Behaviours of *Medicago truncatula*–*Sinorhizobium meliloti* Symbioses Under Osmotic Stress in Relation with the Symbiotic Partner Input: Effects on Nodule Functioning and Protection

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Introduction

Legumes in symbiosis with rhizobia have the ability to support production of protein-rich seeds and fodder by fixing atmospheric nitrogen, in addition to their beneficial effect on productivity of cereals and other crops in agricultural rotations (Graham and Vance 2003). However, this association is often compromised by severe environmental stresses, mainly salinity and drought (Zahran 1999). As much as 60 % of legume production in the developing world occurs under conditions of significant drought stress (Graham and Vance 2003, Zhang et al. 2007). It is therefore important to elucidate the drought tolerance mechanisms of these species in order to improve their agronomic performance. Nevertheless, the variability of legume responses to osmotic stress and the complexity of their genetic control make it difficult to find ways to alleviate water deficit-induced stress. Moreover, the interaction of plant with the micro-symbiont further convolutes the understanding of symbiosis tolerance mechanisms. The symbiosis is an associative mode-life between two partners where each one contributes to the energy charge and benefits from the produced nutrients. Plant behaviour is a determinant factor for the success of symbiosis under stressful conditions (Sadiki and Rabih 2001, Djilianov et al. 2003). The infection process is highly sensitive to stress, although rhizobia are tolerant even to stress conditions that inhibit plant growth (Zahran 1999), and have a

Keywords

antioxidant enzymes; nitrogen-fixing capacity; osmotic stress; symbiotic interactions; tolerance

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Abstract

Three genotypes of the model legume *Medicago truncatula* were assessed for symbiotic effectiveness in cross inoculation with two strains of *Sinorhizobium meliloti* under mannitol-mediated osmotic stress. Symbioses showed different tolerance levels revealed on plant growth, nitrogen-fixing capacity and indices of nodule functioning and protection. The variability of stress response was essentially correlated with performance at non-stressful conditions. Symbiosis attitude depended on bacterial partner, host-plant genotype and their interaction. Plant genotype manifested the highest contribution to symbiotic efficiency indices under osmotic stress, even for nodulation and nitrogen fixation where the bacterial strain effect is highly pronounced. Contrasting (tolerant/sensitive) associations were identified for tolerance behaviours, involving the same plant genotype with different rhizobial strains and vice versa. In nodules, osmotic stress leads to accumulation of oxidized lipids and decrease in total protein and leghaemoglobin contents. Antioxidant responses were manifested as induction of guaiacol peroxidase (POX, E.C. 1.11.1.7) and superoxide dismutase (E.C. 1.15.1.1). POX induction was higher in tolerant symbioses and both enzymes were suggested as contributors to the protection of nodule integrity and functioning under osmotic stress. In conclusion, symbiotic efficiency in *M. truncatula*–*S. meliloti* combinations under osmotic stress is determined by each symbiont’s input as well as the plant–microbe genotype interaction, and POX induction could prove a sensitive marker of tolerant symbioses.
significant input into the tolerance level (Mhadhbi et al. 2004, 2008, Zacarias et al. 2004, Mnasri et al. 2007). In nodules, one of the earlier results of osmotic constraint is the over-production of reactive oxygen species (ROS) (Figueiredo et al. 2007, Naya et al. 2007, Zhang and Nan 2007). ROS lead to damage of tissue integrity and nodule function through oxidative attack to lipids, proteins and nucleic acids (Becana et al. 2000, Matamoros et al. 2003, Porcel et al. 2003, Naya et al. 2007). Symbiosis response to oxidative stress includes morphological modifications such as the change of nodule cortex structure (Matamoros et al. 1999), and biochemical adaptations such as the modulation of antioxidant enzyme expression in nodules. Several reports underline the intimate relationship between enhanced or constitutive antioxidant enzyme activities and increased symbiosis resistance to environmental stress (Mhadhbi et al. 2004, Tejera et al. 2004, Marino et al. 2007, Zhang and Nan 2007). After Medicago truncatula was chosen as a suitable plant model for studying legume-rhizobia interactions, numerous studies of molecular, genetic, proteomic and physiological aspects have focused on its symbiosis with Sinorhizobium meliloti (Barker et al. 1990, Rolfe et al. 2003, Badri et al. 2007, Lopez et al. 2008, Rose 2008). Therefore, understanding the physiological and biochemical mechanisms conferring drought tolerance to this symbiosis is very important for the development of selection and breeding strategies.

In the present work, we examined the effect of mannitol-mediated osmotic stress on the behaviour of different M. truncatula–S. meliloti associations. The analyses were performed in order to investigate the interaction of plant–bacteria under stressful conditions, and to subsequently identify symbioses with contrasting (tolerant/sensitive) response to osmotic stress. Antioxidant enzyme activities in the nodules of the contrasting symbioses were estimated in order to identify potential biochemical markers of specific responses to osmotic stress.

Material and Methods

Biological material

This study was performed with three M. truncatula genotypes: a reference one (Jemalong J6) and two local genotypes (TN8.20 and TN6.18) from different Tunisian regions (Mhadhbi et al. 2005). Plants were inoculated with two S. meliloti strains: one reference (RCR2011) and one native (TII7) strain (Zribi et al. 2004).

Glasshouse trial

Medicago truncatula seeds were sterilized in concentrated sulphuric acid for 6 min, rinsed several times with sterile distilled water and then germinated on 0.9 % agar in Petri plates at 4 ºC for 4 days. Germinated seeds were transferred onto sterile perlite, and then inoculated (approximately 10⁶ cfu). One week later, each seedling was transferred into 0.5 l syrup bottles containing nutrient solution (Mhadhbi et al. 2005). The seedlings were then re-inoculated (10⁶ cfu). The nutrient solution was aerated with airflow of 400 ml min⁻¹. The experiment was conducted in a glasshouse at 25 ºC and 16/8 h (day/night) photoperiods. Osmotic stress was induced with the addition of 75 mM mannitol to the nutrient solution (~215 kPa). Plants were harvested at the flowering stage.

Nitrogen-fixing assay

Nitrogenase (E.C. 1.7.9.92) was assayed by acetylene reduction activity (ARA; Hardy et al. 1968) using a gas chromatograph (HP 4890A; Hewlett Packard, Palo Alto, CA, USA) with a Porapak-T column. Nodule-bearing roots were incubated in 10 % C₂H₄ atmosphere. After 60 min, three replicates of 0.5 ml samples were withdrawn from the root atmosphere of each plant (three plants/association) and ethylene production was measured by gas chromatography. Pure acetylene and ethylene were used as internal standards (Mhadhbi et al. 2005).

Leghaemoglobin content

The content of leghaemoglobin in nodules was determined by the extraction of the pigment in Drabkin’s solution using the method described by Shiffmann and Lobel (1970) with few modifications. Fresh nodules (100 mg) were homogenized in 3 ml Drabkin’s solution. The homogenate was centrifuged at 5000 g for 15 min. The supernatant was added to 10 ml of Drabkin’s solution, homogenized and centrifuged for 30 min at 15 000 g. The supernatant was then collected and absorbance was determined at 540 nm. The content of leghaemoglobin was estimated referring to a standard curve determined from samples of bovine haemoglobin monitored at the same conditions.

Lipid peroxidation assay

Lipid peroxidation in nodules was assayed using the thiobarbituric acid (TBARS) method modified according to Singh et al. (2007). This test determines malondialdehyde (MDA) as an end product of the TBARS reaction. Nodules (300 mg) were homogenized in 3 ml of 0.1 % TCA solution. The homogenate was centrifuged at 10 000 g for 20 min and 0.5 ml of the supernatant was added to 1 ml of 0.5 % TBA in 20 % TCA. The mixture was incubated in boiling water for 30 min, and the reaction stopped by...
placing the reaction tubes in an ice bath. The samples were then centrifuged at 10 000 g for 5 min, and the absorbance of the supernatant was read at 532 nm. The value for nonspecific absorption at 600 nm was subtracted. The amount of MDA was calculated using the extinction coefficient $\varepsilon = 155 \text{ mm}^{-1} \text{ cm}^{-1}$.

Preparation of enzyme extracts

All operations were performed at 4 °C to maintain enzyme activity. Extracts were prepared by homogenizing 0.2 g of nodules in a mortar with 10 % (w/w) polyvinylpolypyrrolidone and 1 ml of 50 mM phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1 % (v/v) Triton X-100, 1 mM phenylmethylsulphonyl fluoride (PMSF) as an inhibitor of proteases, added with 5 mM ascorbate for APX activity. Extracts were centrifuged at 13 000 g for 20 min and the supernatant was used to determine enzyme activities. Protein rate was measured according to the method of Bradford (1976) using bovine serum albumin as a standard.

Enzyme assays

Superoxide dismutase (SOD) activity was determined spectrophotometrically according to Beauchamp and Fridovich (1971) by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50 % at 25 °C.

Spectrophotometric assays of the other antioxidant enzymes were performed by monitoring the formation of tetraguaiacol from guaiacol at 470 nm for POX ($\varepsilon = 26.6 \text{ mm}^{-1} \text{ cm}^{-1}$), the decomposition of H$_2$O$_2$ at 240 nm for catalase (CAT; $\varepsilon = 36 \text{ m}^{-1} \text{ cm}^{-1}$) and the disappearance of ascorbate at 290 nm for ascorbate peroxidase (APX; $\varepsilon = 2.8 \text{ mm}^{-1} \text{ cm}^{-1}$) as described previously (Mhadhbi et al. 2005).

Table 1: Effect of mannitol-mediated osmotic stress (75 mm) on shoot dry biomass production (SDW, g/plant), root dry biomass (RDW, mg/plant), number of nodules (NN) and nodule fresh biomass (NFW, mg/plant) of Medicago truncatula genotypes Jemalong J6, TN8.20 and TN6.18 inoculated by RCR2011 and TII7 Sinorhizobium meliloti strains

<table>
<thead>
<tr>
<th></th>
<th>Jemalong J6</th>
<th></th>
<th>TN 8.20</th>
<th></th>
<th>TN 6.18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RCR2011</td>
<td>TII7</td>
<td>RCR2011</td>
<td>TII7</td>
<td>RCR2011</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Stressed</td>
<td>Control</td>
<td>Stressed</td>
<td>Control</td>
</tr>
<tr>
<td>SDW</td>
<td>1.85c</td>
<td>0.87i</td>
<td>1.97b</td>
<td>1.59d</td>
<td>2.30a</td>
</tr>
<tr>
<td>% Decrease</td>
<td>52.97</td>
<td>19.29</td>
<td>27.83</td>
<td>21.86</td>
<td>66.67</td>
</tr>
<tr>
<td>RDW</td>
<td>414.70a</td>
<td>336.50b</td>
<td>360.53b</td>
<td>303.30b</td>
<td>378.22a</td>
</tr>
<tr>
<td>% Decrease</td>
<td>18.86</td>
<td>15.58</td>
<td>34.19</td>
<td>27.56</td>
<td>206.80c</td>
</tr>
<tr>
<td>NN</td>
<td>109a</td>
<td>75c</td>
<td>86b</td>
<td>67c</td>
<td>78b</td>
</tr>
<tr>
<td>% Decrease</td>
<td>31.14</td>
<td>32.09</td>
<td>33.33</td>
<td>30.72</td>
<td>72b</td>
</tr>
<tr>
<td>NFW</td>
<td>183.12c</td>
<td>95.45s</td>
<td>215.7a</td>
<td>174.32a</td>
<td>438.1a</td>
</tr>
<tr>
<td>% Decrease</td>
<td>47.88</td>
<td>19.18</td>
<td>15.51</td>
<td>9.83</td>
<td>9.83</td>
</tr>
<tr>
<td>Values are means of 10 replicates. Means denoted with different superscript letters differ significantly at P ≤ 0.05 based on Duncan’s multiple-range test.</td>
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</tbody>
</table>
activity. Plants of Jemalong J6 genotype inoculated with RCR2011 reference strain also showed high sensitivity to mannitol-mediated osmotic stress.

We performed a two-way ANOVA to estimate the impact of each partner on symbiosis behaviour. The variance of symbiotic effectiveness indices was dependent on the genotype of plant and rhizobia, as well as the interaction between the two partners. For biomass production, the contribution of plant genotype to the total variance was high (Fig. 1), with an average of 82 % for SDW and RDW, whereas it reached 66 % for NFW. A similar figure was observed in non-stressed plants. However, for the number of nodules and mainly ARA, the bacterial partner – that was the essential factor controlling the total variance of these parameters at non-stressful conditions – remained highly significant (30 %) even under osmotic stress. The interaction effect was slightly pronounced under stress conditions except for nitrogen-fixing activity (ARA) where it averaged 30 %.

Nodule metabolism modulation by osmotic stress

Osmotic stress effect was assessed on nodules, the central organs of symbiosis. In addition to assessment of nitrogenase activity – the key enzyme present in nodules – by ARA analysis, we analysed some indices of structure stability (lipid peroxidation) and function (leghaemoglobin). The total protein rate was decreased for all symbioses (Table 2). The ARA and the leghaemoglobin content also declined, and decrease in the former was positively correlated with decrease in the latter (r = 0.87, P ≤ 0.001). In stressed nodules there was an accumulation of MDA as final product of lipid peroxidation, mainly in sensitive symbioses (Table 2).

Analysis of enzymatic antioxidant defence, the main protective system in nodules (Becana et al. 2000), showed that osmotic stress differently affected the examined enzymes (Table 3). CAT activity was slightly lower but the decrease was nonsignificant in many cases. SOD and POX activities were stimulated by mannitol application. The maximum level of SOD increase (64 %) was found in nodules of the sensitive *M. truncatula* genotype TN6.18. However, POX activity was mainly induced in those involving the tolerant genotype TN8.20 (up to 65 %). APX activity was slightly but non-significantly increased by the osmotic stress in nodules of the overall analysed symbioses.

### Discussion

**Modulation of symbiotic performances by osmotic stress**

Mannitol application affected all parameters used as indices to estimate symbiosis effectiveness. Nevertheless, ARA

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**Table 2. Osmotic stress effect on nitrogen-fixing capacity (ARA, μmol h⁻¹ plant⁻¹), nodule protein content (mg g⁻¹ nfw), lipid peroxidation [malondialdehyde (MDA), μmol g⁻¹ nfw] and leghaemoglobin content (lghb, mg g⁻¹ nfw) of *Medicago truncatula* genotypes Jemalong J6, TN8.20 and TN6.18 inoculated with RCR2011 and TII7 *Sinorhizobium meliloti* strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDW</td>
<td>C</td>
<td>S</td>
</tr>
<tr>
<td>RDW</td>
<td>C</td>
<td>S</td>
</tr>
<tr>
<td>NN</td>
<td>C</td>
<td>S</td>
</tr>
<tr>
<td>NFW</td>
<td>C</td>
<td>S</td>
</tr>
<tr>
<td>ARA</td>
<td>C</td>
<td>S</td>
</tr>
<tr>
<td>Strain</td>
<td>Control</td>
<td>Stressed</td>
</tr>
<tr>
<td>ARA</td>
<td>11.18</td>
<td>5.88</td>
</tr>
<tr>
<td>% Decrease</td>
<td>47.41</td>
<td>39.28</td>
</tr>
<tr>
<td>% Decrease</td>
<td>17.38</td>
<td>9.75</td>
</tr>
<tr>
<td>MDA</td>
<td>8.6</td>
<td>12.16</td>
</tr>
<tr>
<td>% Increase</td>
<td>41.39</td>
<td>27.21</td>
</tr>
<tr>
<td>Lghb</td>
<td>3.75</td>
<td>2.6</td>
</tr>
<tr>
<td>% Decrease</td>
<td>30.67</td>
<td>23.53</td>
</tr>
</tbody>
</table>

Values are means of nine replicates for ARA and six for MDA and lghb. Means denoted with different superscript letters differ significantly at P ≤ 0.05 based on Duncan’s multiple-range test.
was the most affected parameter. This sensitivity of nitrogenase activity compared to plant growth was reported previously (Verdoy et al. 2004, Lopez et al. 2008) and is explained by the complexity of interaction and the need for energy required by the nodule. Efficient symbioses at standard conditions remained the best performing ones under osmotic stress. Such results suggest that high biomass production and nitrogen-fixing capacity under osmotic stress is a consequence of high potential biomass production under non-stressful conditions (Mhadhbi et al. 2007, Pimratch et al. 2008).

The host-plant genotype was the major contributor to the total variance of the parameters used to estimate symbiotic effectiveness (shoot and root dry weight, nodule number and dry weight and nitrogen-fixing capacity) as was observed at non-stressful conditions in our previous study (Mhadhbi et al. 2005). This agrees with Fesenko et al. (1994), Robinson et al. (2000) and Pimratch et al. (2008) who reported the dependence of shoot biomass production on plant genotype, both at standard conditions and under water deficit. Otherwise, the contribution of inoculated rhizobial strain into ARA and nodulation observed in our results was reported earlier (Fesenko et al. 1994, Robinson et al. 2000, Kantar et al. 2003, Mhadhbi et al. 2007). ARA is known to strongly depend on the interaction between the two symbiotic partners (Mhadhbi et al. 2005, 2007) and the effect of environmental constraints (Serraj et al. 1999). Consequently, it could be concluded that each partner contribute to the variability of the symbiosis behaviour. Indeed, in this work we identified contrasting symbioses related to plant genotype – tolerant associations with TN8.20 and sensitive ones involving TN6.18, as well as contrasting behaviours of symbioses involving the same plant genotype (Jemalong J6) but different bacterial partner RCR2011 (sensitive) or TII7 (tolerant).

### Stress effects and antioxidant system in nodules

Osmotic stress suppresses enzymes of metabolic pathways involved in nitrogen fixation such as sucrose synthase, phosphoenolpyruvate carboxylase, glutamate synthase, etc. (Figueiredo et al. 2007, Naya et al. 2007). This effect appears in the form of general decrease in ARA, nodule protein rate and leghaemoglobin content and an increase in lipid peroxidation. The correlation between the level of decrease in ARA and leghaemoglobin is explained by the key role of the latter – as oxygen transporter – in the process of bacteroids and cell respiration and, therefore, the adequate functioning of nitrogenase. A similar result was found in nodules submitted to salt stress (Nandwal et al. 2007, Lopez et al. 2008). The limitation in metabolic capacity of bacteroids and oxidative damage of cellular components are contributing factors to the inhibition of nitrogenase activity in nodules (Naya et al. 2007). One of the indices of cellular damage is the accumulation of MDA (Singh et al. 2007, Zhang et al. 2007) that was increased in stressed nodules. Lipid peroxidation was differentially affected depending on symbiosis tolerance levels. Taken together, the above results suggest that the level of nitrogen-fixing activity under stressful conditions is related to stability of membrane lipids and maintenance of sufficient amount of key metabolic proteins such as leghaemoglobin.

Catalase activity decreased under osmotic stress in nodules as reported previously (Mhadhbi et al. 2004, 2007, Nandwal et al. 2007). CAT is not regarded as the foremost of antioxidant defence under osmotic stress because of the low affinity to its substrate (Matamoros et al. 2003, Tejera et al. 2004), although this enzyme plays an important role in the nodulation process and nodule functioning (Jamet et al. 2003). SOD also plays a crucial role in symbiosis functioning (Santos et al. 2000), but it was not modulated by
moderate stress (Rubio et al. 2002). Present results showed an increase in SOD under stressful conditions, mainly in nodules of the most affected symbioses. Consequently, the protective role of SOD, for nitrogenase functioning, is essentially solicited when the physiological or even the structural state of nodules is greatly damaged by stress. POX is another important enzyme in the antioxidant defence of nodules under osmotic stress (Mhadhbi et al. 2004, 2007). The induction and protective role of peroxidases under salt and water osmotic stresses, as well as mineral toxicity, were widely reported in legume-rhizobia nodules (Mhadhbi et al. 2004, Jebara et al. 2005, Zhang and Water osmotic stresses, as well as mineral toxicity, were widely reported in legume-rhizobia nodules (Mhadhbi et al. 2004, Jebara et al. 2005, Zhang and Nan 2007) and free-living rhizobia (Barloy-Hubler et al. 2004). Our data indicate that the increase in POX activity is significant in all stressed symbioses. Thus, POX induction could provide a sensitive marker for distinguishing tolerant symbioses. The high level of POX induction in tolerant nodules could protect nodule integrity via lignification, supporting the relative observed SOD increase.

In conclusion, symbiotic responses to osmotic stress are dependent on both symbiotic partners. Contrasting tolerant and sensitive symbiotic responses were identified for each host-plant genotype in association with different bacterial strains, as well as for each bacterial strain inoculated in different plant genotypes. Tolerance of the symbiotic association is primarily determined by the degree of host-plant tolerance. The bacterial partner has an input related to its potential efficiency under stress conditions. The forefront of antioxidant defence under osmotic stress in nodules is primarily an increase in POX activity while SOD involvement is more evident in nodules showing high levels of decrease in nitrogen-fixing capacity.

References


Symbiotic Interactions under Osmotic Stress


Research article

Characterization and expression analysis of AGAMOUS-like, SEEDSTICK-like, and SEPALLATA-like MADS-box genes in peach (Prunus persica) fruit

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ABSTRACT

MADS-box genes encode transcriptional regulators that are critical for flowering, flower organogenesis and plant development. Although there are extensive reports on genes involved in flower organogenesis in model and economically important plant species, there are few reports on MADS-box genes in woody plants. In this study, we have cloned and characterized AGAMOUS (AG), SEEDSTICK (STK) and SEPALLATA (SEP) homologs from peach tree (Prunus persica L. Batsch) and studied their expression patterns in different tissues as well as in fruit pericarp during pit hardening. AG- STK- and SEP-like homologs, representative of the C-, D-, E-like MADS-box gene lineages, respectively, play key roles in stamen, carpel, ovule and fruit development in Arabidopsis thaliana. Sequence similarities, phylogenetic analysis and structural characteristics were used to provide classification of the isolated genes in type C (PPERAG), type D (PPERSSTK) and type E (PPERSEP1, PPERSEP3, PPERFB9) organ identity genes. Expression patterns were determined and in combination with phylogenetic data provided useful indications on the function of these genes. These data suggest the involvement of MADS-box genes in peach flower and fruit development and provide further evidence for the role of these genes in woody perennial trees that is compatible with their function in model plant species.

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1. Introduction

MADS-box genes comprise a multigene family of transcription factors regulating different aspects of development in higher plants. The MADS-box family is divided into several subfamilies regarding the primary sequences and expression patterns [17]. Due to the fact that a general co-evolution occurs between the MADS-box and the rest of the genes, the MADS-box genes are considered as a model for the study of evolution [37]. The application of genetic and molecular analysis of floral homeotic mutants of Arabidopsis (Arabidopsis thaliana L. Heynh) and snapdragon (Antirrhinum majus L.) has helped in the discovery of the first MADS-box genes [34]. These initial genetic studies led to the proposal of the ABC model of flower development [6]. In Arabidopsis, the action of a-C class MADS-box gene, named AP1 together with a non-MADS-box gene, the AP2, form sepalas. The aforementioned genes in cooperation with the B-class MADS-box genes AP3 and PI determine the identity of petals. Stamens are determined by the B function genes together with the C function gene AG, and the identity of the carpel is determined by AG. In snapdragon the functional homologs of AP3, PI, and AG are DEF, GLO, and PLE respectively [23].

Studies on arabidopsis and petunia (Petunia hybrida Vilm.) indicated that the ABC model should include the ovule identity genes (STK from arabidopsis and FBP7, FBP11 from petunia) and be extended to an ABCD model, where D function specifies ovule development [7]. The C- and D-gene groups are sister lineages as they evolved from a duplication event during early angiosperm evolution [21]. In arabidopsis ovule development is promoted by a complex collaboration of the C-type (AG and SHP), along with the D-type (STK) genes [27].

The ABCD model has been further extended with an additional class indicated as class E genes or SEP genes. In arabidopsis this class consists of four members, SEP1, SEP2, SEP3 and SEP4, encoding MADS-box factors that show partial redundant functions in floral organ identity determination. The homologous genes from petunia are FBP5, PHMADS12, FBP2 and PHFBP4 respectively. Results from Ditta et al. [8], Honma and Goto [14] and Pelaz et al. [26], show that
the SEP genes are necessary for the function of class A, B and C genes and discuss the molecular basis for the genetic relation among floral organ identity. According to their experiments, the single sep3 mutants displayed a partial transformation of the petals into sepals, therefore the requirement of SEP3 for the development of petals is larger than that of SEP1, SEP2, or SEP4. Interaction studies in yeast showed that SEP3 proteins interact with A, B and C MADS-box factors forming multimeric complexes. The formation of these complexes would allow, at the same time, the transcriptional activation of the target genes [14]. Similar genes have now also been studied in other dicotyledonous plants [33], and very similar structures and expression patterns have been found in more distantly related species.

Despite the progress made studying the MADS-box genes involved in flowering and flower organ formation in annual plants less is known for their role in fruit trees. There have been extensive reports on ABC model of MADS-box genes in annual flowering plants [20] but not in woody plants apart from studies on characterization of A-, B-, C-type MADS-box genes in apple flowers (Malus domestica Borkh.) [40], on five MADS-box genes in citrus [10] and on two C-type MADS-box genes in poplar [4]. Peach has become a model tree species for genetic studies within the Rosaceae [1]. The diploid genome (2n = 16), the small genome size of 300 Mb and the relatively short reproductive time for a fruit tree (2–3 years until flowering) facilitate genetic studies. Since peach and arabidopsis belong to the same clade of Rosids, and they have anatomical and physiological similarities between their flowers and fruits (Fig. 1), it is applicable to transfer the knowledge obtained from a model plant such as arabidopsis to a crop tree cultivated for its fruit.

Our group has cloned and characterized the peach MADS-box genes PPERFFRUITFUL (PPERFUL) (A-clade) and PPERSHATTERPROOF

Table 1
Primer sequences used in the experiments. Regarding the primers used for in situ hybridization experiments, consensus T3 sequence is shown in bold letters, preceded by a 9 base pair leader sequence in italic and followed after the slash (/) by the gene-specific sequence.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
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<td>PPERACTIN-2-F</td>
<td>5'-GTGGGCGATGGCAGAGACTGG-3'</td>
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<td>PPERACTIN-2-R</td>
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<td>5'-AGGTGTCCTCAGTCCTCCCT-3'</td>
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<td>PPERSEP1-F</td>
<td>5'-GTCAATGGAAGCTCAAACTTG-3'</td>
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<td>PPERSEP1-R</td>
<td>5'-CACTTCTTCGACCTCCACATT-3'</td>
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<td>PPERSEP3-F</td>
<td>5'-CTAAGGCTCTACGCAATTGG-3'</td>
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<td>PPERSEP3-R</td>
<td>5'-CAGTGTCCCATGAAACGCAG-3'</td>
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<tr>
<td>PPERFBP9-F</td>
<td>5'-TGTAGAGCTGGATGGAAGGA-3'</td>
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<td>PPERFBP9-R</td>
<td>5'-GTCAATGGAAGCTCAAACTTG-3'</td>
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<td>PPERSEP1-F-INSITU</td>
<td>5'-GCAACCTCCATTAAACCTCAAAAGGG</td>
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<td>PPERSEP1-R-INSITU</td>
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<td>PPERFBP9-F-INSITU</td>
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<td>PPERFBP9-R-INSITU</td>
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Fig. 1. The arabidopsis flower (A) in comparison with the peach flower (B). The arabidopsis fruit (C) and a transverse section of the wild-type arabidopsis fruit (D) in comparison with the transverse section of a peach fruit (E). The different parts of the fruits are indicated. (F) Split-pit formation in peach endocarp at the dehiscence zone is indicated with an arrow.
Fig. 2. Alignment of the C-terminal domains of the predicted amino acid sequences of PPERAG (A), PPERSTK (B), PPERSEP1 (C), PPERSEP3 (D), PPERFBP9 (E), and all PPERSEP-like (F) and with the most similar sequences identified in BLAST. C-motifs characteristic of AG, STK, SEP1, SEP3, FBP9 sequences are boxed.
In the same study we showed that differential temporal regulation of PPERFUL and PPERSHIP genes in the split-pit sensitive (Andross) and resistant (Katherine) varieties, might affect split pit formation—the opening of the pit and sometimes splitting of the fruit—which causes deterioration of canned fruit quality and great economic losses. Recent studies in peach have characterized A- and B- type MADS-box genes [42] as well as C-type MADS-box genes [35]. However there are no reports for the total group of C-, D-, E-type of MADS-box genes and their interaction in controlling fruit development in woody plants. We report here the cloning of C-, D-, and E-type PPERAG, PPERSTK and PPERSEP genes from peach and the study of their structure, phylogenetic relationship and expression during peach flower, ovule and fruit development.

2. Materials and methods

2.1. Plant material

Developing fruits were collected from peach trees growing in Veria, northern Greece, which is the main area of peach cultivation, in spring 2004, every week after anthesis until fruit maturity. Samples were taken from the varieties ‘Andross’ and ‘Katherine’, frozen in liquid nitrogen and stored at -80 °C until used.

2.2. Cloning of PPERAG, PPERSTK, and PPERSEP genes

A peach mesocarp library (cultival name Loring) curated at Clemson University [19] was searched for MADS-box gene ESTs, and clones BU043610, AJ825116, BU040997, BU047980, BU043415, and BU046256, with high similarity to arabidopsis AG, STK, SEP3, petunia FBP9, and arabadopsis SEP1/2, respectively were obtained. In addition, a clone (BU044765) with high similarity to arabidopsis ACTIN-2 was found (http://www.genome.clemson.edu/gdr/projects/prunus/abbott/PP_LEa/index.shtml). Clones were fully sequenced and primers were designed based on their sequence. Based on the information from the partial PPERSTK cDNA, we performed an improved inverse-RACE method, called Rolling Circle Amplification RACE (RCA-RACE), which allows the simultaneous isolation of the unknown 3' and 5' ends [28,36]. In order to obtain the internal site between the two inverse primers, we designed two additional primers, namely PPERSTK-F3, PPERSTK-R3 (Table 1).

2.3. RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was isolated from mesocarp, endocarp and embryo (week 5 after full anthesis), leaves, petals sepalas, carpels (including ovules) and stamens of fully developed flower from both varieties using the RNeasy plant RNA isolation kit (Qiagen, Crawley, U.K.). RNA was also isolated from the pericarp of the varieties ‘Andross’ and ‘Katherine’ from 8 to 12 week after full anthesis. For the RT-PCR, 1 μg of total RNA was used for first strand cDNA synthesis. The cDNA was synthesized using 0.2 pmol gene-specific primers, 0.2 mM dNTPs and 1 U

![Fig. 3. Phylogenetic relationships of the PPERMADS proteins shown with white letters in grey background with other MADS-box proteins. The tree was generated by the Neighbor-Joining method using the p-distance correction. Numbers next to the nodes are bootstrap values from 1000 replications. Scale indicates number of amino acid substitutions. The sequences homologous to Prunus persica MADS-box genes are given in Table 3. The sequence names of the proteins belonging to the PLE clade and FUL clade have been given in our previous study [35].]
DyNAzyme II DNA polymerase (Finzymes, Espoo, Finland), PCR primers for AG, STK and SEP homologous genes in peach were designed from the aforementioned ESTs found at the Clemson University’s EST library, and their sequence is shown in Table 1. PPERACTIN-2 was used as the RT-PCR control. The thermocycler program was 30 cycles of: 30 s at 94 °C; 1 min at 53 °C (or 60 °C for PPERSEP1); and 1 min at 72 °C, which were preceded by 2 min at 94 °C and followed by 10 min at 72 °C. The thermocycler program for PPERACTIN-2 was 22 cycles. Fragments were of predicted lengths. Control PCR reactions contained the RNA that was used as a template in the cDNA synthesis.

2.4. Characterization of peach AGAMOUS-like gene (PPERAGAMOUS)

To extend the characterization of peach AGAMOUS-like genes and in order to reveal any other AG-like genes apart from PPERAG, a PCR amplification was performed with template the RCA-RACE product used in cloning of PPERSTK and the primer pair PPERAG-F5end and AUAP. The primer PPERAG-F5end starts with the starting codon of PPERAG. A ~1050 bp PCR fragment was isolated from the agarose gel and was then cloned into pCR 2.1-TOPO vector using the EBI database. Among the best BLAST hits, genes for which there were published reports were selected for comparison. Sequence names were changed to include initials where needed and capitalized. The deduced amino acid sequences of each set of genes were aligned using the multiple sequence alignment program ClustalW [38]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA3.1 software [22] by the Neighbor-Joining Method with p-distance correction [29].

2.5. Protein sequence comparisons and phylogenetic analysis

The nucleotide and deduced amino acid sequences of PPERAGAMOUS, PPERSTK and PPERSEP-like were used for BLAST analysis on the EBI database. Among the best BLAST hits, genes for which there were published reports were selected for comparison. Sequence names were changed to include initials where needed and capitalized. The deduced amino acid sequences of each set of genes were aligned using the multiple sequence alignment program ClustalW [38]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA3.1 software [22] by the Neighbor-Joining Method with p-distance correction [29].

2.6. Southern hybridization

10 μg of genomic DNA (derived from developing fruits from both varieties 4 weeks after full anthesis) was digested with EcoRI, EcoRV, DraI, HindIII and BamHI, (TaKaRa, Otsu, Japan) and transferred to a positively charged Nylon membrane. The digoxigenin labeled gene-specific probes (the same primers were used for RT-PCR analysis) were prepared by PCR amplification using the PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany). Hybridization was performed with DIG Easy Hyb buffer at 42 °C according to the manufacturer and stringent washes at 68 °C in
0.5 × SSC containing 0.1% SDS (twice). Detection was performed with the DIG Luminescent Detection Kit and chemiluminescence was detected with the GeneGenome Bio Imaging System (Syngene, Cambridge, U.K.). The membrane was stripped and re-probed twice (for all PPERSEP-like genes). For detection of PPERAG and PPERSTK genes a fresh membrane was used.

2.7. Lignin staining

Lignin formation in the peach endocarp was detected by staining with phloroglucinol–HCl reagent [2]. Andross and Katherine fruits were sampled at 8 weeks after full anthesis. Phloroglucinol (2.0 g) was dissolved in 80 ml of 20% ethanol and then 20 ml of 12 N HCl was added.

2.8. In situ hybridization

In situ hybridization experiments were performed as previously described [12]. Prunus persica fully developed buds were fixed in 4% (w/v) paraformaldehyde supplemented with 0.25% (v/v) glutaraldehyde in 10 mM sodium phosphate buffer (pH 7.4) for 4 h in a vacuum aspirator. Fixed buds were block-stained in 0.5% (w/v) safranin, dehydrated through ethanol series, embedded in paraffin and cut into 8 μm-thin sections. Antisense RNA probes labeled with digoxigenin-11-rUTP (Boehringer Mannheim, Mannheim, Germany) were originated from PCR-generated templates incorporating T3 polymerase sites [13]. The primers used are presented in Table 1. Each probe was designed close to the 3’ end of the genes and their length was as follows: PPERAG probe-214 bp, PPERSTK probe-206 bp, PPERSEP3 probe-155 bp, PPERSEP1 probe-177 bp, PPERFBP9 probe-254 bp. Sections were prepared for hybridization according to Scheres et al. [31] and hybridized overnight at 42°C in 50% (v/v) formamide, 300 mM NaCl, 1 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.025% (w/v) bovine serum albumin (BSA), 10% (v/v) dextran sulfate and 60 mM DTT. After hybridization the sections were treated with a solution containing 500 mM NaCl, 1 mM EDTA, 10 mM Tris–HCl and 50 lg/ml RNase A. Finally, sections were washed several times.
in a 2xSSC solution. Hybridization signals were visualized with anti-digoxigenin antibodies conjugated with alkaline phosphatase. Images were processed using Photoshop 6 software (Adobe Systems Inc., San Jose, CA, USA).

3. Results

3.1. Amino acid sequence comparisons and phylogenetic analysis

Complete sequencing from both ends of the EST clones selected for further analysis revealed that the sequence of PPERAG, PPERSEP1, PPERSEP3 and PPERFBP9 have already been deposited in GenBank (GenBank accession nos. AAU29513, ABO27622, ABO27621 and AAZ16241 respectively). PPERSTK was not found in Genbank and the EST was incomplete. PPERSTK gene was cloned by the newly developed RCA-RACE method [28]. The PPERSTK transcript was 1040 bp and contained a 735 bp ORF encoding for 244 amino acids. The sequence was deposited in the GenBank (GenBank accession nos. EF602037).

Similarity of the predicted amino acid sequence of PPERAG, PPERSTK, PPERSEP1, PPERSEP3, PPERFBP9, with other MADS-box proteins was examined by sequence alignment and phylogenetic analysis was performed to identify phylogenetic relationships. PPERAG fell into the euAG lineage of C-type, MADS-box proteins along with other AG-like proteins from P. hybrida (PMADS3), M. domestica (MDMADS15), and Rosa rugoza L. (MASAKO C1) [21]. One characteristic commonly found in C-lineage members of MADS-box genes is the presence of an N-terminal extension preceding the MADS domain [18]. The MADS domain of the aligned proteins was highly conserved and conserved regions were also observed in the I and K domains, whereas the C-terminal domain was more divergent. However, at the very C-terminal end of the proteins, there were two short, highly conserved regions, the AG motif I and the AG motif II, which are boxed (Fig. 2A).

PPERSTK fell into the D-lineage/AGL11-lineage along with other D-type MADS-box proteins like Prunus dulcis (Mill.) D.A. Webb (PDMADS1), M. domestica (MDMADS10), and Lotus japonicus (Regel) K. Larsen (LJAGL11) [9]. The conserved C-terminus of the D lineage sequences is characterized by a fairly conserved extension (YET/AKA/DDXX) following the stop codon position of the AG motif II (Fig. 2B).

PPERSEP1 possesses two motifs conserved between members of the SEP1/2 clade, an internal motif NPTLQIGY and a C-terminal motif GFIPGWML [24]. These motifs were almost identical to those of related species such as M. domestica, Cucumis sativus L. and Vitis vinifera L. (Fig. 2C). PPERSEP3 shows an internal motif EPTLQIGY and a C-terminal motif YMAGWLP. These motifs were found in homologous proteins of species such as Gossypium hirsutum, P. hybrida and Antirrhinum majus (Fig. 2D). Finally PPERFBP9 protein harbours the motifs NSTSQIGY (internal motif) and GFIPGWML (C-terminal motif) which are identical in homologous proteins of M. domestica and P. hybrida (Fig. 2E).

Deduced amino acid sequences from all cloned peach genes were aligned with the closest matching homologous proteins from other plant species. The phylogenetic tree included the amino acid sequence of the PPERAG protein and the selected AG-like proteins and other C-type MADS-box proteins belonging to the PLENA clade using the Neighbor-Joining Method (Fig. 3). The closest homologs to the peach MADS-box proteins and the percentages of similarity with proteins of other species are presented in Table 2.

3.2. Southern hybridization

Results from restriction enzyme digestions for the number of PPERAG copies in peach were inconclusive (Fig. 4A) since multiple bands were observed and further experiments were performed (see below). Southern blots provided strong indication for the presence of one putative STK-like and SEP3-like ortholog in peach since for both genes a single band could be detected after digestion with each enzyme tested (Fig. 4B,D). PPERSEP1 and PPERFBP9 gene-specific probes hybridized with one band for all but one enzyme, since the former hybridized with two bands after EcoRV digestion,
Fig. 7. Detection of the expression of PPERAG (A,B) PPERSTK (C,D), PPERFBP9 (E,F) PPERSEP1 (G,H), and PPERSEP3 (I,J). Hybridization signal was visible as a blue-purple precipitate. Expression of PPERAG is most prominent at the ovary, style and along the filaments of the stamens (the signal is ambiguous in the anthers) (A). PPERAG transcription is reduced to a weak signal in the sepals whereas no signal is detected in petals (B). On the other hand, detection of the PPERSTK transcript is strong in the ovary (C) and almost no signal is detected in any other tissues apart from a faint signal in the perianth which is not detectable in the transverse section (D). PPERFBP9 expression is localized at low levels in ovary and perianth (E). On the other hand PPERSEP1 and PPERSEP3 transcripts are visible in all flower organs (G,H,I,J) although PPERSEP3 hybridization signal is low for the sepals and petals in the transverse section (J). Finally, detection of PPERSEP1 transcript is very strong in all floral organs, especially in the perianth (H). A close-up in the anthers clearly demonstrated that PPERSEP1 is the only gene strongly expressed (M). As a negative control, transverse and longitudinal sections (K,L) were hybridized to sense RNA probes transcribed from pGEM-luc Vector (Promega, Madison, WI, U.S.A.). In this case, no hybridization signal was visible. Size bars 2 mm (A,C,E,G,I,L), 1.6 mm (B,D,F,H,J,K) and 1.2 mm (M). A, Anthers; F, filament; O, ovary; P, petals; Pe, perianth; S, Sepals; St, style.
3.3. Characterization of peach AGAMOUS-like sequences

Four bands were observed in Southern hybridization with the PPERAG gene-specific probe after digestion of the genomic DNA with the restriction enzymes EcoRI and HindIII and two bands with EcoRV, XhoI and BamHI (Fig. 4A). It should be mentioned that the probe was designed outside the MADS domain and that there was no recognition site for the restriction enzymes used inside the PPERAG probe. Southern blot analysis revealed that the PPERAG probe at the high stringency conditions employed did not cross-hybridize with PPERSHP and PPERSTK which share high similarity with PPERAG (data not shown). To obtain information regarding expressed AG-like sequences in peach we performed PCR amplification of RCA-RACE products using an AG-specific primer. A ~1050 bp band was cloned and 17 clones were sequenced. Sequence information revealed 12 single nucleotide polymorphism (SNP) differences, in addition to a 3 bp insertion within the coding region which was found in two clones. These SNPs produced 8 modifications in amino acid sequence and the 3 bp insertion produced an extra glutamine found in two clones. The clone which was found with the 3 bp insertion also had 4 SNPs, indicating that is a different PPERAG allele. Furthermore, five clones were found to have different 3’ terminus, indicating that PPERAG transcripts have multiple polyadenylation sites. Some of the SNPs resulted in the production of new recognition sites (in particular new HindIII recognition sites). These data confirm the existence of different PPERAG alleles, and strongly support the presence of additional AG-like genes in peach. We also tried to find other AG-like genes in databases by blast search using PPERAG as query. Several similar sequences were obtained but phylogenetic analysis suggested that they belong to the SHATTERPROOF-like lineage.

3.4. Expression analysis

Initial RT-PCR experiments were carried out on cDNAs derived from peach mesocarp, endocarp and embryo 30 days after full anthesis as well as on cDNAs from leaves, sepals, petals, carpels and stamens of a fully developed flower. All 5 genes were expressed in mesocarp, endocarp and embryo except from PPERFBP9 which was not expressed in embryo. Results from RT-PCR experiments have shown that PPERAG and PPERSTK transcripts were mainly accumulated in carpels. PPERSTK mRNA was not detectable in any other tissue whereas PPERAG transcripts were detected in all other tissues apart from the petals. PPERFBP9 expression was very low in leaves, sepals and petals, whereas no transcripts were detected in stamens. On the other hand PPERSEP3 and PPERSEP1 mRNA was detectable in all tissues examined (Fig. 5). The localization of the expression of the aforementioned genes in fully developed buds was also studied with in situ hybridization (Fig. 7A–I). Expression of PPERAG is most prominent at the ovary and along the stamens (Fig. 7A). PPERAG transcripts are detected along the sepals (Fig. 7B), whereas PPERAG transcription is reduced to a weak signal in the petals (Fig. 7B). On the other hand, detection of the PPERSTK transcript is strong in the ovary (Fig. 7C) and almost no signal is detected in any other tissues apart from a weak signal visible in the perianth (Fig. 7C). PPERFBP9 expression resembles PPERSTK (a weak signal is detected in the ovary and the perianth) (Fig. 7E). On the other hand both PPERSEP1 and PPERSEP3 are expressed in all flower organs (Fig. 7G–J) although PPERSEP1 expression is stronger in all organs, especially in petals, sepals and anthers (Fig. 7H, M).

Expression of PPERSEP-like genes, PPERAG and PPERSTK was compared in Andross and Katherine varieties in pericarp during the crucial developmental stages of pit hardening (8–12 weeks after full anthesis) (Fig. 6A). PPERAG and PPERSEP1 were stably expressed in both varieties. However, PPERFBP9 transcript was not detected in Andross while PPERSEP3 transcript was detected neither in Andross nor in Katherine, 10 and 11 weeks after full anthesis. Moreover PPERSTK expression was not detected in Andross 8 weeks and in both Andross and Katherine, 9 weeks after full anthesis. Together with PPERFUL and PPERSHP mRNA expression profiles (data not shown), Fig. 8 gives a sum of mRNA expression patterns of all MADS-box genes examined in our lab so far.

3.5. Lignin staining

Lignin formation was examined during the period of initiation of pit hardening. Eight weeks after full anthesis (2 weeks after initiation of pit hardening), the endocarp of Andross fruit was not fully stained, whereas the endocarp of Katherine was clearly stained (Fig. 6B).

4. Discussion

The economic value of clingstone peach varieties depends on their resistance to split-pit. In a previous study we demonstrated that temporal regulation of a FRUITFUL-like (PPERFUL) and a SHATTERPROOF-like (PPERSHP) gene in a split-pit sensitive (Andross) and a resistant (Katherine) variety, might affect split-pit formation [36]. Here we report the characterization of five additional MADS-box genes, namely PPERAG, PPERSTK, PPERSEP1, PPERSEP3 and PPERFBP9 in order to provide a better understanding of flowering and fruit development in peach. Each gene had a unique pattern of expression in the tissues and developmental stages examined. Localization of expression with in situ hybridization was in good agreement with RT-PCR expression patterns in most cases. The minor differences among the two methods in few specific cases were likely due to difficulties in sampling where tissues could not be very well distinguished and/or differences in the stage samples were derived. For example, the RT-PCR data derived from later stages of floral development (open flower) because tissue separation can be easier, whereas in situ hybridization data are presented for earlier stages of floral development (closed buds) where all organs can be stained on the same sample. PPERAG and PPERSEP1 were expressed in almost all organs and stages examined while PPERSTK and PPERFBP9 appeared to have a more restricted expression profile.
Three of the characterized genes, PPERSEP1, PPERSEP3, and PPERFBP9, are single copy genes (Fig. 4) that encode putative proteins that phylogenetically belong to the SEP1/2, SEP3 and FBP9 subclades of the SEPALLATA family, respectively [24]. PPERSEP1 was expressed at high levels in all tissues examined which is in agreement with previous data showing that all SEP1/2 genes are expressed in inflorescences and also that these genes are expressed in the second, third and fourth whorls, and in the fruit [24]. PPERSEP3 was expressed at low levels in all fruit tissues examined, whereas it was clearly detectable in all flower tissues, especially in the outer parts of the flower (sepals, petals) and also in leaves. SEP3-like genes are mostly expressed in inflorescences and the inner three floral whorls of all species examined, but expression in leaves has not been observed [24]. Thus, PPERSEP3 has a distinct expression pattern and could be essential for the development of the outer parts of peach flower and the fruit as well. PPERFBP9 expression in all the tissues examined resembled the expression of genes belonging to the FBP9/23 subclade. Proteins of the FBP9/23 subclade, which is lost in arabidopsis, appear to function redundantly with other SEP proteins during floral development but might have other roles during transition to flowering [15]. In snapdragon PhFBP23 interacts with AP1/FUL proteins [16] and a similar interaction of PPERFBP9 with PPERFUL if exists, may be very important in split-pit formation given the possible role of PPERFUL in this phenomenon [36]. SEP genes range from developmentally redundant, as in arabidopsis, to nonredundant, with unique roles for several developmental processes in other plants [24]. Expression patterns of the peach SEP-like genes where coincident may indicate functional redundancy while when differ may indicate unique and specific roles that remain to be examined.

The PPERAG gene characterized in this report is homologous to arabidopsis AG and to snapdragon FARINELLI (FAR). Our expression data is in accordance with expression analysis of Tadiello et al. [35] and demonstrated that PPERAG expression is strong in both stamen and carpel tissues similarly to AG [3]. Furthermore we have detected it although at low levels in sepals and in leaves. Apple AG-like (MdMADS15) is also expressed in sepals suggesting that it may be essential for further development of sepal tissue into fruit flesh [40]. Transcripts of poplar PTAG1 and PTAG2 were detected in vegetative tissues as well as in stamens and carpels of developing flowers [4]. Accumulation of citrus CitMADS1 mRNA was also detected in all flower organs [10]. Although there are species containing two AG homologs that in some cases exhibit diversification in function and/or expression [25,39] in most woody plants examined such as apple and citrus, a single copy of AG-like gene was found. However, peach may contain more than 1 AG-like gene since Southern hybridization revealed multiple bands and cDNA cloning resulted in the isolation of two clones different for an in-frame insertion at the coding region. Whether peach has two AG genes as Tadiello et al. [35] mentioned is still under question and could be clarified only after the analysis of syntenic loci conservation between arabidopsis and peach.

In model species D-class genes (STK of arabidopsis and FB11 of petunia) show specific expression in carpels–ovules whereas the phylogenetically related C-class genes are also expressed in stamens. STK is required for ovule development and the normal development of the funiculus, the cord-like structure in arabidopsis that connects the developing seed to the fruit [30]. PPERSTK was expressed in carpel-ovary, embryo and the fruit tissues pericarp and endocarp, but not in stamens (although a faint signal was detected in anthers) (Fig. 7). This pattern of expression closely resembled D-class gene expression and might indicate that PPERSTK has a role in ovule development and also at later stages in embryo and fruit development of peach. Similar results have been obtained in D. thrysiflorum by in situ localization experiments revealing high expression of the AG ortholog DthyAG1 at the initial stage of placenta- and ovule development, while the D-type gene DthyAG2, like PPERSTK in peach, is expressed in the ovary and throughout ovule development [33], suggesting a role in ovule development as could be the case in peach as well. Interestingly, PPERSTK transcription was detected at low levels in the perianth as well, and could be an ancestral pattern of expression that is no longer needed today. PPERSTK occurs as a single copy gene, like AVAG2 a putative D-class gene from an ornamental asparagus (Asparagus virgatus Baker) [41], and EScuAGL11 from California poppy (Eschscholzia californica Cham.) [5]. The overlapping functions of C/D-type genes in core eudicots are reviewed in [30]. Finally, the differential mRNA accumulation of the five MADS-box genes during pit hardening (8–12 weeks after full anthesis) suggests they may play roles in other aspects of peach fruit development since several studies in other plants demonstrate their homolog interactions withFUL/SHP genes [11,32]. Previous results showed differential temporal regulation of PPERFUL and PPERSHP that might affect split-pit formation [36]. Interestingly, different extent of lignin formation was observed at the same stage of the beginning of pit hardening in the two peach varieties that differ also in split-pit formation (Fig. 6B). A synergistic effect of MADS-box gene regulation of fruit development and lignification could be responsible for split-pit formation in the sensitive variety but this should be further examined.

In summary, we have presented here five new members of the MADS-box gene family from P. persica. Sequence similarities, phylogenetic analysis and structural characteristics were used to provide classification in type C (PPERAG), type D (PPERSTK) and type E (PPERSEP1, PPERSEP3, PPERFB9) organ identity genes. Expression patterns were determined and combined with phylogenetic data was used to predict function of these genes. However, because of the presence of paralogues, functional conservation may be difficult to determine and expression does not always correlate with function. Thus, further experiments are planned to determine putative functional roles of the C-, D-, and E-type genes mainly in peach fruit development.

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**References**

REVIEW

Aox gene structure, transcript variation and expression in plants

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Alternative oxidase (Aox) has been proposed as a functional marker for breeding stress tolerant plant varieties. This requires presence of polymorphic Aox allele sequences in plants that affect plant phenotype in a recognizable way. In this review, we examine the hypothesis that organization of genomic Aox sequences and gene expression patterns are highly variable in relation to the possibility that such a variation may allow development of Aox functional markers in plants. Aox is encoded by a small multigene family, typically with four to five members in higher plants. The predominant structure of genomic Aox sequences is that of four exons interrupted by three introns at well conserved positions. Evolutionary intron loss and gain has resulted in the variation of intron numbers in some Aox members that may harbor two to four introns and three to five exons in their sequence. Accumulating evidence suggests that Aox gene structure is polymorphic enough to allow development of Aox markers in many plant species. However, the functional significance of Aox structural variation has not been examined exhaustively. Aox expression patterns display variability and typically Aox genes fall into two discrete subfamilies, Aox1 and Aox2, the former being present in all plants and the latter restricted in eudicot species. Typically, although not exclusively, the Aox1-type genes are induced by many different kinds of stress, whereas Aox2-type genes are expressed in a constitutive or developmentally regulated way. Specific Aox alleles are among the first and most intensively stress-induced genes in several experimental systems involving oxidative stress. Differential response of Aox genes to stress may provide a flexible plan of plant defense where an energy-dissipating system in mitochondria is involved. Evidence to link structural variation and differential allele expression patterns is scarce. Much research is still required to understand the significance of polymorphisms within AOX gene sequences for gene regulation and its potential for breeding on important agronomic traits. Association studies and mapping approaches will be helpful to advance future perspectives for application more efficiently.

Introduction

Alternative oxidase (AOX) is a component of the mitochondrial respiration pathway present in all higher plants which diverts electrons from the energy conserving cytochrome pathway to catalyze the four-electron reduction of oxygen to water (Siedow and Umbach 2000). Although its presence is not restricted in plants (McDonald 2009, McDonald and Vanlerberghge 2006), much of the recent interest on this enzyme is because of

Abbreviations – AOX, Alternative oxidase; CS, cleavage site; FUE, far upstream element; MFA, monofluoroacetate; miRNA, microRNA; NUE, near upstream element; ROS, reactive oxygen species; SNP, single nucleotide polymorphism; TCA, tricarboxylic acid; UTR, untranslated region.
its potential to serve as a functional marker in molecular plant breeding (Arnholdt-Schmitt et al. 2006).

Accumulating evidence suggests that AOX may play a significant role in cell adaptation under different types of stress. AOX is activated at high respiratory substrate availability and is induced by high levels of endogenous or exogenous reactive oxygen species (ROS) (Millar et al. 2001, Oliver et al. 2008, Vanlerberghe and McIntosh, 1997). AOX is able to prevent excessive reduction of the mitochondrial electron transfer chain, which could result in disruption of glycolysis and the tricarboxylic acid (TCA) cycle under conditions of high substrate availability (Lambers 1982). Thus, AOX activity enables high turnover rates of carbon and operation of TCA cycle under stress conditions, although with a cost in ATP synthesis. High induction of AOX with concomitant dramatic reduction in growth is also evident under nutrient limitation as, for example, was observed in tobacco cells, whereas the growth of transgenic tobacco cells lacking AOX was only moderately reduced by the nutrient deficiencies, and carbon use efficiency values remained the same as under nutrient-sufficient conditions (Sieger et al. 2005). These results indicate that AOX respiration provides an important general mechanism by which plant cells can modulate their growth in response to nutrient availability.

Usually, a specific Aox gene is highly induced by many types of abiotic stress in plants. The expression of over 60 genes encoding components of the classical, phosphorylating respiratory chain and TCA cycle remained largely constant when cells were subjected to a broad range of abiotic stresses, but expression of the alternative components responded differentially to the various treatments (Clifftion et al. 2005). A common aspect of many types of abiotic and biotic stresses is the high production of ROS resulting in a cell damaging condition known as oxidative stress. As has been suggested long ago and shown by the results of many studies, AOX reduces the effects of oxidative stress by preventing accumulation of ROS in mitochondria (Amirsadeghi et al. 2006, Gray et al. 2004, Gupta et al. 2009, Maxwell et al. 1999, Möller 2001, Popov et al. 1997, Purvis and Shewfelt 1993).

Many attempts to elucidate the role of the AOX pathway have been based on the manipulation of Aox gene expression through overexpression and silencing experiments. Results of these reverse genetic strategies mainly regarding Arabidopsis and tobacco and manipulation of only one Aox gene at a time are reviewed in this issue (Vanlerberghe et al. 2009), and a major conclusion is that lack of an Aox allele (which is not compensated by induction of another counterpart allele) has little impact under normal growth conditions but has severe consequences for survival under stress. It is also evident that data from reverse genetic experiments provide support for most of the proposed physiological roles of AOX till date but do not allow the development of a simple model to explain all the observations. Thus, the authors make the hypothesis that AOX may control the mitochondrial stress signaling (perhaps ROS-based) pathway by defining the strength of the signal, that is determining cell death or survival. In doing so, AOX can either enhance or suppress ROS generation that is largely dependent upon a given set of metabolic conditions.

The above data from reverse genetics point to a significant role for AOX under adverse conditions in both modulation of plant growth and development and protection of cells from oxidative stress. More importantly, it is suggested that this role is not just housekeeping but can be regulatory, and evidence in support of this view is accumulating (Van Aken et al. 2009, Vanlerberghe et al. 2009). Thus, development of Aox-based functional markers for stress tolerance should be highly advantageous.

To be useful as a functional marker, Aox should fulfill several requirements: polymorphic sequences within species must be available; polymorphic, functional motifs affecting plant phenotype within Aox genes must be identifiable; and a strong relationship between DNA polymorphisms and variation of the trait of interest should be established. In this review, we provide evidence that organization of genomic Aox sequences and gene expression patterns are variable. This fulfills the first requirement in the path for development of functional markers based on Aox in plants. The second requirement, that is a strong correlation between Aox sequence polymorphism and trait variability, still needs to be broadly established.

**Variation of Aox gene organization**

Plant AOXs are encoded by a small nuclear multigene family that is divided into two subfamilies, Aox1 and Aox2 in eudicots, whereas only Aox1 members have been found in monocots. The number of Aox genes in different plant species is variable. For example, Arabidopsis has five genes: Aox1a, Aox1b, Aox1c, Aox1d and Aox2 (Clifftion et al. 2006, Saisho et al. 1997, 2001b), whereas rice has four: Aox1a, Aox1b, Aox1c and Aox1d lacking the Aox2 as monocot, and Vitis vinifera has three genes: Aox1a, Aox1b and Aox2 (Costa et al. 2009b). Variation has also been detected in copy numbers of each subfamily in different eudicot plants. Arabidopsis has four Aox1-type and one Aox2-type genes whereas soybean has one Aox1-type and two Aox2-type genes.
**Fig. 1.** Intron–exon organization of plant AOX genes. Most genes display the conserved structure of four exons interrupted by three introns. Intron gain and loss have resulted in variations in some species where Aox genes have five or three exons.
Thus, while Aox1-type genes have expanded in Arabidopsis, Aox2-type genes have expanded in legumes such as soybean and cowpea (Costa et al. 2004, McCabe et al. 1998). Usually, only one of the two Aox subfamilies has more than one member with carrot being the only reported exception where both subfamilies expanded having two members (Campos et al. 2009, Costa et al. 2009a). A tandem gene arrangement has been reported for Arabidopsis Aox1b and Aox1a (Saisho et al. 1997) as well as for soybean Aox2b and Aox2a (Thirkettle-Watts et al. 2003) and rice Aox1b and Aox1a (Ito et al. 1997), probably because of gene duplication. However, recently, it has been demonstrated that the two carrot AOX2 genes were linked to two linkage groups (unpublished, see Cardoso et al. 2009). Aox genes in both families present a conserved intron–exon structure that in many species consists of four exons interrupted by three introns at highly conserved splice site positions (Considine et al. 2002). Variations of this structure have been evolved by intron loss or gain and Fig. 1 shows Aox gene intron–exon structure in several species denoting the most prominent categories.

**Sequence variation in Aox genes**

Although Aox gene sequence information is available for several plant species at the genomic and even more at the transcript levels, evidence for variation in allelic sequences of Aox within species or in individual plants is limited. There are few reports for the presence of single nucleotide polymorphisms (SNPs) in Aox genes in rice and tomato, which may be related to differential gene expression and stress tolerance (Abe et al. 2002, Holtzapfel et al. 2003). Recently, an effort has been launched by Arnholdt–Schmitt and coworkers to gather more relevant information, and most of the results are presented in this issue. Variation in the intron length of Aox1b of Hypericum perforatum L. was observed when two fragments of 1408 and 1349 bps were identified for the partial AOX1b gene sequence. This intron length polymorphism was first identified in an individual plant, but was later verified through Exon-Primed Intron-Crossing (EPIC)-PCR in individual plants from six diverse regions in Portugal (Ferreira et al. 2009). In all plants, both fragments showed near identical sequences in all three exon regions. However, both complete intron regions revealed deletions that counted for the difference in the overall fragment sizes. Polymorphic sites were observed in several regions of Aox2 cloned from the olive (Olea europaea L.) cultivars ‘Galega vulgar,’ ‘Cobrançoza’ and ‘Picual.’ Data revealed SNP polymorphisms in introns of the three varieties and variability in the 3’-untranslated region (3’-UTR) region among seven recombinant clones from ‘Galega vulgar’ (Macedo et al. 2009). Repetitive patterns of intron length variation have been observed in the carrot DcAOX2a gene. Polymorphic and identical PCR fragments revealed underlying high levels of sequence polymorphism encompassing insertion/deletion events, SNPs and polymorphism pattern (Cardoso et al. 2009). Variable transcript length of Aox1a and Aox1b was observed among two grape varieties, PN40024 and Pinot Noir. Also Aox2 in PN40024 was found to harbor a retrotransposon rendering the gene sequence 5 kb longer than Aox2 in Pinot Noir (Costa et al. 2009b). It is conceivable that evidence of variation among individual Aox genes in diverse plant species is accumulating and existing polymorphism may support the development of molecular markers for breeding purposes in these species.

**Aox gene 3’-UTR microheterogeneity**

Another aspect of polymorphism in Aox sequences is the observed 3’-UTR microheterogeneity as a result of alternative polyadenylation of Aox transcripts in maize (Polidoros et al. 2005) and olive (Macedo et al. 2009). Many processes of post-transcriptional control of gene expression involve the 3’-UTR and polyadenylation signal of the gene. In animals, the highly conserved AAUAAA signal, about 10–30 nt upstream of the cleavage site (CS), and a downstream U- or GU-rich element define the exact site where the poly(A) tail is added. In plants, unlike animals, the localization of the poly(A) tail addition depends on three major groups of poly(A) signals, namely the far upstream elements (FUEs), the near upstream elements (NUEs) – a functional equivalent to the vertebrate AAUAAA element – and the CS itself (Hunt 2007). It is a general phenomenon that plant transcripts are heterogeneous regarding the length of their 3’-UTR. This may affect as much as about 50% of the rice genes having at least two poly(A) sites that are 30 or more nucleotides apart (Shen et al. 2008). Also a total of 27.9% of 62 811 expressed sequence tags having an intact 3’ end of V. vinifera were found to contain alternative polyadenylation sites (Cai et al. 2008). Alternative poly(A) site selection may be accomplished by two different hypothetical mechanisms, the one involving a single polyadenylation signal defining multiple 3’ ends depending on changes in the relative efficiencies of the NUEs and/or CSs associated with the signal, and the other entailing different polyadenylation signals (complete combinations of FUE and associated NUEs and CSs) that might be selected differentially (Hunt 2007). Consequence of this phenomenon that may be widespread throughout the plant kingdom is that a high percentage of mRNAs transcribed from the same gene
are polymorphic at their 3’ ends. The nature of the 3’-UTR and the choice of polyadenylation site in genes with multiple sites may play a role in the expression of a gene, with important physiological consequences.

There are examples of several mechanisms capable of affecting the polyadenylation processing depending on signaling events as responses to environmental and developmental cues. Modification of various polyadenylation factors affect their activity and might contribute to alternative poly(A) site choice (Hunt 2007). For example, poly(A) synthesis in yeast is controlled by cycles of phosphorylation and dephosphorylation that require the action of the phosphatase Glc7 (He and Moore 2005). In Arabidopsis, signaling cascades stimulate alteration of the activity of the polyadenylation machinery components, as for instance the calcium dependent interaction of the cleavage and polyadenylation specificity factor AtCPSF with calmodulin, which inhibits the AtCPSF RNA-binding activity (Delaney et al. 2006). Finally, a relationship between polyadenylation and hormonal signaling was revealed by the abscisic acid (ABA) dependent inhibition of the alternative processing which down-regulates the production of full-length FCA-encoding mRNAs that promote Arabidopsis development (Razem et al. 2006), providing a molecular link between ABA-mediated signaling, alternative polyadenylation and the regulation of flowering time.

These mechanisms could affect the polyadenylation process as a response to signaling and result in the production of transcripts with different 3’-UTR lengths. Such a case involving the maize Aox1a gene was provided by a study revealing alternative polyadenylation after treatment with H2O2, a well-known stress signaling molecule (Polidoros et al. 2005). Although the two major classes of 3’-UTRs in the transcript of maize Aox1a differing about 80 nt were also observed in control untreated tissue, the relative abundance of the two classes was different (unpublished observations). Interestingly, this difference could have a functional role if the deleted sequence in one class had a regulatory role(s).

There are several examples of regulatory elements residing in 3’-UTRs. A riboswitch (metabolite-sensing gene control element) function in the 3’-UTR of the thiamin biosynthetic gene THIC mediates feedback regulation of expression in response to changes in cellular thiamin pyrophosphate levels (Wachter et al. 2007). In this example, 3’-UTR length positively correlates with transcript accumulation, thereby establishing a basis for gene control by alternative 3’ end processing. In a more broad example, 3’-UTRs play an important role in post-transcriptional regulation that is mediated by microRNAs (miRNAs) in animals (Stark et al. 2005). Animal miRNA binding sites occur typically in the 3’-UTRs of the target genes, and a large set of genes involved in basic cellular processes avoid miRNA regulation because of short 3’-UTRs that are specifically depleted of miRNA binding sites (Stark et al. 2005). In plants, unlike animals, the complementary sites can exist anywhere along the target mRNA rather than exclusively at the 3’-UTR (Zhang et al. 2006). Regarding Aox genes, a repetitive deletion in intron 3 of the carrot Aox2a was found to affect a putative pri-miRNA site (Cardoso et al. 2009). Several examples exist where the 3’-UTR harbor miRNA targets in plants (Rhoades et al. 2002). Examining the maize Aox1a 3’-UTR for the presence of miRNA target sites, we identified a putative miR163 target motif (Fig. 2) having characteristics of a canonical site with good pairing to both 5’ and 3’ ends of the miRNA (Brennecke et al. 2005) and an overall pairing energy ΔG = −22.36 kcal mol⁻¹. Although the functional significance of this motif in Aox1a is obscure and may be unlikely, as miR163 has been reported only in Arabidopsis and its putative target (S-adenosyl-L-methionine:carboxyl methyltransferase family members) is not related to Aox, the presence of this motif in the maize Aox 3’-UTR can be suggested as an example of how modulation of the 3’-UTR length can have significant effects of the regulation of Aox genes. miRNAs are regulatory RNAs with a mature length of about 21 nucleotides that are processed from hairpin precursors by Dicer-like enzymes and can negatively regulate gene expression by attenuating translation or by directing mRNA cleavage (Dugas and Bartel 2004). If the miRNA target site is between two CSs in the 3’-UTR, as is the case for maize Aox1a (Fig. 2), only transcripts with the long UTR will be affected by miRNA mediated silencing. Short UTR transcripts avoid this mechanism by not displaying the miRNA target site in their sequence. About half of the cloned maize Aox1a transcripts in H2O2 treated maize seedlings had short 3’-UTRs (Polidoros et al. 2005). Preferential expression of the Aox1a short UTR class points to a poly(A) site selection mechanism aiming to avoid

Fig. 2. (A) The structure of the maize Aox1a 3’-UTR showing the position of two different cleavage sites (CS1, CS2) with the neighboring near upstream elements (NUE1, NUE2) and the far upstream element (FUE). The position of the putative miRNA target site (miR163) between CS1 and CS2 is indicated. (B) Alignment of miR163 with its putative target site at the maize Aox1a 3’-UTR.
negative regulation through the miRNA or any other element that might reside in the long UTR transcript.

Aox 3′-UTR microheteroge neity may be not restricted to maize, as this phenomenon was also observed in olive Aox2 that contained seven classes of short and long UTR variants (Macedo et al. 2009). A search for miRNA sites in the olive 3′-UTR revealed five putative miRNA targets that had an overall pairing energy of $\Delta G < -20 \text{ kcal mol}^{-1}$. Three of these targets were present in all seven variants but the other two were absent in the shorter two 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 possibly because of a yet unknown regulatory function. Discovery of other Aox genes with variable 3′-UTR length of their transcripts will provide the tools to investigate the putative signifi cance of these findings.

**Regulatory element variation in Aox gene promoters**

Different Aox gene family members display differential expression patterns depending on the plant species, tissues, growth, development and environment. This can be an effect of within-gene polymorphism and subsequent differences in gene regulation as discussed above, or as a consequence of differences in the structure of the promoter regions of these genes. Extensive promoter characterization has been conducted in Arabidopsis as well as in soybean (Ho et al. 2007, Thirkettle-Watts et al. 2003). These studies have revealed positive as well as negative regulatory regions that function in a species-specific manner and sequence motifs that are common between the two species. It was also found that the expression patterns observed between Arabidopsis and soybean were not conserved with gene orthology. Comparisons made between promoters driving the expression of genes with similar expression profiles revealed that the promoter region of soybean GmAOX2b contained seven sequence elements in common with the promoter region of AtAOX1c, pointing to the putative significance of these elements in co-regulation of the two genes. It should be noted that these genes are not induced by oxidative stress. Nevertheless, both genes share three out of the seven elements with ZmAox1a, which is highly responsive to oxidative stress (Polidoros et al. 2005). Thus, identification of common elements in the promoter region is not enough to potentiate common gene regulation. Identification of common sequence elements directing the co-expression of Aox1a and NDB2 observed in Arabidopsis under a number of treatments suggested that common motifs arranged hierarchically in the upstream promoter regions of these genes may be related to similar responses (Clifton et al. 2005). It is then possible that multiple common regulatory elements with similar organization may be important to support the necessary specificity and selectivity for common gene regulatory context. Such a similarity was found between the maize ZmAox1a and the rice OsAox1a promoters (Polidoros et al. 2005). Particularly, a 90 bp upstream the TATA-box region was 73.4% homologous and a TGACG motif (as inverse repeat) within this region was conserved between the two promoters. The TGACG motif is conserved in the promoters of soybean GmAox2b and Arabidopsis AtAox1a and AtAox1b genes, and represents the binding motif of the TGA1 protein known to interact with the redox-activated NPR1 protein (Despres et al. 2003) important in plant responses to pathogens. In conclusion, there are many similarities in regulatory motifs among different Aox promoters, although their relative significance in common regulation of the harboring genes is dependent upon the presence of other motifs and the local hierarchical organization of the regulatory elements in these promoters.

**Aox gene expression patterns**

Aox gene expression in plants was investigated after cloning the first Aox cDNA in 1991 (Rhoads and McIntosh 1991). In the following years, a wealth of information has been accumulated regarding Aox expression patterns in various species. Data indicated that Aox genes are expressed either constitutively at specific stages, organs and tissues during development or are highly induced under stress. Thus, it is conceivable that AOX isoforms could be involved in two major functions: maintenance of mitochondrial respiratory capacity during plant development and preservation of mitochondrial functionality as a consequence of the high induction of Aox expression under various stresses. Aox genes are distributed in two subfamilies in eudicots in which the Aox1 subfamily is regarded as the inducible one whereas Aox2 is usually thought as constitutively or developmentally regulated (Considine et al. 2002, Juszczuk and Rychter 2003). Differential tissue, developmental and stress regulation of Aox genes that fits to the above model has been reported in Arabidopsis (Clifton et al. 2006, Saisho et al. 2001a, 2001b) and soybean (Considine et al. 2002, Djajanegara et al. 2002, Finnegan et al. 1997, McCabe et al. 1998). Orthologous genes tend to have a similar regulation as, for instance, the soybean and Vigna unguiculata Aox2a and Aox2b genes (Costa et al. 2004) but there are examples that are not compatible to this
model in other species (Considine et al. 2002). Developmentally regulated Aox genes although belonging to the AoxI subfamily have been reported in monocots, for example rice (Saika et al. 2002), wheat (Takumi et al. 2002) and maize (Karpova et al. 2002). Aox genes in various species also respond differentially to biotic and abiotic stresses (Maxwell et al. 1999, 2002, McIntosh et al. 1998, Mizuno et al. 2008, Simons et al. 1999, Vanlerberghe and McIntosh 1997), mitochondrial mutations (Karpova et al. 2002), treatments that interrupt mitochondrial functions, hormones and signaling molecules, particularly ROS, salicylic acid and nitric oxide (Djajane-gara et al. 2002, Fung et al. 2006, Huang et al. 2002, Li et al. 2008, Maxwell et al. 1999, McIntosh et al. 1998, Millar and Day 1996, Polidoros et al. 2005, Rhoads and McIntosh 1992, Wagner 1995). In Arabidopsis, several studies comparing Aox1a expression with other stress defense genes reported Aox1a to be the most stress responsive (Clifton et al. 2005, Huang et al. 2002, Saisho et al. 1997). It should be noted, however, that in experiments with different species conflicting results have been reported (Clifton et al. 2006, Frederico et al. 2009).

There are multiple signaling pathways leading to Aox induction that can be either ROS dependent or ROS independent (Gray et al. 2004). ROS dependent pathways are probably very significant in stress related studies, as many types of biotic and abiotic stresses induce ROS production and accumulation at much higher than normal levels (Mittler 2002). In Arabidopsis, a network of at least 152 genes is involved in managing the level of ROS. In a comparative analysis of microarray expression data for the different genes of this network in three different knockout or antisense lines (with reduced or lacking ascorbate peroxidase 1, Cu/Zn superoxide dismutase 2 and catalase 2) that were overproducing ROS and in plants subjected to five different abiotic stress conditions (heat, drought, salt, cold or high light), the Arabidopsis Aox1a expression was induced to all but one condition, exposure to high light, where it was actually slightly reduced (Mittler et al. 2004). No other gene of this network displayed such a wide range of induction and responses were more specific. The regulation of the transcriptional induction of Aox1a was studied by the analysis of the Aox1a promoter using deletion and mutagenesis, and a common region especially important for strong induction by both the mitochondrial electron transport chain inhibitor antimycin antimycin A (AA) and the TCA cycle inhibitor monofluoroacetate (MFA) was identified (Dojcinovic et al. 2005). Although ROS production by AA is well documented and could be possibly responsible for the induction of the gene, the ROS related induction by the concentration of MFA used in these experiments looked unlikely. The authors concluded that induction of Aox1a in response to perturbation of mitochondrial function relies on a complex set of interactions at the level of promoter, rather than simple transcription factor–transcription factor binding site interaction. The above data suggest that Aox may play a significant role not only in preventing ROS buildup, but also in sensing metabolic perturbations and in the coordination of stress responses. This is furthermore supported by expression studies using Aox overexpression, suppression or knockout lines mainly in Arabidopsis where altered Aox expression results in an altered cellular metabolic state (Umbach et al. 2005), pointing also to the significance of Aox function outside the mitochondria, in the organism level, because altered Aox expression could inflict more significant extramitochondrial metabolic or antioxidant defense effects than mitochondrial ones (Clifton et al. 2006, Fiorani et al. 2005, Giraud et al. 2008, Smith et al. 2009, Van Aken et al. 2009).

Aox alleles are regulated independently of each other (Clifton et al. 2006) but studies in knockout Arabidopsis plants lacking Aox1a showed increased induction of Aox1d, although not enough to fully compensate for the lack of AOX1a under restricting cytochrome pathway conditions (Strodtkrotter et al. 2009). Overall, the above studies suggest that differential regulation of Aox genes is correlated with specific roles each gene has to fulfill in normal development or under stress and multiple stress defense, and metabolic pathway signals are integrated in a ‘regulatory apparatus’ responsible to effect specificity in Aox expression.

Given the well-documented Aox induction at the transcriptional level under various stress conditions, there are two important questions regarding the significance and the role of this response. The first is whether there is a direct correlation between Aox expression, protein abundance and its activity or engagement in respiration. This is an issue that will be covered thoroughly elsewhere in this special issue (Florez-Sarasa et al. 2009, Rasmusson et al. 2009). The second, in relation to the main theme of this review, that is assessment of Aox polymorphisms and use as a tool for plant improvement, is whether Aox expression and induction under stress is variable in different genotypes and related with phenotypic differences and adaptation to stress. In other words, are there any data pointing to a significant differential Aox expression between a tolerant and a sensitive variety under a certain stress and is there any relationship of variation in Aox expression with differential response to stress? This is a fundamental question (Arnholdt-Schmitt et al. 2006), although almost totally unexplored up to now.
There is only one published study that has undertaken the task to examine variation of Aox expression in contrasting genotypes regarding their response to stress (Costa et al. 2007). In that study the VuAox1, VuAox2a and VuAox2b gene expression and AOX protein level and capacity were examined in roots of a sensitive and a tolerant V. unguiculata variety after hydroponic exposure to high salt concentration or osmotic stress. The results indicated differential VuAox2b expression among the two varieties under both conditions whereas VuAox1 and VuAox2a remained unchanged. In the tolerant cultivar (Vita 3), the expression of VuAox2b gene was induced by an osmotic stress but it was underexpressed in salt stress conditions. In the sensitive cultivar (Vita 5), the transcript level of the VuAox2b was unchanged in response to Polyethylene glycol (PEG) treatment, and upon salt stress, it was overexpressed.

Arnholdt-Schmitt et al. (2006) have pointed to the importance of considering species- and genotype-specific AOX expression and its kinetics. Additionally, a theoretical strategy was developed that highlighted the significance of designing molecular functional markers for agronomic traits from important target tissues in a top-down approach starting at whole plant level (Arnholdt-Schmitt 2005a, 2005b). Recent data confirm this view concerning genotype dependent AOX expression on salt treatments (Mhadhby, Fotopoulos, Mylona, Aouani and Polidoros unpublished results). Examining the differential response of a set of antioxidant genes with real-time RT-PCR in Medicago truncatula leaves and roots to high salt concentration in one sensitive and two tolerant varieties, a different picture regarding Aox expression was recorded than in V. unguiculata. The response of an Aox1a ortholog in this species was dependent on salt concentration and showed differences depending on the duration of the treatment (24 or 48 h) and the organs studied in interaction with the genotype.

Conclusions

Many lines of evidence suggest that AOX may act as a central regulator of plant growth and development as well as an integrator of stress signals for defense deployment under stress. Therefore, it has been proposed that this gene could be a promising candidate for functional marker-assisted breeding strategies for stress tolerance. The use of Aox as a marker should be based on the presence of polymorphic sequences and the identification of polymorphic motifs related to altered gene expression, and ultimately modified AOX engagement and capacity, leading to phenotypic variation for the trait under investigation. In this review, we presented evidence documenting that Aox genes are highly polymorphic regarding the gene family organization and gene sequence, and display variable expression patterns in normal plant development and as a response to stress. However, limited data are available and still do not allow an unequivocal link of sequence polymorphism with phenotypic variation regarding stress tolerance. The possibility to establish the missing link exists as sequence variation has been observed at all levels and gene regions encompassing SNPs, indels and 3’-UTR microheterogeneity. This variation can have immense effects on both Aox expression and AOX function. Initial reports show that different genotypes displaying various degrees of tolerance under a certain stress condition have also differences in Aox expression responses to the stress. Many factors seem to affect the course and magnitude of the response. Further studies are needed to establish which of the different parameters affecting Aox gene expression will be critical for a strong correlation between Aox gene expression and the stress response phenotype. Understanding the potential of using Aox as a functional marker for stress breeding will be largely dependent upon our ability to recognize these factors and integrate their effects into a system correlating Aox sequence polymorphisms, the kinetics and variability of gene expression at transcript level, and AOX activity and capacity to the stress response. However, from the perspective of a breeder, it will be sufficient to closely associate or link identified AOX polymorphic sequences to target agronomic traits. This would allow initiating marker-assisted breeding strategies but broad establishment of this association or link is still not documented by the limited available results and is a question of increasing interest for plant breeders and plant biologists.

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

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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 ‘Cobrancosa’ could be significantly reduced by treatment with salicyl-hydroxamic acid, 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 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’, ‘Cobrancosa’ 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; H₂O₂, 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, Vanlerbergh 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 H₂O₂ (e.g. Amirsadeghi et al. 2006, Gray et al. 2004, Popov et al. 1997, Umbach et al. 2005, Vanlerbergh et al. and McIntosh 1996). Consequently, inhibitors of AOX activity, such as salicyl-hydroxamic acid (SHAM), were found to interact with H₂O₂ cell levels. In SHAM-treated *Arabidopsis thaliana* roots, peroxidase was inhibited and root length was strongly reduced. H₂O₂ disappeared from the root hair zone, but was accumulated in cell walls in the meristemic region (Dunand et al. 2007). H₂O₂ 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 H₂O₂ 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 *aoo1a* 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 H₂O₂ 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 ‘Cobrancosa’, and screening of a partial sequence of *OeAOX2* from cultivars ‘Cobrancosa’ 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, H₂O₂ 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 ‘Cobrancosa’. Shoot cuttings of about 14 cm length were collected from the medium portion of 1-year-old branches. The ‘Cobrancosa’ 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, H2O2 (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.

Table 1. Treatments of SHAM, H2O2 and pyruvate applied to semi-hardwood olive shoot cuttings (for details see section Materials 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>
<td>3 h</td>
</tr>
<tr>
<td>7</td>
<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>
<td>0.01</td>
<td>3 h</td>
</tr>
<tr>
<td>10</td>
<td>Pyruvate</td>
<td>0.1</td>
<td>3 h</td>
</tr>
<tr>
<td>11</td>
<td>Pyruvate</td>
<td>0.01</td>
<td>20 s</td>
</tr>
<tr>
<td>12</td>
<td>Pyruvate</td>
<td>0.1</td>
<td>20 s</td>
</tr>
</tbody>
</table>

Each replication with 20 semi-hardwood cuttings was used as an experimental unit for statistical data analysis. The trials with H2O2 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.

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 (T0), 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 Transcriptase (Fermentas, Ontario, Canada) using the oligo(dT) primer 5′-GACCA-GCGGTATCGATGTCGACTTTTTTTTTTV (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 mM dNTP (Fermentas, Ontario, Canada), 0.05 μg μl⁻¹ of Actinomycin D (Roche, Mannheim, Germany), 2 mM 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 μM of each primer OeAOX2.1111Fw: 5′-CCACTAGTTGTTGGTGCTTG-3′ and OeAOX2.12-46Rev: 5′-AGTAAGTGGGATCATTTCTTAG-3′ (both from Eurofins MWG Operon, Ebersberg, Germany). RT-PCR was normalised by O. evnopeal Actin using the primers Oe AF: 5′-TTGCTCTCGACTATGAACGG-3′ and Oe AR: 5′-CTCCTCGGCCCATAATAATA-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).

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′-ATTGCATCTAGGGTGCTTTGCC-3′ and OAOX2.6-47Rev: 5′-CAGGCATAAGTGAAGGCTG-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 x manufacturer supplied Phusion HF buffer containing 1.5 mM MgCl₂, 0.2 mM 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 x manufacturer supplied (NH₄)₂SO₄ buffer, 2.5 mM MgCl₂ and 0.2 mM 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 EcoRI. Recombinant clones were

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), ‘Cobrancosa’ and ‘Picual’ was isolated. The trees were grown in fields in Montemor-o-Novo (‘Galega vulgar’ and ‘Cobrancosa’) 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.

Fig. 1. Structure of the partial OeAOX2 gene from O. europaea cv. Galega vulgar.
used for commercial sequencing: 1 from ‘Galega vulgar’, 7 from ‘Cobrancosa’ 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 μM 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 precursors and mature miRNAs in intron sequences

Putative miRNA precursors were searched in eight intron sequences of the OeAOX2 gene from ‘Galega vulgar’, ‘Cobrancosa’ 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.bioinfo.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/arna-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).
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%.

**H₂O₂ treatment of shoot cuttings**

The effect of H₂O₂ 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 mM. 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 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).

<table>
<thead>
<tr>
<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><strong>Cv.</strong></td>
<td><strong>Origin</strong></td>
<td><strong>M</strong></td>
</tr>
<tr>
<td>Galega vulgar</td>
<td>gDNA</td>
<td>1</td>
</tr>
<tr>
<td>Montemor</td>
<td>cDNA</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
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<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Cobrançososa</td>
<td>gDNA</td>
<td>1</td>
</tr>
<tr>
<td>Montemor</td>
<td>cDNA</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
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<td>4</td>
</tr>
<tr>
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</tr>
</tbody>
</table>

Physiol. Plant. 137, 2009
Fig. 5. Alignment of the translated amino acid 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 with 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. 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 mM 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 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). 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 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 ‘Cobrancosa’, 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 (Table 2). The stop codon is located 160 bp upstream the di-iron binding site of the LEEEA box (Fig. 5).

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 (Kaide et al. 1998).

The intron of ‘Cobrancosa’ 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 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 ‘Cobrancosa’ (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 ‘Cobrancosa’ 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′-GAAAUAAGAUAUUUGUGA-3′) was identified with homology to a miRNA of A. thaliana ath-miR417 (78-GAAGGUGAAUUUGUGA-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-GAAUGUGAUAUUUGUCCA-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 ‘Cobrancosa’ this region demonstrated five
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 OeAOX2 through four different OeAOX2 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).

<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>Gal_8</td>
<td>AUAGAUAAUUGUCAUUUUUGUCGCCCUAUAUCCCACCCCUUUUCGCUGCUUAUUUGUGCAAGUGUGCCAGAUGCUAU</td>
<td>82</td>
<td>−13.22</td>
<td>No</td>
</tr>
<tr>
<td>Cob_2</td>
<td>UUUUUUGUGUCGAAUAAGGAAUUGGAUUGGAUUUCAAUAAAUAUAUAGUGUCAUUUGUGCAAGUUGGUGCAAGUGCUAU</td>
<td>73</td>
<td>−16.30</td>
<td>65.9%</td>
</tr>
<tr>
<td>Cob_3</td>
<td>AUAGAUAAUUGUCAUUUUUGUCGCCCUAAUCCCCUUUUGCCUGCUUAUUUGUGCAAGUGUGCCAGAUGCUAU</td>
<td>81</td>
<td>−11.72</td>
<td>No</td>
</tr>
<tr>
<td>Cob_5</td>
<td>AUAGAUAAUUGUCAUUUUUGUCGCCCUAUAUCCCUUUUGGCCCUGCUUAUUUGUGCAAGUGUGCCAGAUGCUAU</td>
<td>82</td>
<td>−13.12</td>
<td>No</td>
</tr>
<tr>
<td>Cob_9</td>
<td>AUAGAUAAUUGUCAUUUUUGUCGCCCUUAAUCCCUUUUGGCUGCUUAUUUGUGCAAGUGUGCCAGAUGCUAU</td>
<td>81</td>
<td>−11.72</td>
<td>No</td>
</tr>
<tr>
<td>Pic_1</td>
<td>AUAGAUAAUUGUCAUUUUUGUCGCCCUUAAUCCCUUUUGGCUGCUUAUUUGUGCAAGUGUGCCAGAUGCUAU</td>
<td>81</td>
<td>−11.72</td>
<td>No</td>
</tr>
<tr>
<td>Pic_2</td>
<td>AUAGAUAAUUGUCAUUUUUGUCGCCCUUAAUCCCUUUUGGCUGCUUAUUUGUGCAAGUGUGCCAGAUGCUAU</td>
<td>81</td>
<td>−11.72</td>
<td>No</td>
</tr>
<tr>
<td>Pic_3</td>
<td>AUAGAUAAUUGUCAUUUUUGUCGCCCUUAAUCCCUUUUGGCUGCUUAUUUGUGCAAGUGUGCCAGAUGCUAU</td>
<td>81</td>
<td>−11.72</td>
<td>No</td>
</tr>
</tbody>
</table>

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 GUUUGGUU includes the patterns UUGUUG (1st), UUUUGU (2nd) and GUUUGG (22th); the FUE.3 region UUGUUGGU is related to the 21st and 23rd patterns identified in A. thaliana (UUGUUG and UUGUGG, respectively); the FUE.4 region, UAUUUGUUUAAGUUUGUUUCUU, 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.
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. 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\(_2\) 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).

### 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>

(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 AAAAUU. 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\(_2\)O\(_2\) 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\(_2\)O\(_2\) 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\(_2\)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.

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 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 Gal₃ (software MIRANDA v3.0).

<table>
<thead>
<tr>
<th>miRNA identification</th>
<th>nt position</th>
<th>Alignment</th>
<th>Energy (kcal mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ath-miR401 (Sunkar and Zhu 2004) 2-23</td>
<td>Query: 3' acAGCCAGC-UGUGGUCAAAgc 5'</td>
<td>Ref: 5' tgTTGGTGTGCTAGTTcat 3'</td>
<td>-22.10</td>
</tr>
<tr>
<td>ppt-miR1212 (Talmor-Nieman et al. 2006) 35-51</td>
<td>Query: 3' acAGCCACGUGUGUCAAAAgc 5'</td>
<td>Ref: 5' aTTGGTCGTTTTg 3'</td>
<td>-20.10</td>
</tr>
<tr>
<td>smo-miR1110 (Axtell et al. 2007) 27-47</td>
<td>Query: 3' gcGUAAGAUACGACAGGGUgc 5'</td>
<td>Ref: 5' cgCGTTTTAATTGGTCCCGgt 3'</td>
<td>-21.36</td>
</tr>
<tr>
<td>tae-miR1125 153-171</td>
<td>Query: 3' agGAACUGGUGACGGGGAUCg 5'</td>
<td>Ref: 5' gatCTGCCGCTAGT-TTGGTc 3'</td>
<td>-22.56</td>
</tr>
<tr>
<td>tae-miR1125 158-177</td>
<td>Query: 3' ggcGGCGUCAACCAGAGCAAACCAa 5'</td>
<td>Ref: 5' gatCTGCCGCTAGT----TTGGTc 3'</td>
<td>-23.97</td>
</tr>
</tbody>
</table>

Galega vulgar 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 RADE₃ 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 LEEEA 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 ‘Cobranc¸osa’ 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 Gal1-2, Gal1-3 and Gal1-4 (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 (Gal1-1) 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 $\Delta G < -20 \text{ kcal mol}^{-1}$. 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.

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