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Involvement of a cinnamyl alcohol dehydrogenase of *Quercus suber* in the defence response to infection by *Phytophthora cinnamomi*

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Abstract

A gene encoding a potential NADPH-dependent cinnamyl alcohol dehydrogenase (QsCAD1) (GenBank accession no: AY362455) was identified in *Quercus suber* (cork oak). Its complete cDNA sequence was obtained by RACE-PCR, starting from total RNA extracted from roots of seedlings of *Q. suber*, infected with *Phytophthora cinnamomi*, the causal agent of the decline and sudden death of *Q. suber* and *Quercus ilex* subsp. *rotundifolia* in the Iberian Peninsula. Sequence information to perform the RACE-PCR was acquired from a polymorphic fragment (C9), specifically identified by cDNA-AFLP, in leaves of epicormic shoots of a cork oak tree that suffered sudden death. RT-PCR and hybridization analysis showed that the *QsCAD1* gene is up-regulated in root seedlings of *Q. suber* infected with *P. cinnamomi*. QsCAD1 has a high structural homology with VR-ERE (*Vigna radiata*), an enzyme that detoxifies eutypine (produced by *Eutypa lata*, the causal agent of eutypa dieback of grapevines), to eutypinol, and with QrCAD1 (*Q. ilex* subsp. *rotundifolia*), EgCAD1 (*Eucalyptus gunnii*), MdCAD1 (*Malus x domestica*). Taken together, these results suggest that these enzymes, and namely QsCAD1 belong to a new group of CAD potentially involved in deactivation of toxins produced by phytopathogens.
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Keywords: Oak tree; *Phytophthora cinnamomi*; Cinnamyl alcohol dehydrogenase; Defence response

1. Introduction

The involvement of *Phytophthora cinnamomi* in the decline disease of cork oak (*Quercus suber*) and holm oak (*Quercus ilex* subsp. *rotundifolia*) in the Iberian Peninsula was hypothesized 10 years ago [1,2]. Recent extensive field surveys and greenhouse experiments provided evidence that *P. cinnamomi* infects the roots of these evergreen oaks. The reduction of the amount of roots available for water and nutrient uptake, caused by *Phytophthora* root rot, leads to widespread deaths of cork oak and holm oak, either directly, or by predisposing the trees to other biotic and abiotic stresses, factors usually nondeleterious to healthy plants [1,3,4]. In affected areas, some cork oaks exhibit a range of symptoms and a variable rate of disease expression, while others die suddenly. Those that show evidence of chronic disease show a gradual deterioration of the crown that starts with leaf chlorosis and dieback of leaf bearing branches. As disease progresses, anomalous leaf fall occurs. New leaves, formed on apparently dead shoots, have reduced size, resulting in thinning of the crown and reduction of leaf area. Some affected trees show stem fluxes indicative of root malfunction. Trees showing such symptoms may survive for several years, depending on their resistance to disease progression and adaptation to environmental conditions. In contrast to chronic disease decline, the sudden death process is characterized by a quick drying of the leaves and death of the tree, within few months. The causes of these contrasting behaviours, slow decline and sudden death, are not known. Until now, no efficient methods have been found that will limit the threat of the disease to the oak ecosystem.

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Toxins produced by some fungi and bacteria play an important role in the expression of disease in plants. Toxin tolerance or resistance has been often associated with the capacity of the plant to metabolically detoxify the toxin [5–7]. As association between toxin tolerance and resistance to the disease has been frequently described [8,9], it is important to explain the mechanism underlying detoxification, and characterize the enzymes involved in this process.

A novel NADPH-dependent aldehyde reductase gene (*VR-ERE*) from *Vigna radiata,* that confers resistance to the grapevine fungal toxin eutypine [4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzyl aldehyde], was recently described [10]. Eutypine is produced by the mycelium of *Eutypa lata,* the causal agent of eutypa dieback of grapevines [11–14]. It induces a number of symptoms, including dwarfed and withered shoots, marginal necrosis of the leaves, desiccation of the inflorescences and death of branches. It was suggested [15] that eutypine is transported from the trunk through the sap to the herbaceous parts of the vine, where it exerts its toxic action. The recombinant form of VR-ERE was shown to have a high affinity for 3-substituted benzaldehydes, reducing them to alcohols. Its reducing activity was namely confirmed with eutypine, that it converts into the nontoxic corresponding benzyl alcohol, eutypinol [16]. The tolerance of some cultivars to this disease was correlated with their capacity to achieve this conversion.

The aim of the present work is to disclose cork oak genes involved in the defence response to infection by *P. cinnamomi,* namely in connection with the sudden death disease expression. As a first step in attempting this objective, we looked for polymorphic fragments present in mRNA profiles of leaves from adult asymptomatic trees and from trees that either exhibited symptoms of slow decline or underwent sudden death. In a second step, we have looked for the corresponding genes in roots of cork oak seedlings. The outcome of this approach was the identification of four genes, namely one that encodes a cinnamyl alcohol dehydrogenase (CAD), highly homologous to *VR-ERE*. The expression of CAD was analysed in infected and noninfected roots of *Q. suber* seedlings. The potential involvement of the uncovered CAD in the detoxification of a putative toxin produced by *P. cinnamomi* is discussed.

2. Material and methods

2.1. cDNA-AFLP analysis

2.1.1. Plant material

Fully expanded young leaves were collected in May, from five adult cork oak trees (BS222A-4, BS250HL-0, BS110HL-3, AS17BV-0 and AS21BV-3) located in infested sites from decline areas of Alentejo and Algarve regions of Portugal, and immediately stored at −80 °C. *P. cinnamomi* had previously been isolated from roots and associated soil of oak trees located in those sites [3]. Trees were selected on the basis of the defoliation degree (DG), ranging from 0 (healthy tree) to 4 (dead tree), according to the scale proposed by Cadahia et al. [17]. The last figure, included in the designation of the trees, corresponds to the DG attributed at the moment of the collection of the leaves. Leaves from a cork oak tree affected by sudden death (BS222A-4) were collected from epicormic shoots present in the trunk.

2.1.2. Extraction and purification of total RNA

Total RNA was extracted from 50 mg of leaves with the RNeasy Kit from Qiagen, according to the instructions supplied by the manufacturer [18]. Total RNA was then treated with DNase I (1 U/μL; Gibco), in the presence of 2 μL of RNaseout (40 U/μL; Gibco) [19]. The reaction occurred in a final volume of 100 μL, for 30 min at 37 °C. After DNA digestion, the total RNA was purified with the RNeasy Kit. The quality and the quantity of total RNA present in the samples were evaluated by UV spectrophotometry and by electrophoresis in denaturing agarose gel.

2.1.3. cDNA synthesis

The synthesis of cDNA was accomplished with the cDNA synthesis system (Roche), with modifications to the original protocol. Synthesis of the first cDNA strand was initiated with the degenerate primer [5′AGTGAATT CT12V (V = A; C; G], comprised of an equimolar mixture of the three oligonucleotides [20]. Synthesis of the second cDNA strand and digestion of residual RNA were performed according to the kit protocol [21,22]. The double strand cDNA was then purified with the Qiaquick PCR purification kit (Qiagen). This methodology is designed to purify double-stranded DNA fragments from enzymatic reaction mixtures. Fragments ranging from 100 bp to 10 kb were separated from polymerases, RNases, ligases, nucleotides and salts. In the process DNA binds to the QIAquick column (silica-gel membrane), and proteins or compounds resulting from the enzymatic reactions are eliminated by washing the column with buffers. In the last step of the protocol, the DNA is eluted from the column with 50 μL of the elution buffer (10 mM Tris–Cl, pH 8.5). The volume of cDNA samples, eluted from the column, was reduced to a final volume of 20 μL by centrifugation under vacuum. cDNA samples (20 μL) were then subjected to the standard AFLP template production [23]. The cDNA was first digested with *Eco*RI and *Mse*I (2.5 U/μL each) in a restriction buffer (50 mM Tris–HCl pH—7.5, 50 mM magnesium acetate, 250 mM potassium acetate), in a total volume of 25 μL for 2 h at 37 °C (AFLP Core Reagent Kit, GIBCO BRL). After enzyme inactivation at 70 °C for 15 min, *Eco*RI (5 pmol) and *Mse*I (50 pmol) adaptors were ligated to cDNA digested fragments. The reaction was performed for 2 h at 37 °C in ligase buffer, 1 U of T4 DNA ligase and ATP (0.4 mM) (AFLP Core Reagent Kit, GIBCO BRL). The cDNA ligated to the adaptor was pre-amplified using the following cycling parameters: 28 cycles of 30 s at 94 °C; 60 s at 56 °C and 60 s

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