein *de novo* synthesis as well as adaptive global genome regulation (see e.g. in Arnholdt-Schmitt 2004, Zavattieri et al. 2009). Restructuring of the inner and outer shapes of target cells, a change in the rate of growth and/or development and induction of adventitious organs such as roots, shoots or hairs are visible signs of an acclimation strategy. Thus, stress acclimation is not physically possible without a change in energy allocation and spatial changes in metabolism (see discussion in Arnholdt-Schmitt et al. 2006a). Physiologic and morphologic plasticity are not occurring in a random way, but show components of reproducibility that needs coordinated events at target cell levels, such as for example the formation of cluster roots or root hairs during phosphorous depletion stress (Shane et al. 2004), or of roots in olive shoots upon auxin treatment or the initiation of somatic embryogenesis by a reduction of the concentration of auxin in the medium (Frederico et al. 2009).

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 (ROS), such as hydrogen peroxide (H_2O_2). H_2O_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 (Amirsadeghi et al. 2006). In guard cells auxin seems to limit H_2O_2 levels through preventing its generation, but unlike cytokinin it did not reduce exogenous H_2O_2 levels (Song et al. 2006). Several reports are now available that point to a critical role of ROS signaling in root induction and development (Dunand et al. 2007, Nag et al. 2001). Seed germination of rice seedlings could be promoted by applying exogenous H_2O_2 (Sasaki et al. 2005). Treatment of olive shoot cuttings with H_2O_2 could increase early rooting and the number of roots across genotypes and environments. However, the effects of the treatment differed clearly among olive cultivars and different years. Quantitative and qualitative differences in rooting between genotypes and varying environmental conditions during tree growth could not be equalized by H_2O_2 application. No interaction between years or cultivars and 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 by hypothesis-driven research approaches (Arnholdt-Schmitt 2005). Candidate gene 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 a functional marker candidate, three pre-requisites 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 physiologic responses related to the target trait, through functional genomics, including transgenic strategies, linkage mapping and/or association studies. Once a gene is identified as a 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.

Respiration plays a central role in cell acclimation. Therefore, all genetic components directly involved in adaptive respiration can serve as promising candidates for functional marker development related to any stress reaction. The present paper focuses on the development of alternative oxidase (AOX) as a functional marker for adventitious rooting of olive shoot cuttings. AOX is nuclear encoded, but active in the alternative

respiration pathway in mitochondria. It is positioned upstream to important energy and carbon turnover regulation. AOX to cytochrome oxidase (COX) rate is an important measure for carbon efficiency rates related to environmental factors, growth and development (Hansen et al. 2009). The hypothesis that AOX can be critical for a stress reaction, such as adventitious rooting studied in this paper, was recently developed by Arnholdt-Schmitt et al. (2006a) (see also Arnholdt-Schmitt et al. 2006b, Santos Macedo et al. 2006a,b). This view got support through current knowledge on the central role of mitochondria and AOX in stress perception and cell signaling upon stress (Amirsadeghi et al. 2007, Vanlerberghe et al. 2009). 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 the complex interaction between AOX activity and H_2O_2 (e.g. Amirsadeghi et al. 2006, Gray et al. 2004, Popov et al. 1997, Umbach et al. 2005, Vanlerberghe and McIntosh 1996). Consequently, inhibitors of AOX activity, such as salicyl-hydroxamic acid (SHAM), were found to interact with H_2O_2 cell levels. In SHAM-treated *Arabidopsis thaliana* roots, peroxidase was inhibited and root length was strongly reduced. H_2O_2 disappeared from the root hair zone, but was accumulated in cell walls in the meristemic region (Dunand et al. 2007). H_2O_2 treatment of rice seedlings promoted germination and seedling growth, and *AOX1a* was upregulated by this treatment at an early stage (Sasaki et al. 2005). An upregulation of *AOX* transcription upon H_2O_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, members of the *AOX2* gene subfamily are thought to have a closer relationship with tissue specificity and development. However, this correlation is not fixed, as a relationship with stress induction was also indicated for *AOX2* in *A. thaliana* and *Vigna unguiculata* (see the review Polidoros et al. 2009). Alternative respiration can be promoted through blocking the cyanine-sensitive respiration pathway via COX. Potassium cyanide (KCN) treatments of dormant grapevine cuttings induced an increase of bud break and rooting (Mizutani et al. 1994), which can point not only to a role for COX but also to the importance of AOX. *AOX* expression can be upregulated 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_2O_2 to test its effect as an external stimulator for olive root induction.

The importance of *AOX* genes in affecting physiologic responses under determined conditions must be verified at species level as 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 displays already the overall characteristics of a commercially important variety for breeding. A later introgression of additional genetic characteristics, such as quality traits of olive oil or other agronomic important tree traits, will be possible by testing the stability of the potential functional marker for rooting efficiency. 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 encourage further efforts to work on the hypothesis that *AOX* gene sequences may serve as a source for functional markers for efficient adventitious rooting of olive shoot cuttings. However, the link between polymorphic *AOX* gene sequences, altered gene regulation and a rooting phenotype still needs to be approved.

Materials and methods

Rooting assays: treatment of shoot cuttings with IBA, SHAM, H_2O_2 and pyruvate

Plant material

Olive semi-hardwood shoot cuttings were obtained from 10-year-old, field-grown mother plants of the easy-to-root Portuguese olive cultivar 'Cobrançosa'. Shoot cuttings of about 14 cm length were collected from the medium portion of 1-year-old branches. The 'Cobrançosa' orchard has a polyclonal origin grown in a nursery company located in Montemor-o-Novo, Alentejo, in the southern part of Portugal. The orchard was established on a range of 1 m rows and 3 m between rows. The plants had been trained as shrubs. Annually, the orchard is submitted to a vigorous pruning to maximize the production of vegetative material for tree propagation.

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 27–28°C. 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 the rooting substrate.

Shoot cutting treatments

Shoot cuttings were treated with indol-3-butyric acid (IBA; Sigma-Aldrich, St Louis, MI), which is used in commercial olive propagation as a root promoting auxin, the inhibitor of AOX activity SHAM (Sigma-Aldrich, St Louis, MI) and two stimulators of AOX activity, H_2O_2 (Sigma-Aldrich, St Louis, MI) and pyruvate (Sigma-Aldrich, St Louis, MI). All compounds were applied as water solutions through immersion of the cutting bases (4–6 cm). Because of their insolubility in water, IBA was previously soluted in a few drops of NaOH 1N and SHAM in 95% ethanol. For detailed information on product concentrations used and contact times with the cuttings bases see Table 1.

Experimental design and data analysis

The experimental conditions applied to SHAM trials correspond to a fully randomized scheme within a three-factorial design: three experimental periods × four treatments × three replications. Independent experiments were performed during March/April and October/November in 2007 and during May/June in 2008. Each replication with 20 semi-hardwood cuttings was used as an experimental unit for statistical data analysis.

The trials with H_2O_2 and pyruvate were also conducted applying a completely randomized scheme within a simple factorial assay with six treatments and three replications. 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 were submitted to variance analysis using STATISTICA V.7.0 as software and the results are presented as 95% confidence interval charts.

Table 1. Treatments of SHAM, H_2O_2 and pyruvate applied to semi-hardwood olive shoot cuttings (for details see section Materials and Methods).

Code	Treatment	Concentration (m*M*)	Contact time
1	Negative control	–	–
2	IBA (positive control)	17	20 s
3	SHAM	10	20 s
4	IBA + SHAM	17 + 10	20 s
5	H_2O_2	10	1 h
6	H_2O_2	10	3 h
7	H_2O_2	10	6 h
8	H_2O_2	10	14 h
9	Pyruvate	0.01	3 h
10	Pyruvate	0.1	3 h
11	Pyruvate	0.01	20 s
12	Pyruvate	0.1	20 s

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

Plant material

Shoot cuttings randomly collected from polyclonal 'Cobrançosa' trees were used for *OeAOX2* gene expression studies. The samples consist of olive tissue rings, each about 1 cm high, taken from the basal portion of 10 shoot cuttings as a bulked sample. The samples were collected in the field (*T*0), 12 h and 14 days after IBA treatment. Ten days after IBA application, root primordial induction can typically be observed by using histologic techniques (Peixe et al. 2007a).

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, Hilden, Germany). Single-strand cDNA was synthesized with the help of the enzyme *RevertAid™ HMinus M-MuLV Reverse Transciptase* (Fermentas, Ontario, Canada) using the oligo(dT) primer 5′-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTV (V = A,C or G)-3′ (Roche, Mannheim, Germany). The primer (2 μM) was incubated with 5 μg of total RNA for 5 min at 70°C. After this procedure, the mixture was placed on ice for 5 min and then the following reagents were added: 1× enzyme buffer *RevertAid HMinus M-MuLV Reverse Transcriptase* (Fermentas, Ontario, Canada), 1 mg ml^{-1} of BSA (Ambion, Austin, TX), 1 m*M* dNTP (Fermentas, Ontario, Canada), 0.05 μg μl^{-1} of Actinomycin D (Roche, Mannheim, Germany), 2 m*M* of dithiothreitol (DTT) (Fermentas, Ontario, Canada), 0.8 U μl^{-1} of RNase inhibitor (Fermentas, Ontario, Canada) and 6 U μl^{-1} of the enzyme *RevertAid HMinus M-MuLV Reverse Transcriptase* (Fermentas, Ontario, Canada). This reaction mixture was incubated for 90 min at 42°C.

Gene expression

RT-PCR was performed in final volumes of 25 µl with pure Taq Ready-to-go PCR Beads (GE Healthcare, Little Chalfont, England) using 1 µl of 1:10 cDNA dilution and 0.2 µ*M* of each primer OeAOX2_1111Fw: 5′-CCACTAGTTGTTGGTCGTGTGC-3′ and OeAOX2_1246Rev: 5′-AGTAAGTGGCATGTTTCTGTAG-3′ (both from Eurofins MWG Operon, Ebersberg, Germany). RT-PCR was normalised by O. evnopeal Actin using the primers Oe AF: 5′-TTG CTCTCGACTATG AACA GG-3′ and Oe AR: 5′-CTC TCGGCCCCA ATA GTA ATA-3′ (Eurofins MWG Operon, Ebersberg, Germany). PCR was carried out for 35 cycles in the 2720 Termalcycler (Applied Biosystems, Foster City, CA). Each cycle consisted of 30 s at 94°C for denaturation, 30 s at 55°C for primers annealing and 30 s at 72°C for DNA synthesis. An initial denaturation step at 94°C for 5 min and a final step at 72°C for 10 min were included. PCR products were analyzed by electrophoresis in 1.4% (w/v) agarose gel after staining in an ethidium bromide (EtBr) solution (2 ng ml^{-1}) using the Gene Flash Bio Imaging system (Syngene, Cambridge, UK).

Gene diversity and 3′-UTR sequence and length variability

Plant material

To initiate studies on *OeAOX2* gene diversity at genomic DNA (gDNA) level, the DNA of young leaves from one tree of each of the three cultivars 'Galega vulgar' (clone 1053), 'Cobrançosa' and 'Picual' was isolated. The trees were grown in fields in Montemor-o-Novo ('Galega vulgar' and 'Cobrançosa') and Elvas ('Picual'), Alentejo, Portugal.

For cDNA studies, leaves of five microshoots were extracted as a bulked sample. The microshoots originated from an unknown number of trees of the clone 1053 of the cultivar 'Galega vulgar'. The establishment of microshoot cultures was performed as previously described by Peixe et al. (2007b).

DNA extraction

gDNA was extracted using the DNEasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA integrity and quantification were assessed by electrophoresis in 1% agarose gel. DNA quantification was performed using defined amounts of lambda DNA as a standard. Nucleic acids were visualized through EtBr staining as described before.

RNA extraction and cDNA synthesis

All steps were performed as described under Molecular studies: *OeAOX2* expression in root-inducible tissues of shoot cuttings.

AOX2-specific fragment isolation from three cultivars

AOX2-specific primers were designed in the region of exon 3 (OAOX2_33Fw: 5′-ATTGCATCTCAGGTCTCTT CGC-3′) and 3′-untranslated region (3′-UTR; OAOX2_647Rev: 5′-CAGGCATAAGTAAGTGGCATG-3′) (both from Eurofins MWG Operon, Ebersberg, Germany) (see Fig. 1). For the PCR mix, 0.02 U of a *Phusion™ High-Fidelity DNA Polymerase* (Finnzymes, Espoo, Finland) were used with 1× manufacturer supplied Phusion HF buffer containing 1.5 m*M* $MgCl_2$, 0.2 m*M* of each dNTPs (Fermentas, Ontario, Canada) and 0.2 µM of each primer. The PCR was carried out with 0.2 ng µl^{-1} of gDNA as template running an initial step of 30 s at 98°C followed by 35 cycles, each consisting of 10 s at 98°C, 15 s at 58°C and 60 s at 72°C.

PCR fragments were analyzed in 1.4% agarose gels and visualized as described. The single PCR fragment generated from each cultivar was purified using the GFX PCR DNA and Gel Purification kit (GE Healthcare, Little Chalfont, England). The addition of adenines to the 3′ end of amplicons required for the cloning procedure was made by adding 0.1 U µl^{-1} of *Taq* polymerase (Promega, Madison, WI), 1× manufacturer supplied $(NH_4)_2SO_4$ buffer, 2.5 m*M* $MgCl_2$ and 0.2 m*M* dATP (Fermentas, Ontario, Canada). The final mix was incubated for 30 min at 72°C in a 2720 Termalcycler (Applied Biosystems, Foster City, CA). The amplicons were cloned into a pGEM®-T Easy System I vector (Promega, Madison, WI) and subsequently used for the transformation of competent cells JM109 (Promega, Madison, WI). Plasmid DNA of selected white clones was extracted using the alkaline lyses protocol (Sambrook et al. 1989) and was characterized by the restriction enzyme *Eco*RI. Recombinant clones were

Fig. 1. Structure of the partial *OeAOX2* gene from *O. europaea* cv. Galega vulgar.

used for commercial sequencing: 1 from 'Galega vulgar', 7 from 'Cobrançosa' and 4 from 'Picual'.

3′-UTR characterization in cDNAs of 'Galega vulgar'

3′-UTR sequence and length variability were analyzed in transcripts of microshoots (clone 1053). Amplicons were produced by the forward primer OAOX2_33Fw combined with the reverse primer Vial9: 5′-GACCACGCGTATCGATGTCGAC-3′ (Roche).

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

The PCR was carried out with an initial step of 5 min at 94°C followed by 35 cycles, each consisting of 30 s at 94°C, 90 s at 68°C and 10 min at 72°C. PCR fragments were analyzed in 1.4% agarose gels, purified, cloned and selected as described above. Nine recombinants bacterial clones from 'Galega vulgar' provided from a PCR using DNA from a bulked sample of five microshoots were used to study 3′-UTR variability.

Sequence analysis and sequence alignment

Selected clones were sequenced by commercial services through the MACROGEN company (www.macrogen.com). All sequences resulted from at least two sequencing cycles of each plasmid DNA in sense and antisense directions, using specific primers located within the vector (T7 and SP6, Promega, Madison, WI). Sequence homologies in the NCBI database (National Center for Biotechnology Information, Bethesda, MD, USA) were studied using the Blast algorithm (Altschul et al. 1990) (http://www.ncbi.nlm.nih.gov) (BLASTX and BLASTN).

gDNA and cDNA sequences were analyzed and compared using the programs EditSeq (Lasergene 7, GATC Biotech, Konstanz) and Bioedit (Hall 1999). For sequence alignment, the Clustal W algorithm of Megalign (Lasergene 7, GATC Biotech) was applied.

The level of variability was calculated for each gene region (exon, intron and 3′-UTR) according to: (no of nts with variation*100)/size of the gene region.

For polymorphism analysis in the partial open reading frame (ORF) of 'Galega vulgar', the available gDNA sequence was aligned with nine cDNA sequences.

Computational prediction and validation of miRNA percursors 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. 2008).

Prediction of the secondary structure of pre-miRNA was run on the web-based software MFOLD 3.1, which is available at http://mfold.bioinfo.rpi.edu/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.

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

SHAM treatment of shoot cuttings

Fig. 2 shows the rooting rates of shoot cuttings treated with SHAM for an average value of three independent experiments with 'Cobrancosa'. The positive control (code 2), which corresponds to the treatment with IBA, gave the highest rooting percentages, around 60% in average. SHAM treatment either alone (code 3) or in

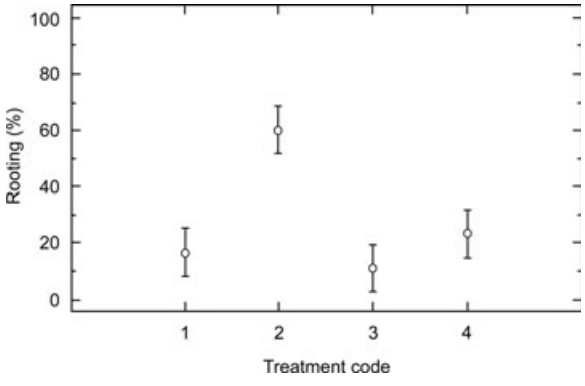

Fig. 2. Rooting rates observed in SHAM trial (LSD = 95%). (1: Control; 2: 17 m*M* IBA during 20 s; 3: 10 m*M* SHAM during 20 s; 4: 10 m*M* SHAM + 17 m*M* IBA during 20 s).

combination with IBA (code 4) significantly reduced the level of root induction to the values observed for the negative control (code 1). Rooting rates in the presence of SHAM reached a maximal of 28%.

H_2O_2 treatment of shoot cuttings

The effect of H_2O_2 was time-dependent (Fig. 3). The 14-h 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 is a common practice in commercial olive propagation. A trend for increased rooting rates was recognizable from third hour onward, but it reached a degree near to statistical significance for the difference to the control only after a treatment for 14 h.

Pyruvate treatment of shoot cuttings

Pyruvate was applied at two different concentrations and contact times. A short pulse of 20 s (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 m*M*. However, when the time was prolonged to 3 h, 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 10).

OeAOX2 expression in shoot cuttings

Fig. 1 shows the scheme of the partial *OeAOX2* sequence. The genomic sequence consists of a partial

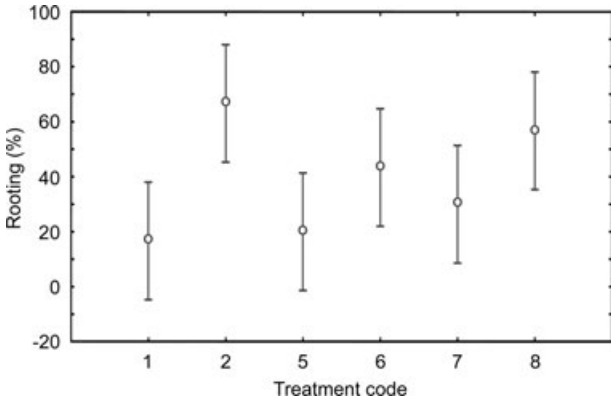

Fig. 3. Rooting rates observed in the H_2O_2 trial (LSD = 95%). (1: Control; 2: 17 m*M* IBA during 20 s; 5: 10 m*M* H_2O_2 during 1 h; 6: 10 m*M* H_2O_2 during 3 h; 7: 10 m*M* H_2O_2 during 6 h; 8: 10 m*M* H_2O_2 during 14 h).

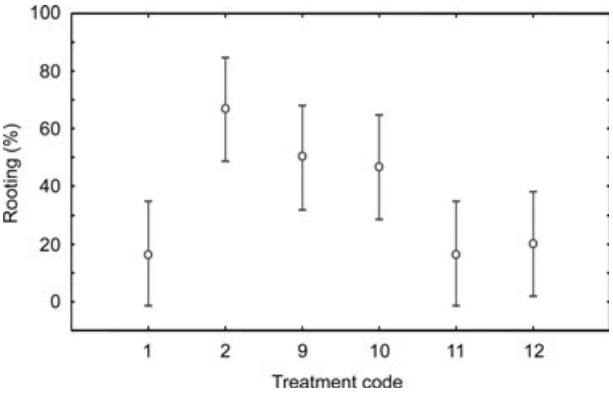

Fig. 4. Rooting rates observed in the pyruvate trial (LSD = 95%). (1: Control; 2: 17 m*M* IBA during 20 s; 9: 0.01 m*M* pyruvate during 3 h; 10: 0.1 m*M* pyruvate during 3 h; 11: 0.01 m*M* pyruvate during 20 s; 12: 0.1 m*M* pyruvate during 20 s).

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 the more conserved regions of the AOX (NERMHL, LEEEA and RADE_H region, Berthold et al. 2000), which include five 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 (Andersson and Nordlund 1999, Berthold et al. 2000, Siedow et al. 1995).

Fig. 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 histologic evidence for root induction can typically be found (Peixe et al. 2007a), an increase in *OeAOX2* transcript level was obtained.

Gene diversity revealed in *OeAOX2*

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

Out of the 10 ORF sequences identified from 'Galega vulgar' (9 provided from cDNA and 1 from gDNA), 6 presented variations between each other (Table 2). Twelve single nucleotide polymorphisms

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).

				Nucleotide position in the partial ORF (475 bp until the stop codon)															*Nucleotide position in the intron (total size of 499 bp)*										*Nucleotide position in the 3'-UTR (159 bp after the stop codon)*								
Cv.	*Origin*	*M*	*bc*	*23*	*25*	*114*	*118*	*122*	*134*	*141*	*178*	*196*	*245*	*248*	*249*	*370*	*382*	*399*	*429*	*500*	*537*	*627*	*641*	*651*	*703*	*754*	*815*	*889*	*903*	*996*	*1006*	*1057*	*1058*	*1063*	*1064*	*1070*	*1073*	*1076*
Galega vulgar	Montemor	cDNA	1	A	G	A	T	C	G	T	C	T	C	T	C	T	C	A	T											C	C							
			2	A	G	A	T	T	G	C	C	A	C	T	C	T	C	A	T											T	C	T	A	-	-	T	G	T
			3	A	G	A	T	T	G	T	C	T	C	T	C	T	C	A	T											T	C	T	A	A	-	T	G	T
			4	A	G	A	T	T	G	T	C	T	C	T	C	T	C	A	C											T	C	T	A	A	-	T	G	T
			5	C	T	A	T	T	G	T	C	T	C	T	C	C	C	G	T											T	C	T	A	A	-	T	G	T
		gDNA	8	A	G	G	A	T	C	T	T	T	C	T	C	T	T	A	T	T	T	C	G	T	G	G	T	A	T	T	C	G	A	A	A	C	A	C
Cobrançosa	Montemor	gDNA	1	A	G	A	T	T	G	T	C	T	A	-	-	T	T	A	T	T	-	A	G	A	G	T	T	G	C	T	T	G	A	A	A	C	A	C
			2	A	G	A	T	T	G	T	C	T	A	-	-	T	C	A	T	C	-	C	A	A	C	G	C	G	T	T	C	T	A	A	-	T	G	T
			3	A	G	G	A	T	C	T	T	T	C	T	C	T	T	A	T	T	T	A	G	A	G	T	T	G	C	T	T	G	A	A	A	C	A	C
			4	A	G	A	T	T	G	T	C	T	A	-	-	T	C	A	T	C	-	C	A	A	C	G	C	G	T	T	C	T	-	A	A	C	A	C
Picual	Elvas	gDNA	1	A	G	A	T	T	G	T	C	T	A	-	-	T	C	A	T	C	-	C	A	A	C	G	T	A	T	T	C	G	A	A	A	C	A	C
			2	A	G	A	T	T	G	T	C	T	A	-	-	T	C	A	T	C	-	C	A	A	C	G	C	G	T	T	C	T	A	A	-	T	G	T
			3	A	G	A	T	T	G	T	C	T	A	-	-	T	C	A	T	C	-	C	A	T	G	G	T	A	T	T	C	G	A	A	A	C	A	C

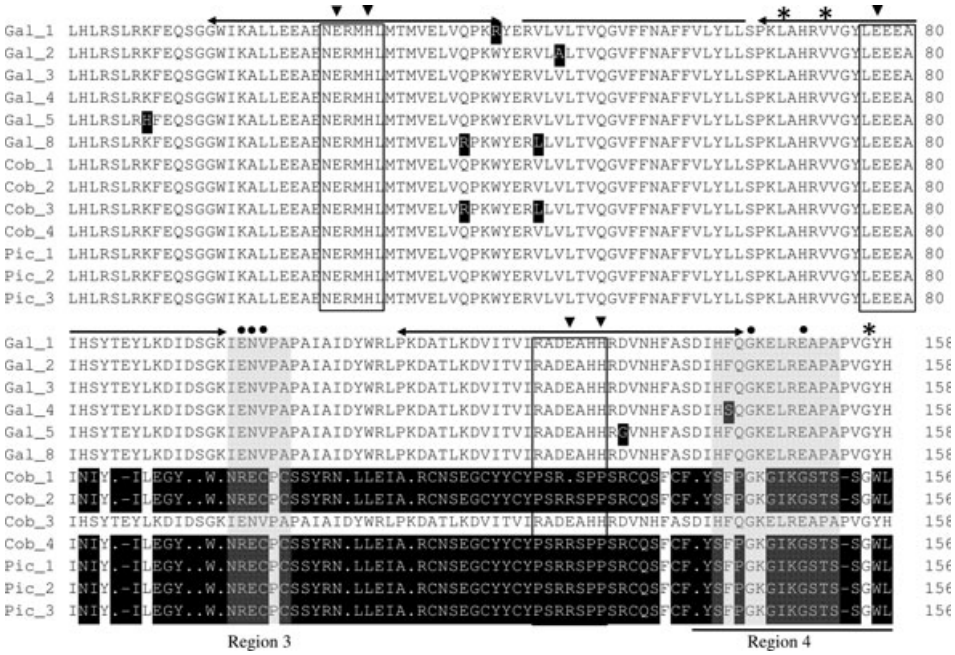

Fig. 5. Alignment of the translated amino acidic sequences obtained from the three genotypes of *O. europaea*. The underlined sequence corresponds to the peptide sequence of exon 4, whereas all the other peptide sequences correspond to exon 3 in reference to *A. thaliana*. Differing amino acid residues are shown in 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 SHAM (Berthold 1998). Two structural elements proposed to influence AOX regulatory behavior are shown in gray boxes (regions 3 and 4 proposed by Crichton et al. 2005), and 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 a line above the amino acid sequences. The peptide sequences presented in this figure refer to the ORF translation of the sequences given in Table 2.

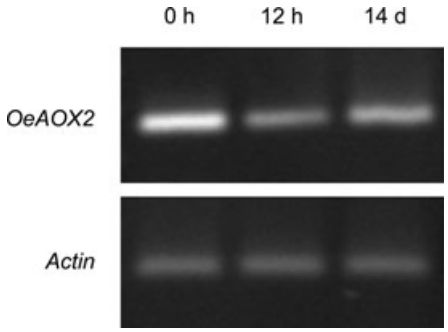

Fig. 6. Differential expression of *OeAOX2* in *O. europaea* cv. Cobrançosa at shoot cutting and during root induction in a bulked sample of 10 shoot cuttings (immersion during 20 s in a solution with 17 m*M* of IBA). RT-PCR was individually controlled by amplification of *Actin* mRNA.

(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) because of the SNP at position 429 occurred within one of the structural elements in the fourth helical region proposed to influence AOX regulation (Andersson and Nordlund 1999, Crichton et al. 2005, Siedow et al. 1995). In sequence 5 the Asp (D)/Gly (G) substitution because of an 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 (Andersson and Nordlund 1999, Siedow et al. 1995). A multiple alignment made with the peptide sequences of plant AOXs available at the NCBI database showed at this position an Asp in all sequences (data not showed). In sequence 8 a Gln (Q)/Arg (R) substitution by an SNP in position 114 was identified eight positions upstream of the di-iron binding NERMHL box, included in the second helical

region (Andersson and Nordlund 1999, Siedow et al. 1995). Variations at the possible membrane-binding domain were observed in the sequences 8 and 2 as a result of a Val (V)/Leu (L) (SNP at the position 134) and a Val (V)/Ala (A) (SNP at the position 141) substitutions, respectively.

From seven gDNA sequences identified as *OeAOX2* from 'Cobrançosa', four presented variations (Table 2). Among these sequences, six SNPs were identified. All SNPs are repetitive, as they were already observed in 'Galega vulgar'. Additionally, an insertion/deletion (InDel) of two nucleotides (nts) was identified at positions 248 and 249. From these eight sources of variation, six were responsible for synonymous translations and two 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 (Andersson and Nordlund 1999, Siedow et al. 1995). Sequence 3 presented a mutation because of Val (V)/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 five of the seven sequences from this cultivar. Two sequences of the seven were identical to sequence 2 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 two 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 three 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 has 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. Most are repetitive within sequences from one single cultivar and between cultivars. However, 'Galega vulgar' is the only culture, which showed unique SNPs (positions 23, 25, 122, 141, 196, 370, 399 and 429). Any nonsense mutation was observed in the 10 sequences of this cultivar as described for the other two cultivars. However, it must be considered that nine of the sequences were deduced from actively transcribed sequences (cDNA) (Table 2).

Intron

All intron sequences from the three cultivars show the typical nuclear DNA exon–intron junctures 5′-GT/AG-3′ (Saisho et al. 1997). 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 vs 26% U on average for introns (Deutsch and Long 1999, Goodall and Filipowicz 1989, Ko et al. 1998). It was suggested that U-richness can be important for intron recognition (Ko et al. 1998).

The intron of 'Cobrançosa' presented seven SNPs and one InDel among the four sequences (Table 2). In the intron of 'Picual', five SNPs and one 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). However, after computational validation using the software MiPred, only two sequences of 'Cobrançosa' (1 and 3) presented a potential miRNA precursor with a higher probability of 65.9% (P-value = 0.004 considering a shuffle time of 1000x) (Table 3). Analysis of the predicted region allows the identification of variability between these two sequences of 'Cobrançosa' and all the other sequences analyzed. Two substitutions in the sequences Cob_1 and Cob_3 were identified: a substitution of C by G (position 703) combined with the substitution of G by T (position 754) (see Table 2).

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 in Fig. 7.

The sequence between 13 and 32 nt (5′-GAAAAUA-GGAAUUUGUUUGA-3′) was identified with homology to a miRNA of *A. thaliana* ath-miR417 (78-GAAG-GUAGUGAAUUUGUUCGA-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 an miRNA of *Oryza sativa* osa-miR417 (44-GAAUGUAGUGAAUUUGUUCCA-64) with a score of 82.5 (see Fig. 8).

3′-Untranslated region

Among the 3′-UTRs of 'Galega vulgar' sequences five SNPs and two single nt InDels were identified (Table 2). In the cultivar 'Cobrançosa' this region demonstrated five

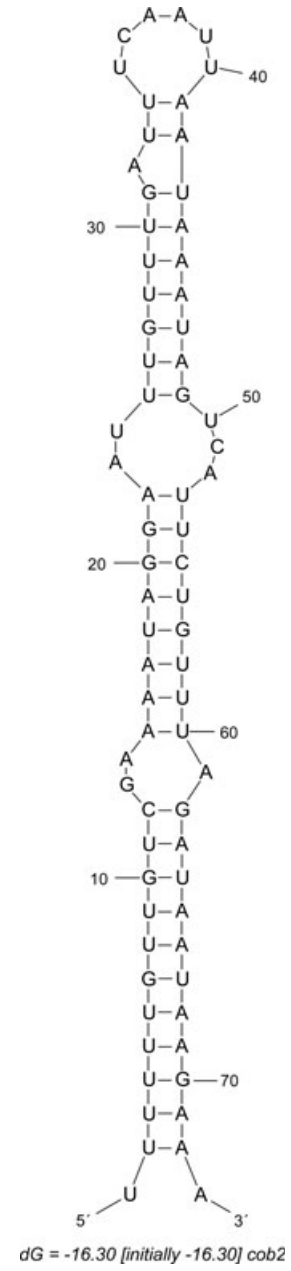

Fig. 7. Secondary structure of miRNA precursor predicted in the *OeAOX2* intron sequence of *O. europaea* cv. Cobrançosa.

SNPs and two single nt InDel. In 'Picual' the 3′-UTRs of sequences 1 and 3 are identical; however, between these and sequence 2 four 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).

The available sequence data for individual trees are restricted by the low number of studied bacterial clones (seven bacterial clones analyzed in 'Cobrançosa' and four in 'Picual'; for 'Galega vulgar' only one sequence was available). However, the data for the tree of 'Cobrançosa' and 'Picual' reveal the existence of heterogeneity for *OeAOX*2 through four different *OeAOX*2 sequences in 'Cobrançosa' and three varying sequences in 'Picual'. In both cultivars, the number of polymorphic gene sequences was enhanced by considering the intron level: 'Cobrançosa' showed three different sequences in the ORF and also three different sequences in the 3′-UTR; however, four different sequences were identified in the intron. 'Picual' demonstrated one identical sequence for the ORF, but two different sequences in the 3′-UTR and three polymorphic sequences considering the intron. Olive can be expected to be diploid. However, because no genetic analyses were performed at this stage in this study, the results should be seen as varying sequences, but cannot be interpreted as allelic variation.

3′-UTR sequence and length variability in transcripts of *OeAOX2*

Transcript analyses of the *OeAOX2* were performed from a bulked sample of five microshoots from cv. Galega vulgar. The data obtained highlight potential mechanisms for *OeAOX2* regulation and were included for demonstration. Table 4 shows the length of the 3′-UTR and the number of clones detected for each 3′-UTR size in 'Galega vulgar'. Transcripts of variable length resulted from alternative polyadenylation (AP). AP affects a large number of higher eukaryote mRNAs, producing mature transcripts with 3′-ends of variable length.

AP 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 cleavage site (CS), including the YA dinucelotide (CA or UA) in the CS and two U-rich regions, one before and another after the CS, both spanning about 5–10 nt (Loke

Table 3. Computational prediction of intronic miRNA precursors in *OeAOX2* of three *O. europaea* cultivars: 'Galega vulgar' (Gal), 'Cobranosa' (Cob) and 'Picual' (Pic) (bp corresponds to the length of the pre-miRNA sequence in bp, MFE corresponds to minimal free energy in kilocalories per mol, Prob. corresponds to probability to be a real pre-miRNA sequence with p-value = 0.004 and considering a shuffle time of 1000x).

Genotype	Putative pre-miRNA sequence	bp	MFE	Prob.
Gal_8	AUAGUAUUUUGUCAUUUUUUUGUCCCCUAUUAUCCACCCUUUUGC CUGCUUAUAUUUUGCAAGAUGAUGUGCCAGAUGCUAU	82	−13.22	No
Cob_2	UUUUUUGUUGUCGAAAAUAGGAAUUUGUUUGAUUUCAAUUAAUA AAUAGUCAUUCUGUUUAGAUAAUAAGAAA	73	−16.30	65.9%
Cob_3	AUAGUAUUUUGUCAUUUUUUUGUCCCCUAUUAUCCACCCUUUUGCC UGCUUAUAUUUUGCAAGAUGAUGUGCCAGAUGCUAU	81	−11.72	No
Cob_5	AUAGUAUUUUGUCAUUUUUUUUGUCCCCUAUUAUCCACCCUUUUGCC UGCUUAUAUUUUGCAAGAUGAUGUGCCAGAUGCUAU	82	−13.12	No
	UUUUUUGUUGUCGAAAAUAGGAAUUUGUUUGAUUUCAAUUAAUA AAUAGUCAUUCUGUUUAGAUAAUAAGAAA	73	−16.30	65.9%
Cob_9	AUAGUAUUUUGUCAUUUUUUUGUCCCCUAUUAUCCACCCUUUUGCC UGCUUAUAUUUUGCAAGAUGAUGUGCCAGAUGCUAU	81	−11.72	No
Pic_1	AUAGUAUUUUGUCAUUUUUUUGUCCCCUAUUAUCCACCCUUUUGCC UGCUUAUAUUUUGCAAGAUGAUGUGCCAGAUGCUAU	81	−11.72	No
Pic_2	AUAGUAUUUUGUCAUUUUUUUGUCCCCUAUUAUCCACCCUUUUGCC UGCUUAUAUUUUGCAAGAUGAUGUGCCAGAUGCUAU	81	−11.72	No
Pic_3	AUAGUAUUUUGUCAUUUUUUUGUCCCCUAUUAUCCACCCUUUUGCC UGCUUAUAUUUUGCAAGAUGAUGUGCCAGAUGCUAU	81	−11.72	No

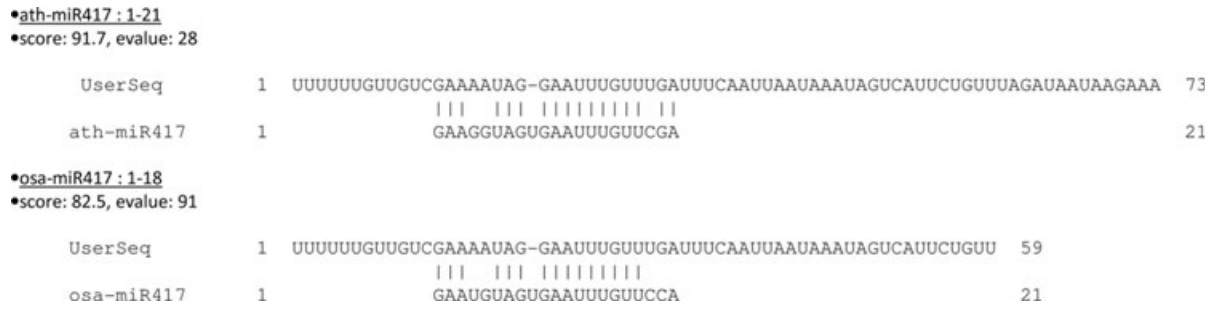

Fig. 8. Identification of a putative miRNA in *O. europaea*. Alignment of the predicted pre-miRNA of *OeAOX2* with ath-miR417 and osa-miR417.

et al. 2005). NUE is an A-rich region and spans about 6–10 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 25–125 nt upstream of the NUE (Loke et al. 2005). The alternative sites for the poly(A) signal are indicated in Fig. 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) includes the pattern UUGUUG found as 25th most frequent pattern in *A. thaliana*; the FUE.2 GUUUUGUU includes the patterns UUUGUU (1st), UUUUGU (2nd) and GUUUUG (22th); the FUE.3 region UGUUGUGU is related to the 21st and 23rd patterns identified in *A. thaliana* (UGUUGU and UUGUGU, respectively); the FUE.4 region, UAUUUUUGUAAGUUUGUUCUUU, is the largest region and belong to a group of seven patterns previously identified in *A. thaliana*: 2nd, 7th (UUUUUG), 9th (UUCUUU), 14th (UUUGUA), 16th (AUUUUU), 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 also includes the pattern UUGUAA referred by Hunt (1994) as typical for the FUE.

NUE are A-rich and three were identified in *OeAOX2*. NUE.1 is common to the second NUE pattern

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.

3-UTR size (bp)	Number of clones	Sequence identification
76	1	Gal_1
113	1	Gal_6
151	1	Gal_5
259	1	Gal_7
297	1	Gal_2
298	2	Gal_3
301	2	Gal_4

(UUAAUU) identified in *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, for Gal_5 in 151, for Gal_7 in 259, for Gal_2 in 297, for Gal_3 in 298 and for Gal_4 in 301. In all sequences a T-rich region was observed upstream the CS element that is characteristic for the CE.

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 (Axtell and Bartel 2005, Floyd and Bowman 2004). 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 targets sites of the plant miRNAs are mostly located in the protein-coding sequence acting in a way similar to RNA interference (Wang et al. 2004), but can also be found in the 3′-UTR (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 *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 *AOX* genes in root induction of olive shoot cuttings and (2) to reveal whether *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, H_2O_2 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. It was not the aim of this study to quantify electron partitioning between both respiration pathways (Lambers et al. 2005), but to observe the effect on a physiologic response. 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, b). *OeAOX2* was highly expressed 10 min after shoot cutting before the IBA treatment, decreased rapidly and was then increased at the time when root primordia started to be visible at histologic level (Peixe et al. 2007a). Further, application of H_2O_2 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, and 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 (Amirsadeghi et al. 2006, Clifton et al. 2006, Fiorani et al. 2005, Giraud et al. 2008, Umbach et al. 2005, Van Aken et al. 2009). The experiments presented indicate an involvement of both AOX and H_2O_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.

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

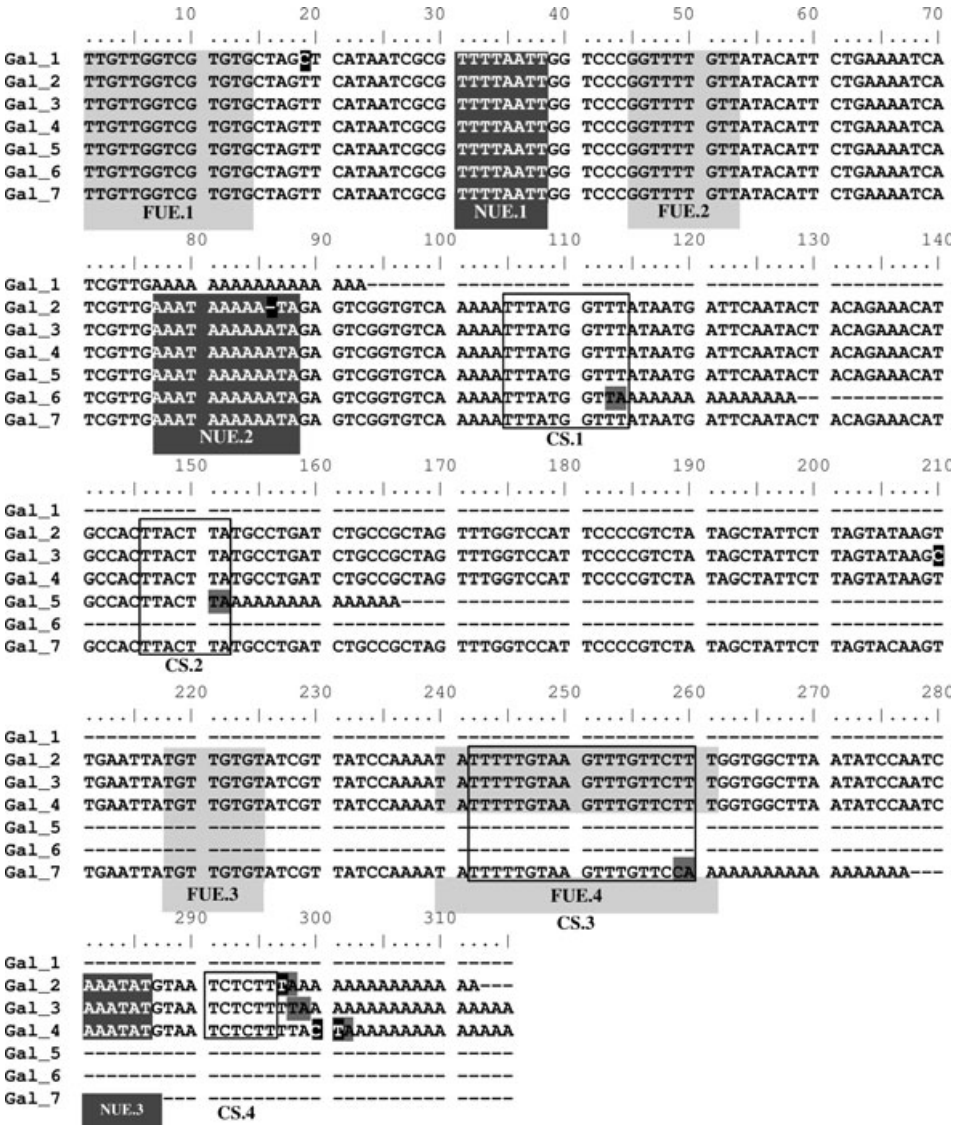

Fig. 9. Alignment of the 3′-UTR sequences identified in seven different *OeAOX2* transcripts of *O. europaea* cv. Galega vulgar.

ORF, one intron and the 3′-UTR regions. Recent knowledge of *AOX* gene regulation suggests that differential regulation cannot be sufficiently explained by defined motifs in the promoter region (see review Polidoros et al. 2009). Variation in within-gene sequences at genome level and 3′-UTR microheterogeneity 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. Sequence 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, as introns are typically known to exhibit a higher variability than protein-coding sequences (Gibbs 2003). As most parts of the ORF consist of the highly conserved region related to exon 3 from *A. thaliana* (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 found again in 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

Table 5. miRNA target sites predicted at the 3-UTR region of the sequence Gal_3 (software MIRANDA v3.0).

miRNA identification	nt position	Alignment			Energy (kcal mol^{-1})
ath-miR401 (Sunkar and Zhu 2004)	2-23	Query :	3' acAGCCAGC–UGUGGUCAAagc 5'		–22.10
			| : | | | | | : : : | : | | | |	
		Ref :	5' tgTTGGTCGTGTGCTAGTTcat 3'		
	35-51	Query :	3' acAGCCAGCUGUGGUCAAAGc 5'		–20.10
			| : | | | | | | : | | | | | :	
		Ref :	5' aaTTGGTC----CCGGTTTTg 3'		
ppt-miR1212 (Talmor-Nieman et al. 2006)	27-47	Query :	3' gcGUAAGAUACGACAGGGUgc 5'		–21.36
			| : | | : | | : | | | | | :	
		Ref :	5' cgCGTTTTAATTGGTCCCGgt 3'		
smo-miR1110 (Axtell et al. 2007)	153-171	Query :	3' agGAACUGGUGACGGGGAUCg 5'		–22.56
			| | | : | | | | | | | |	
		Ref :	5' tgCCTGAT-- CTGCCGCTAGt 3'		
tae-miR1125	158-177	Query :	3' ggcGGCGUCAACCAGAGCAACCAa 5'		–23.97
			| : | | | | | | | | | | | | |	
		Ref :	5' gatCTGCCGCTAGT----TTGGTc 3'		

cultivar. This is of special interest for our approach, as 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 *AOX1a* of *O. sativa* (*OsAOX1a*) leading to a non-synonymous translation, which was completely linked to the presence of the quantitative trait locus (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 di-iron binding site of RADE_ _H box where Asp is found to be highly conserved among diverse species. The effect of substitutions in neighbor residues of the di-iron binding sites had already been demonstrated. Nakamura et al. (2005) reported in *Trypanosoma 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 L*E*EEA 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 *Sauromatum 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 four 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 heterogenous 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 physiologic transcripts (Lew et al. 1998, Culbertson 1999). Nonsense mutations are related to a decrease of correspondent gene transcript accumulation (Aung et al. 2006, Nawrath et al. 2002). Hori 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 junction are recognized as substrates for NMD. In both cultivars of olive the premature stop codon is located at 160 nts upstream of the 3′-most exon–exon junction. 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 (Aung et al. 2006, Olsson et al. 2004, Sattler et al. 2009).

Introns are known to participate in NMD (Frischmeyer and Dietz 1999) as a mechanism of gene expression control. This knowledge confirmed the important role of plant introns in the control of gene expression (Fiume et al. 2004, Gianì et al. 2003, Rose 2002) and contributed that recently introns are gaining new credit in the scientific community (Rodríguez-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, because of 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 with 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 the 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 also be related with nonsense mutations (Aoufouchi et al. 1996), which can be caused by SNPs (Aung et al. 2006, Isshiki et al. 2001, Nawrath et al. 2002, 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, 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. 2004), 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 an SNP at position 703 (G/C) combined with an SNP at the position 754 (T/G). This is an example of how a polymorphism can influence prediction of regulatory sequences. A similar example, where predictability of a pre-miRNA site was also influenced by DNA polymorphism, was reported for intron 3 of the carrot *AOX2a* gene (Cardoso et al. 2009) and intron 1 of St John's Wort *AOX1b* (Ferreira et al. 2009). In both cases the existence of an InDel event was related with the predictability of the pre-miRNA site. 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' microshoots, heterogeneity in 3′-UTR size was revealed as a result of both local microheterogeneity and AP. 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 five clones in sequences Gal_2, Gal_3 and Gal_4 (Table 4). AP could be considered for 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 CS is missing that was found in all other 3′-UTRs. The presence of five classes of AP in *OeAOX2* in a single cultivar raises the possibility for differential regulation of this gene in any given tissue or organ (Polidoros et al. 2009). 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). 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 suggest how the modulation of the 3′-UTR length can have significant effects on the regulation of *AOX* genes. A search for miRNA sites in olive 3′-UTR revealed five putative miRNA targets that had an overall pairing energy of $\Delta G < -20$ kcal mol^{-1}. 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. There are several methodologies for biologic validation of predicted miRNA targets that can be applied in the future, which include reported-gene constructs, mutation studies, gene-silencing techniques, rescue assays and classic genetic studies.

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 lengths have 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.

Acknowledgements – The authors highly appreciate funding of the Marie Curie Chair project through the European Commission. Research is further supported by national fellowships from Fundação para a Ciência e a Tecnologia (SFRH/ BD/22061/2005; SFRH/BI/15993/2006 and SFRH/BPD/27016/2006). The authors would like to thank Jorge Boehm, Viveiros Plansel Lda., for providing us olive plant material of the cultivars 'Galega vulgar' and 'Cobrançosa'.

References

Abe F, Saito K, Miura K, Toriyama K (2002) A single nucleotide polymorphism in the alternative oxidase gene among rice varieties differing in low temperature tolerance. FEBS Lett 527: 181–185

Achard P, Herr A, Baulcombe DC, Harberd NP (2004) Modulation of floral development by a gibberellin-regulated micro-RNA. Development 131: 3357–3365

Albury MA, Affourtit C, Crichton PG, Moore AL (2002) Structure of the plant alternative oxidase: site-directed mutagenesis provides new information on the active site and membrane topology. J Biol Chem 277: 1190–1194

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410

Amirsadeghi S, Robson CA, McDonald AE, Vanlerberghe GC (2006) Changes in plant mitochondrial electron transport alter cellular levels of reactive oxygen species and susceptibility to cell death signaling molecules. Plant Cell Physiol 47: 1509–1519

Amirsadeghi S, Robson CA, Vanlerberghe GC (2007) The role of the mitochondrion in plant responses to biotic stress. Physiol Plant 139: 253–266

Andersson ME, Nordlund P (1999) A revised model of the active site of alternative oxidase. FEBS Lett 449: 17–22

Aoufouchi S, Yélamos J, Milstein C (1996) Nonsense mutations inhibit RNA splicing in a cell-free system: recognition of mutant codon is independent of protein synthesis. Cell 85: 415–422

Arnholdt-Schmitt B (2004) Stress-induced cell reprogramming. A role for global genome regulation? Plant Physiol 136: 2579–2586

Arnholdt-Schmitt B (2005) Functional markers and a 'systemic strategy': convergency between plant breeding, plant nutrition and molecular biology. Plant Physiol Biochem 43: 817–820

Arnholdt-Schmitt B, Costa J.H., Fernandes de Melo, D. (2006a) AOX–A functional marker for efficient cell reprogramming under stress? Trends Plant Sci 11: 281–287

Arnholdt-Schmitt B, Macedo ES, Peixe A, Cardoso HCG, Cordeiro AM (2006b) AOX–a potential functional marker for efficient rooting in olive shoot cuttings. Proceedings of 2nd International Seminar Olivebioteq, Marsala, Mazara del Vallo, Italy, Vol I, pp 249–254

Aung K, Lin S-I, Wu C-C, Huang Y-T, Su C-I, Chiou T-J (2006) *pho2*, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. Plant Physiol 141: 1000–1011

Axtell MJ, Bartel DP (2005) Antiquity of microRNAs and their targets in land plants. Plant Cell 17: 1658–1673

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281–297

Berthold DA (1998) Isolation of mutants the *Arabidopsis thaliana* alternative oxidase (ubiquinol: oxygen oxidoreductase) resistant to salicylhydroxamic acid. Biochim Biophys Acta 1364: 73–83

Berthold DA, Andersson ME, Nordlund P (2000) New insight into the structure and function of the alternative oxidase. Biochim Biophys Acta 1460: 241–254

Byers PH (2002) Killing the messenger: new insights into nonsense-mediated mRNA decay. J Clin Invest 109: 3–6

Camacho A, Moreno-Sanchez R, Bernal-Lugo I (2004) Control of superoxide production in mitochondria from maize mesocotyls. FEBS Lett 570: 52–56

Cardoso HG, Campos MD, Costa AR, Campos MC, Nothnagel T, Arnholdt-Schmitt B (2009) Carrot alternative oxidase gene AOX2a demonstrates allelic and genotypic polymorphisms in intron 3. Physiol Plant 137: 592–608

Chiou TJ, Aung K, Lin SI, Wu CC, Chiang SF, Su C (2006) Regulation of phosphate homeostasis by microRNA in Arabidopsis. Plant Cell 18: 412–421
Clifton R, Millar AH, Whelan J (2006) Alternative oxidases in Arabidopsis: a comparative analysis of differential expression in the gene family provides new insights into function of non-phosphorylating bypasses. BBA-Bioenergetics 1757: 730–741
Collins NC, Tardieu F, Tuberosa R (2008) Quantitative trait loci and crop performance under abiotic stress: where do we stand? Plant Physiol 147: 469–486
Conti E, Izaurralde E (2005) Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species. Curr Opin Cell Biol 17: 316–325
Crichton PG, Affourtit C, Albury MS, Carré JE, Moore AL (2005) Constitutive activity of *Sauromatum guttatum* alternative oxidase in *Schizosaccharomyces pombe* implicates residues in addition to conserved cysteins in α-keto acid activation. FEBS Lett 579: 331–336
Culbertson MR (1999) RNA surveillance: unforeseen consequences for gene expression, inherited genetic disorders and cancer. Trends Genet 15: 74–80
Deutsch M, Long M (1999) Intron–exon structures of eukaryotic model organisms. Nucleic Acids Res 27: 3219–3228
Dietz HC, Valle D, Francomano CA, Kendzior RJ Jr, Pyeritz RE, Cutting GR (1993) The skipping of constitutive exons *in vivo* induced by nonsense mutations. Science 259: 680–683
Dunand C, Crèvecoeur M, Penel C (2007) Distribution of superoxide and hydrogen peroxide in Arabidopsis root and their influence on root development: possible interaction with peroxidases. New Phytol 174: 332–341
Feng H, Li X, Duan J, Li H, Liang H (2008) Chilling tolerance of wheat seedlings is related to an enhanced alternative respiratory pathway. Crop Sci 48: 2381–2388
Ferreira AO, Cardoso HG, Macedo ES, Breviario D, Arnholdt-Schmitt B (2009) Intron polymorphism pattern in *AOX1b* of wild St John's wort (*Hypericum perforatum*) allows discrimination between individual plants. Physiol Plant 137: 520–531
Fiorani F, Umbach AL, Siedow J (2005) The alternative oxidase of plant mitochondrial is involved in the acclimation of shoot growth at low temperature. A study of Arabidopsis AOX1a transgenic plants. Plant Physiol 139: 1795–1805
Fiume E, Christou P, Gianí S, Breviario D (2004) Introns are key elements of rice tubulin expression. Planta 218: 693–703
Floyd SK, Bowman JL (2004) Gene regulation: ancient micro-RNA target sequences in plants. Nature 428: 485–486
Frederico AM, Campos MD, Cardoso HG, Imani J, Arnholdt-Schmitt B (2009) Alternative oxidase involvement in *Daucus carota* somatic embryogenesis. Physiol Plant 137: 498–508
Frischmeyer PA, Dietz HC (1999) Nonsense-mediated mRNA decay in health and diseases. Hum Mol Genet 8: 1893–1900
Gianì S, Morello L, Bardini M, Breviario D (2003) Tubulin intron sequences: multi-functional tools. Cell Biol Int 27: 203–205
Gibbs WW (2003) The unseen genome: gems among the junk. Sci Am 289: 46–53
Giraud E, Ho LH, Clifton R, Carroll A, Estavillo G, Tan YF, Howell KA, Ivanova A, Pogson BJ, Millar AH, Whelan J (2008) The absence of alternative oxidase1a in *Arabidopsis* results in acute sensitivity to combined light and drought stress. Plant Physiol 147: 595–610
Goodall GJ, Filipowicz W (1989) The AU-rich sequences present in the introns of plant nuclear pre-mRNAs are required for splicing. Cell 58: 473–483
Goto Y, Yue L, Yokoi A, Nishimura R, Uehara T, Koizumi S, Saikawa Y (2001) A novel single-nucleotide polymorphism in the 3′-untranslated region of the human dihydrofolate reductase gene with enhanced expression. Clin Cancer Res 7: 1952–1956
Gray G, Maxwell D, Villarimo A, McIntosh L (2004) Mitochondria/nuclear signaling of alternative oxidase gene expression occurs through distinct pathways involving organic acids and reactive oxygen species. Plant Cell Rep 23: 497–503
Hall GW, Thein S (1994) Nonsense codon mutations in the terminal exon of the β-globin gene are not associated with a reduction in β-mRNA accumulation: a mechanism for the phenotype of dominant β-thalassemia. Blood 83: 2031–2037
Hall TA (1999) BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. Nucleic Acids Symp Ser 41, 95–98
Hansen LD, Thomas NR, Arnholdt-Schmitt B (2009) Temperature responses of substrate carbon conversion efficiencies and growth rates of plants tissues. Physiol Plant 137: 446–458
Hori K, Watanabe Y (2007) Context analysis of termination codons in mRNA that are recognized by plant NMD. Plant Cell Physiol 48: 1072–1078
Hull J, Campino S, Rowlands K, Chan M-S, Copley RR, Taylor MS, Rockett K, Elvidge G, Keating B, Knight J, Kwiatkowski D (2007) Identification of common genetic variation that modulates alternative splicing. PLoS Genet 3: 1009–1018
Hunt AG (1994) Messenger RNA 3′end formation in plants. Ann Rev Plant Physiol Plant Mol Biol 45: 47–60
Isshiki M, Yamamoto Y, Satoh H, Shimamoto K (2001) Nonsense-mediated decay of mutant waxy mRNA in rice. Plant Physiol 125: 1388–1395
Jiang J, Gusev Y, Aderca I, Mettler TA, Nagorney DM, Brackett DJ, Roberts LR, Schmittgen TD (2008)

Micro-RNA expression in hepatocellular carcinoma is associated with hepatitis infection, cirrhosis and patient's survival. Clin Cancer Res 14: 419–427
Juszczuk IM, Rychter AM (2003) Pyruvate accumulation during phosphate deficiency stress of bean roots. Plant Physiol Biochem 40: 783–788
Kawase T, Akatsuka Y, Torikai H, Morishima S, Oka A, Tsujimura A, Miyazaki M, Tsujimura K, Miyamura K, Ogawa S, Inoko H, Morishima Y, Kodera Y, Kuzushima K, Takahashi T (2007) Alternative splicing due to an intronic SNP in HMSD generates a novel minor histocompatibility antigen. Blood 110: 1055–1063
Ko CH, Brendel V, Taylor RD, Walbot V (1998) U-richness is a defining feature of plant introns and may function as an intron recognition signal in maize. Plant Mol Biol 36: 573–583
Ladd AN, Cooper TA (2002) Finding signals that regulate alternative splicing in the post-genomic era. Genome Biol 3: 4–16
Lambers H, Robinson SA, Ribas-Carbo M (2005) Regulation of respiration in vivo. In: Lambers H, Ribas-Carbo M (eds) Plant respiration: From Cell to Ecosystem. Advances in Photosynthesis and Respiration, Vol. 18. Springer, Dordrecht, pp 1–15
Lambert JC, Zimmer EL, Merrot S, Hayes A, Thaker U, Desai P, Houzet A, Hermant X, Cottel D, Pritchard A, Iwatsubo T, Pasquier F, Frigard B, Conneally PM, Chartier-Harlin MC, DeKosky ST, Lendon C, Mann D, Kamboh MI, Amouyel P (2003) Association of 3'-UTR polymorphisms of the oxidised LDL receptor 1 (OLR1) gene with Alzheimer's disease. J Med Genet 40: 424–430
Lew JE, Enomoto S, Berman J (1998) Telomere length regulation and telomeric chromatin require the nonsense-mediated mRNA decay pathway. Mol Cell Biol 18: 6121–6130
Li Q, Hunt AG (1995) A near upstream element in a plant polyadenylation signal consists of more than six bases. Plant Mol Biol 28: 927–934
Liu HX, Zhang M, Krainer AR (1998) Identification of functional exonic splicing enhancer motifs recognized by individual SR proteins. Gene Dev 12: 1998–2012
Loio RD, Nakamura K, Moubayidin L, Perilli S, Taniguchi M, Morita MT, Aoyama T, Costantino P, Sabatini S (2008) A genetic framework for the control of cell division and differentiation in the root meristem. Science 322: 1381–1384
Loke JC, Stahlberg EA, Strenski DG, Haas BJ, Wood PC, Li QQ (2005) Compilation of mRNA polyadenylation signals in Arabidopsis revealed a new signal element and potential secondary structures. Plant Physiol 138: 1457–1468
Mallory AC, Reinhart BJ, Jones-Rhoades MW, Tang G, Zamore PD, Barton MK, Bartel DP (2004) MicroRNA control of Phabulosa in leaf development: importance of pairing to the microRNA 5′ region. EMBO J 18: 3356–3364
Mathews DH, Sabina J, Zuker M, Turner DH (1999) Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J Mol Biol 288: 911–940
Mizutani F, Shinohara K, Amano S, Hino A, Kadoya K, Akiyoshi H, Watanabe J. (1994) Effect of KCN and SHAM on bud break and rooting of single-eye cuttings of 'Kyoho' grape. Bull Exp Farm Coll Agr, Ehime Univ, 15: 1–5
Nag S, Saha K, Choudhuri MA (2001) Role of auxin and polyamines in adventitious root formation in relation to changes in compounds involved in rooting. J Plant Growth Regul 20: 182–194
Nakamura K, Sakamoto K, Kido Y, Fujimoto Y, Suzuki T, Suzuki M, Yabu Y, Ohta N, Tsuda A, Onuma M, Kita K (2005) Mutational analysis of the *Trypanosoma vivax* alternative oxidase: the E(X)6Y motif is conserved in both mitochondrial alternative oxidase and plastid terminal oxidase and is indispensable for enzyme activity. Biochem Biophys Res Commun 334: 593–600
Nawrath C, Heck S, Parinthawong N, Métraux J-P (2002) EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in Arabidopsis, is a member of the MATE transporter family. Plant Cell 14: 275–286
Novelli G, Borgiani P, Mango R, Lauro R, Romeo F (2007) Further evidence that polymorphisms of the OLR1 gene are associated with susceptibility to coronary artery disease and myocardial infarction. Nutr Metab Cardiovas 17: 7–8
Oliver SN, Lunn JE, Urbanczyk-Wochniak E, Lytovchenk A, Dongen JTv, Faix B, Schmälzlin E, Alisdair RF, Geigenberger P (2008) Decreased expression of cytosolic pyruvate kinase in potato tubers leads to a decline in pyruvate resulting in an in vivo repression of the alternative oxidase. Plant Physiol 148: 1640–1654
Olsson U, Sirijovski N, Hansson M (2004) Characterization of eight barley xantha-f mutants deficient in magnesium chelatase. Plant Physiol Biochem 42: 557–564
Peixe A, Raposo A, Lourenço R, Cardoso H, Macedo E (2007a) Coconut water and BAP successfully replaced zeatin in olive (*Olea europaea* L.) micropropagation. Sci Hortic 113: 1–7
Peixe A, Serras M, Campos C, Zavattieri MA, Dias MAS (2007b) Estudo histológico sobre a formação de raízes adventícias em estacas caulinares de oliveira (*Olea europaea* L.): a histological evaluation. Rev Ciências Agrárias 30: 476–482

Polidoros AN, Mylona PV, Arnholdt-Schmitt B (2009) AOX gene structure, transcript variation and expression in plants. Physiol Plant 137: 342–353
Polidoros NA, Mylona PV, Pasentsis K, Scandalios JG, Tsaftaris AS (2005) The maize alternative oxidase 1a (Aox1a) gene is regulated by signals related to oxidase stress. Redox Rep 18: 71–78
Popov VN, Simonian RA, Skulachev VP, Starkov AA (1997) Inhibition of the alternative oxidase stimulates H_2O_2 production in plant mitochondria. FEBS Lett 415: 87–90
Potters G, Pasternak TP, Guisez Y, Palme KJ, Jansen MAK (2007) Stress-induced morphogenic responses: growing out of trouble? Trends Plant Sci 12: 98–105
Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP (2002) MicroRNAs in plants. Genes Dev 16: 1616–1626
Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP (2002) Prediction of plant microRNA targets. Cell 110: 513–520
Rodríguez-Trelles F, Tarrío R, Ayala FJ (2006) Origins and evolution of spliceosomal introns. Annu Rev Genet 40: 47–76
Rose AB (2002) Requirements for intron-mediated enhancement of gene expression in Arabidopsis. RNA 8: 1444–1453
Roy SW, Gilbert W (2006) The evolution of spliceosomal introns: patterns, puzzles and progress. Nature Rev Genet 7: 211–221
Rugini E, Francesco D, Muganu G, Astolfi M, Caricato SG (1997) The effects of polyamines and hydrogen peroxide on root formation in olive and the role of polyamines as an early marker for rooting ability. In: Altman A, Waisel Y (eds) Biology of Root Formation and Development. Plenum Press, New York, pp 65–73
Saisho D, Nambara E, Naito S, Tsutsumi N, Hirai A, Nakazono M (1997) Characterization of the gene family for alternative oxidase from *Arabidopsis thaliana*. Plant Mol Biol 35: 585–596
Sambrook J, Fritsch EF, Maniatis TH (1989) Molecular Cloning–A Laboratory Manual, 2nd Edn. ColdSpring Habour Laboratory Press, New York.
Santos Macedo E, Cardoso H, Peixe A, Arnholdt-Schmitt B (2006a). AOX de *Olea europaea* L – Isolamento e clonagem de um multigene. Melhoramento 41: 57–63
Santos Macedo E, Cardoso H, Peixe A, Arnholdt-Schmitt B (2006b). Isolation of alternative oxidase (AOX) genes of *Olea europaea* L. Proceedings of the 2nd International Seminar Olivebioteq, Marsala, Vol. I. Mazara del Vallo, Italy, pp 131–134
Sasaki K, Kishitani S, Abe F, Sato T (2005) Promotion of seedling growth of seeds of rice (*Oryza sativa* L. cv. hitomebore) by treatment with H_2O_2 before sowing. Plant Prod Sci 8: 509–514
Sattler SE, Saathoff AJ, Haas EJ, Palmer NA, Funnell-Harris DL, Sarath G, Pedersen JF (2009) A nonsense mutation in a cinnamyl alcohol dehydrogenase gene is responsible for the sorghum brown midrib-6 phenotype. Plant Physiol 150: 584–595
Sebastiani L, Tognetti R (2004) Growing season and hydrogen peroxide effects on root induction and development in *Olea europaea* L. (cvs 'Frantoio' and 'Gentile di Larino') cuttings. Sci Hort 100: 75–82
Seli E, Yaba A, Guzeloglu-Kayisli O, Lalioti MD (2008) Alternative splicing of the mouse embryonic poly(A) binding protein (Epab) mRNA is regulated by an exonic splicing enhancer: a model for post transcriptional control of gene expression in the oocyte. Mol Hum Reprod 14: 393–398
Shane MW, Cramer MD, Noguchi SF, Cawthray GR, Millar AH, Day DA, Lambers H (2004) Developmental physiology of cluster-root carboxylate synthesis and exudation in harsh hakea. Expression of phosphoenolpyruvate carboxylase and the alternative oxidase 1. Plant Physiol 135: 549–560
Shen Y, Ji G, Haas BJ, Wu X, Zheng J, Reese GJ, Li QQ (2008) Genome level analysis of rice mRNA 3′-end processing signals and alternative polyadenylation. Nucleic Acids Res 36: 3150–3161
Siedow JN, Umbach AL (1995) Plant mitochondrial electron transfer and molecular biology. Plant Cell 7: 821–831
Siedow JN, Umbach AL, Moore AL (1995) The active hypothesis site of the cyanide-resistant oxidase from plant mitochondria contains a binuclear iron center. FEBS Lett 362: 10–14
Song J, Murdoch J, Gardiner SE, Young A, Jameson PE, Clemens J (2006) Molecular markers and a sequence deletion in intron 2 of the putative partial homologue of LEAFY reveal geographical structure to genetic diversity in the acutely threatened legume genus *Clianthus*. Biol Conserv 141: 2041–2053
Stark A, Brennecke J, Bushati N, Russell RB, Cohen SM (2005) Animal microRNAs confer robustness to gene expression and have a significant impact on 3′ UTR evolution. Cell 123: 1133–1146
Talmor-Neiman M, Stav R, Frank W, Voss B, Arazi T (2006) Novel micro-RNAs and intermediates of micro-RNA biogenesis from moss. Plant J 47: 25–37
Umbach AL, Fiorani F, Siedow JN (2005) Characterization of transformed Arabidopsis with altered alternative oxidase levels and analysis of effects on reactive oxygen species in tissue. Plant Physiol 139: 1806–1820
Valentine CR (1998) The association of nonsense codons with exon skipping. Mutat Res 411: 87–117
Van Aken O, Giraud E, Clifton R, Whelan J (2009) Alternative oxidase: a target and regulator of stress responses. Physiol Plant 137: 354–361

Vanlerberghe GC, McIntosh L (1996) Signals regulating the expression of the nuclear gene encoding alternative oxidase of plant mitochondria. Plant Physiol 111: 589–595

Vanleberghe GC, Cvetkovska M, Wang J (2009) Is the maintenance of homeostatic mitochondrial signaling during stress a physiological role for alternative oxidase? Physiol Plant 137: 392–406

Wang JF, Zhou H, Chen YQ, Luo QJ, Qu LH (2004) Identification of 20 microRNAs from *Oryza sativa*. Nucleic Acids Res 32: 1688–1695

Wang X, Zhao X, Zhu J, Wu W (2005) Genome-wide investigation of intron length polymorphisms and their potential as molecular markers in rice (*Oryza sativa* L.) DNA Res 12: 417–427

Xie F, Huang S, Guo K, Xiang A, Zhu Y, Nie L, Yang Z (2007) Computational identification of novel microRNAs and targets in *Brassica napus*. FEBS Lett 581: 1464–1474

Zavattieri MA, Frederico AM, Lima M, Sabino R, Arnholdt-Schmitt B (2009) Induction of somatic embryogenesis as an example of stress-related plant reactions. Electronic J Biotech Available from: http://www.ejbiotechnology.info/content/next/abstract/a12.html

Zhang X, Gureasko J, Shen K, Cole PA, Kuriyan J (2006) An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. Cell 125: 1137–1149

Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31: 3406–3415

Edited by V. Hurry