New Phytologist

Research

# Tolerance of olive (*Olea europaea*) cv Frantoio to *Verticillium dahliae* relies on both basal and pathogen-induced differential transcriptomic responses

**María de la O Leyva-Pérez[1], Jaime Jiménez-Ruiz[1], Carmen Gómez-Lama Cabanás[2], Antonio Valverde-Corredor[2], Juan B. Barroso[1], Francisco Luque[1] and Jesús Mercado-Blanco[2]**

[1]Department of Experimental Biology, Center for Advanced Studies in Olive Grove and Olive Oils, University of Jaén, Jaén 23071, Spain; [2]Department of Crop Protection, Institute for Sustainable Agriculture, Agencia Estatal Consejo Superior de Investigaciones Científicas (CSIC), Campus 'Alameda del Obispo', Avenida Menéndez Pidal s/n, Apartado, Córdoba 14004, Spain

Author for correspondence:
*Jesús Mercado-Blanco*
*Tel: +34 957 499261*
*Email: jesus.mercado@ias.csic.es*

Received: *19 April 2017*
Accepted: *26 August 2017*

*New Phytologist* (2018) **217:** 671–686
**doi**: 10.1111/nph.14833

**Key words:** defence response, defoliating pathotype, olive transcriptome, RNA-seq, tolerance, vascular pathogen, verticillium wilt of olive.

## Summary

- Verticillium wilt of olive (VWO) is one of the most serious biotic constraints for this tree crop. Our knowledge of the genetics of the tolerance/resistance to this disease is very limited. Here we show that tolerance of the cv Frantoio relies on both basal and early pathogen-induced differential transcriptomic responses.
- A comparative transcriptomic analysis (RNA-seq) was conducted in root tissues of cvs Frantoio (VWO-tolerant) and Picual (VWO-susceptible). RNA samples originated from roots of inoculated olive plants during the early infection stages by *Verticillium dahliae* (highly virulent, defoliating pathotype).
- A huge number of differentially expressed genes (DEGs) were found between 'Frantoio' and 'Picual' (27 312 unigenes) in the absence of the pathogen. Upon infection with *V. dahliae*, 'Picual' and 'Frantoio' plants responded differently too. In the early infection stages, four clusters of DEGs could be identified according to their time-course expression patterns. Among others, a pathogenesis-related protein of the Bet v I family and a dirigent-like protein involved in lignification, and several *BAK1*, *NHL1*, reactive oxygen species stress response and *BAM* unigenes showed noticeable differences between cultivars.
- Tolerance of 'Frantoio' plants to VWO is a consequence of a complex and multifaceted process which involves many plant traits.

## Introduction

Olive (*Olea europaea* L.), one of the first domesticated tree species, has undisputed historical, economic and social relevance, particularly in the Mediterranean Basin where it is mainly cultivated. The crop is spreading to other Mediterranean-type climatic regions worldwide (International Olive Council data; http://www.internationaloliveoil.org/estaticos/view/131-world-olive-oil-figures). Among biotic constraints affecting olive, Verticillium wilt (VWO) caused by the soilborne fungus *Verticillium dahliae* Kleb. stands out as one of the most serious diseases affecting this woody crop. This hemibiotrophic, vascular pathogen can infect hundreds of plant species, including high-value annual and perennial crops (Pegg & Brady, 2002; Klosterman *et al.*, 2009; Daayf, 2015). Besides its broad host range, the pathogen's ability to produce quiescent structures (microsclerotia) able to endure in soil for many years and the systemic nature of the infection make *V. dahliae* very difficult to control (Pegg & Brady, 2002). In the particular case of VWO, the alarming spread and increasing prevalence of the highly virulent, defoliating (D) pathotype (lineage 1A) has caused deep concern in many olive-growing regions (Dervis *et al.*, 2010; López-Escudero *et al.*, 2010; Triki *et al.*, 2011; Milgroom *et al.*, 2016). So far, no measure has proven successful when applied individually. The implementation of an integrated disease management strategy for the effective control of VWO is thus strongly recommended (López-Escudero & Mercado-Blanco, 2011).

Within this holistic framework, the use of tolerant/resistant olive varieties is probably the most efficient and environmentally friendly approach to control VWO. Several studies have focused on the search for sources of genetic resistance to *V. dahliae* (López-Escudero *et al.*, 2004; Martos-Moreno *et al.*, 2006; Antoniou *et al.*, 2008; Colella *et al.*, 2008; Trapero *et al.*, 2013, 2015; García-Ruiz *et al.*, 2014; Arias-Calderón *et al.*, 2015a,b; Jiménez-Fernández *et al.*, 2016), although no cultivar/genotype has been reported as entirely resistant to the pathogen. Some genotypes have been qualified as tolerant (i.e. Koroneiki, Empeltre, Changlot Real) to *V. dahliae*, although the vast majority of cultivated varieties are (very) susceptible (i.e. Manzanillo, Cornicabra, Picudo) to VWO (López-Escudero & Mercado-Blanco, 2011). Regrettably, cultivars with the highest economical and historical relevance are among them (Sesli *et al.*, 2010; Erten & Yildiz,

2011; Trapero *et al.*, 2013; García-Ruiz *et al.*, 2015). The term tolerance (Robb, 2007) is used here for olive varieties able to cope with infections of *V. dahliae* without developing severe symptoms of the disease. 'Frantoio' is thus considered as one of the most tolerant cultivars to VWO, in contrast to, for example, the very susceptible cv Picual (López-Escudero *et al.*, 2004; Martos-Moreno *et al.*, 2006; Trapero *et al.*, 2013, 2015; Gómez-Lama Cabanás *et al.*, 2015).

The development of novel and effective disease management strategies relies on, among other considerations, a thorough understanding of the mechanisms deployed by the host plant in response to the pathogen attack. However, genetic bases underlying plant defence responses against vascular and/or root pathogens are poorly understood (Okubara & Paulitz, 2005; Fradin & Thomma, 2006; Yadeta & Thomma, 2013; Häffner & Diederichsen, 2016). With regard to *V. dahliae*, structural (e.g. tylose formation in the xylem) and/or biochemical (e.g. accumulation of phenolic compounds) responses in plant tissue have been reported which can be either constitutive or induced upon pathogen infection (Mueller & Morgham, 1993; Daayf *et al.*, 1997; Baídez *et al.*, 2007; Markakis *et al.*, 2010). Gene expression changes (i.e. transcriptional profiling) induced upon *V. dahliae* attacks have also been studied in several interactions, but mostly involving herbaceous hosts (Gayoso *et al.*, 2010; Derksen *et al.*, 2013; Zhang *et al.*, 2013).

Bubici & Cirulli (2012) proposed that tolerance of cv Frantoio to VWO is mediated by biochemical-based responses rather than by structural mechanisms such as vascular plugging. Moreover, accumulating evidence indicates that these reactions seem to be more obvious in tolerant than in susceptible olive cultivars (Baídez *et al.*, 2007; Markakis *et al.*, 2010; Bubici & Cirulli, 2012; Gharbi *et al.*, 2016, 2017a,b). A similar scenario has also been observed in *V. dahliae*-infected cotton (*Gossypium hirsutum*) plants, these defence responses being more pronounced in tolerant than in susceptible varieties (Daayf *et al.*, 1997). However, our current knowledge of defence responses taking place upon infection by *V. dahliae* and of the genetics of Verticillium wilt tolerance/resistance in woody plants is very limited, including that of olive. Moreover, whether differential genetic responses could be related to the VWO susceptibility/tolerance level of olive cultivars has seldom been investigated. Recently, transcriptomic changes occurring in above-ground tissues during the interaction of *V. dahliae* (D pathotype) with 'Frantoio' roots were reported, many of them related to defence responses against a/biotic stresses (Gómez-Lama Cabanás *et al.*, 2015)

The rapid development of next-generation sequencing (NGS)-based approaches and '-omic' methodologies are boosting our understanding of plant–microbe interactions. Large-scale transcriptomic studies can offer a global picture of transcriptomic events taking place in a biological sample at a specific moment and/or under defined experimental conditions, and more importantly, without previous knowledge of the genome sequence (Strickler *et al.*, 2012; Martin *et al.*, 2013). Thus, RNA-seq has been used to broadly analyse gene expression in diverse *Verticillium*–plant interactions, although mostly involving herbaceous hosts (Xu *et al.*, 2011; Faino *et al.*, 2012; Sun *et al.*, 2013; Zhang *et al.*, 2013; Chen *et al.*, 2015; Tan *et al.*, 2015). In olive, a number of functional genomics studies based on RNA-seq have been carried out, but only a few were related to abiotic (Leyva-Pérez *et al.*, 2014) or biotic (Giampetruzzi *et al.*, 2016) stresses. Recently, an RNA-seq transcriptomic analysis of olive (cv 'Picual', highly susceptible to VWO) roots during the early interaction with *V. dahliae* (D pathotype) was conducted. An olive transcriptome ('Oleup') was thus obtained consisting of 68 259 unigenes (254 252 isoforms/transcripts) (Jiménez-Ruiz *et al.*, 2017).

Understanding the genetic basis underlying olive tolerance/susceptibility to *V. dahliae* will be instrumental for breeding for new VWO-tolerant cultivars, developing novel pathogen diagnostic tools, and/or designing novel tools for VWO management. Therefore, the objectives of this study are to assess whether cvs Frantoio and Picual display basal but differential transcriptomic responses without being challenged by *V. dahliae*; to compare root responses of both cultivars at the transcriptome level upon inoculation with the highly virulent, D pathotype of *V. dahliae* early in the pathogen's infection process; and to identify the presence/absence of specific defence-related gene responses explaining the differential VWO susceptibility level shown by olive cultivars. The hypothesis to be tested is that susceptibility/tolerance of olive cultivars to *V. dahliae* relies on both basal and pathogen-induced differential genetic responses.

## Materials and Methods

### *Verticillium dahliae* culturing conditions and inoculum production

A representative olive-infecting *V. dahliae* isolate (V937I) of the highly virulent, D pathotype (linage 1A) was used in this study (Collado-Romero *et al.*, 2006; Prieto *et al.*, 2009; Maldonado-González *et al.*, 2015). The starting culture and the working inoculum (conidia suspension) of isolate V937I used in the bioassay (see later) were prepared as described by Jiménez-Ruiz *et al.* (2017).

### *Verticillium dahliae*–olive (cvs Frantoio and Picual) bioassay

A bioassay was conducted to study the *V. dahliae* (D pathotype)–olive root interaction at the transcriptomic level, using nongnotobiotic conditions. Four-month-old potted olive plants of cvs Frantoio (for RNA sampling) and Picual (for assessment of VWO symptoms) were purchased from a commercial nursery. Cultivar Frantoio is qualified as tolerant to VWO, while cv Picual is highly susceptible to infections by *V. dahliae* D isolates (López-Escudero & Mercado-Blanco, 2011). Manipulation of acclimated plants and root dip inoculation in a conidial suspension ($1 \times 10^7$ conidia $ml^{-1}$ during 30 min) of isolate V937I were performed as previously described (Mercado-Blanco *et al.*, 2002). Roots of control plants (noninoculated) were dipped in sterile water. These plants were considered as controls for any potential, unintentional damage that roots could undergo during the manipulation and inoculation processes. After that, plants were

individually transplanted into polypropylene pots (7 × 7 × 8 cm) containing an autoclaved sandy substrate (Prieto & Mercado-Blanco, 2008). Plants were randomly distributed and maintained in a growth chamber adjusted to 24 : 21°C, day : night, 60% relative humidity, 14 h photoperiod of fluorescent light (360 μE $m^{-2}$ $s^{-1}$) during 15 d (for RNA sampling) or 2 months (for VWO symptoms observation). To ease plant stress after manipulation, inoculation and transplanting, the photoperiod was increased gradually (Gómez-Lama Cabanás *et al.*, 2015; Jiménez-Ruiz *et al.*, 2017). Roots of each 'Frantoio' plant were harvested at 0, 2 and 7 d (three plants per time point) after V937I inoculation (DAI). Tissue samples were immediately frozen in liquid nitrogen and kept at −80°C until total RNA extraction. An additional group of plants ('Picual' and 'Frantoio' *V. dahliae*-inoculated and noninoculated) were kept for up to 2 months after inoculation to assess VWO symptom development. In this study, samples from 0, 2, and 7 DAI were used for RNA-seq analysis (see later).

### RNA sample preparation and next-generation sequencing

To reduce plant-to-plant variability, we defined three groups of three randomly selected plants within each treatment and time condition. Total RNA samples were extracted from roots of control (nonmanipulated), 2 and 7 DAI, and noninoculated plants, using a Spectrum Plant Total RNA kit (Sigma-Aldrich) according to the manufacturer's instructions. Any DNA contamination was removed with on-column DNase digestion (Roche). The RNA quality tests were performed with the Agilent 2100 bioanalyser (Agilent Technologies, Santa Clara, CA, USA) using an RNA 6000 Pico assay kit (Agilent Technologies). Equimolar amounts of RNA from each group were pooled. Then, cDNA libraries were prepared and NGS sequencing was carried out by Sistemas Genómicos S.L. (Paterna, Valencia, Spain) using an Illumina HiSeq 1000 sequencer. Two replicates per sample were sequenced on different lanes in the flow cell.

### Quantitative real-time polymerase chain reaction analysis

First-strand cDNA was synthesized from 1 μg of total RNA primed with 60 μM of random hexamer primer and Transcriptor Reverse Transcriptase, using the Transcriptor First Strand cDNA Synthesis Kit (Roche), following the manufacturer's instructions. The quantitative real-time polymerase chain reaction (Q-RT-PCR) was performed in the Bio-Rad CFX96 and CFX384 PCR system with master mix SsoFast™ EvaGreen® Supermix (Bio-Rad), in 10 μl of reaction mixture, containing 10 ng of cDNA. Amplifications were performed as follows: initial polymerase activation at 95°C for 30 s, then 40 cycles at 95°C for 3 s, and at 60°C for 7 s, followed by a melting step from 65 to 95°C. An internal control of the constitutive olive actin-coding gene was used for normalization (García-López *et al.*, 2014). Each PCR reaction was performed three times and a pool of three different samples were analyzed at each time point. Oligonucleotides used for amplifications are listed in Supporting Information Table S1.

### Data preprocessing

The raw Illumina RNA-seq reads were first preprocessed using FastqMcf (Aronesty, 2011) by discarding primers and reads with adaptors, unknown nucleotides and poor-quality or short-length reads, increasing the Qscore to > 30 for all the libraries and length > 50 bp (Q30L50). A thorough quality control of sequencing was performed twice using FastQC software v.0.10.1 (Schmieder & Edwards, 2011) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to provide a summary and compare statistics files, before and after preprocessing.

### Gene expression and differentially expressed genes

Gene expression was carried out with RNA-Seq by Arraystar Lasergene 13, using a 95% false discovery rate (FDR). The reference transcriptome was obtained from the *Olea europaea* L. cv Picual (Jiménez-Ruiz *et al.*, 2017) to which 17 205 newly assembled unigenes from cv Frantoio that were absent in the cv Picual transcriptome were incorporated to generate the final 'PicFra' transcriptome consisting of 82 159 unigenes. Assembly and functional annotation of the cv Frantoio unigenes were performed following the same method as that for the 'Picual' transcriptome (Jiménez-Ruiz *et al.*, 2017). The most specific GO-term-enriched analysis was performed with Blast2GO (https://www.blast2go.com) (Conesa *et al.*, 2005).

### Availability of data and materials

The Illumina sequenced read data reported in this article have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive and are available under the following NCBI accession numbers: SRR5330848, SRR5330847, SRR5330842, SRR5330843, SRR5330844, SRR5330845). The Transcriptome project has been deposited under the accession number PRJNA378602.

## Results

### Nonmanipulated, *Verticillium dahliae*-free roots of 'Frantoio' (VWO-tolerant) and 'Picual' (VWO-susceptible) show different basal transcriptomes

A previous reference transcriptome from cv Picual (Jiménez-Ruiz *et al.*, 2017) was completed with 17 205 assembled unigenes from cv Frantoio to produce a newly assembled transcriptome of 82 159 unigenes, namely the 'PicFra' transcriptome, which was used as reference in this work. In order to identify transcriptomic differences related to the susceptibility of a tolerant (Frantoio) and a susceptible (Picual) cultivar to *V. dahliae* infection, the transcriptomes of control (i.e. nonmanipulated, noninoculated) roots of both cultivars were first compared. Remarkably, a huge number of differentially expressed genes (DEGs) were found between 'Frantoio' and 'Picual' roots (27 312) at a 95% FDR in the absence of unintentional root damage and/or pathogen infection. We particularly aimed to identify relevant changes in gene

expression. Thus, the analysis was made selecting DEGs with at least an eightfold change (8FC) between the two cultivars. The result was that 11 219 DEGs were found between them. Indeed, 2715 DEGs showed significant higher expression in 'Picual' roots while 8504 did so in 'Frantoio' roots. In fact, the differences were so outstanding that 1735 highly expressed DEGs in 'Picual' were undetectable in 'Frantoio', while 7961 highly expressed DEGs in 'Frantoio' were untraceable in 'Picual' roots (Fig. S1).

The results obtained after the biological process (BP) most specific GO-term-enriched analysis ($P < 0.001$) showed that overexpressed DEGs corresponding to transposon and retrotransposon elements, several types of stress response genes as well as some other processes, such as cell wall biogenesis, recognition of pollen, photosynthesis in light harvesting or protein unfolding, were significantly more represented in 'Picual' than in 'Frantoio' roots (Fig. 1a). In the latter, however, overexpressed DEGs related to different regulatory processes were more represented (Fig. 1b). Interestingly, these cultivars also showed dissimilar root system architecture, even though they were produced at the same time and with the same propagation procedure. 'Frantoio' roots were always less branched than 'Picual' roots, regardless the age of the sampled plants (Fig. S2).

Differences found between the basal 'Picual' and 'Frantoio' transcriptomes were further verified by carrying out real-time qPCR experiments on total RNA samples from roots of three 'Picual' and three 'Frantoio' *V. dahliae*-free, nonmanipulated nursery-produced plants. Fourteen randomly selected unigenes were analysed. The results corroborated the overall difference found by the RNAseq analysis. Indeed, seven out of the eight up-regulated unigenes in 'Frantoio' and five out of the six up-regulated unigenes in 'Picual' were validated (Fig. S3).

## Transcriptomic responses in 'Frantoio' and 'Picual' roots are dissimilar upon *Verticillium dahliae* infection

The RNA-seq analysis of 'Frantoio' and 'Picual' roots was done by comparing data of infected roots at 7 d after pathogen inoculation with data from manipulated control roots (at the same time

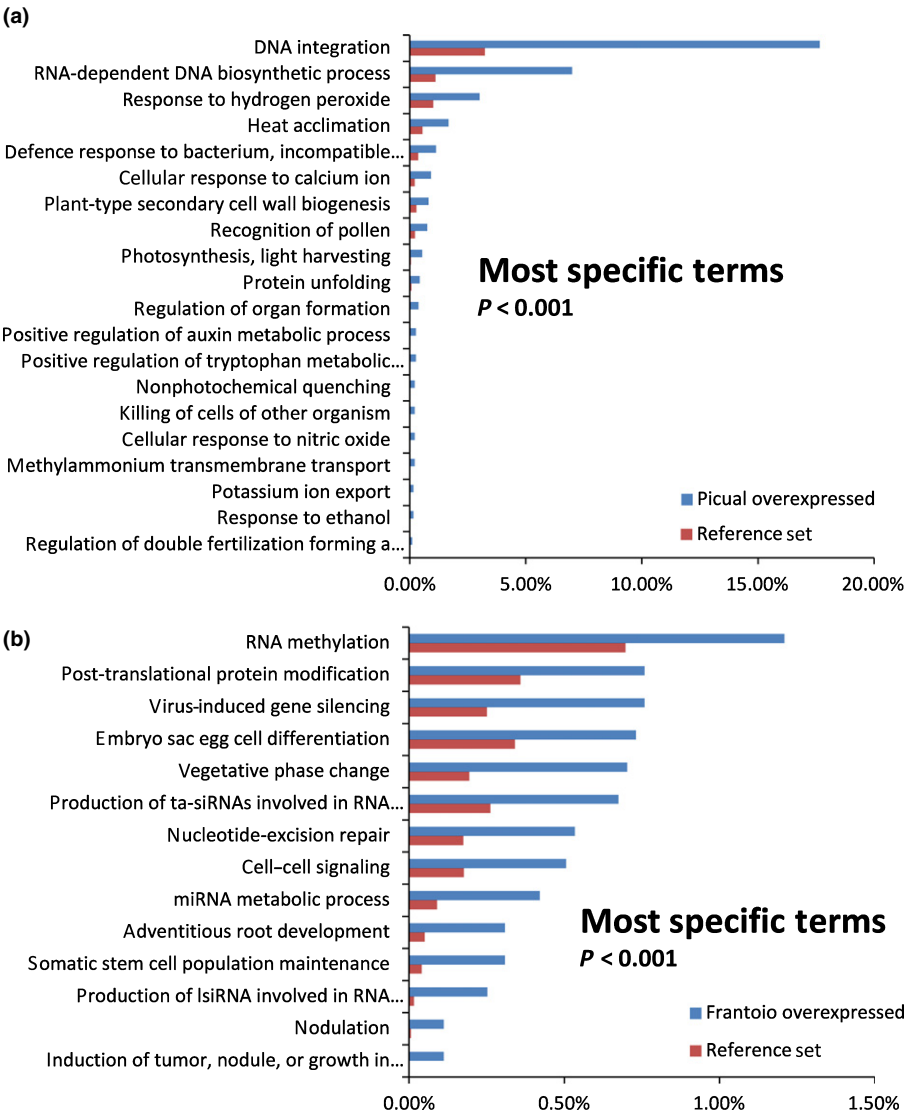


**Fig. 1** Olive 'Picual' and 'Frantoio' roots express very different sets of genes in the absence of unintentional root damage or *Verticillium dahliae* infection. Most specific gene ontology (GO) enrichment terms of overexpressed unigenes in: (a) 'Picual' roots; (b) 'Frantoio' roots.

but without the pathogen) and control, nonmanipulated roots. DEGs were obtained at 8FC and 95% significance. The response to *V. dahliae* infection resulted in a very different set of DEGs for both olive cultivars. First, in 'Frantoio' roots, up- and down-regulation processes were quantitatively very similar (i.e. 1983 and 2311 DEGs, respectively), whereas in 'Picual' roots up-regulation was overwhelmingly predominant and a lot higher than down-regulation (14 530 and 766 DEGs, respectively) (Fig. 2). Second, the vast majority of induced DEGs in both cultivars were different and just 420 DEGs were up-regulated in 'Frantoio' and 'Picual' (Fig. 3a). The BP most specific GO-term-enriched analysis ($P < 0.05$) showed that these 420 unigenes were mostly involved in several biosynthetic routes, root or meristem growth and auxin homeostasis (Fig. 3b; Table S2).

As 'Frantoio' control, nonmanipulated roots expressed a high number of DEGs whose expression was absent in cv Picual (Fig. 1), and 'Picual' roots showed significantly more up-regulated DEGs than 'Frantoio' roots in response to *V. dahliae* infection (Fig. 2), it might be that 'Frantoio' were constitutively expressing genes that in 'Picual' are only expressed in response to this biotic stress. To test this, we checked the presence of potential commonalities between the set of 14 530 up-regulated DEGs in 'Picual' in response to *V. dahliae* inoculation and that of 8504 DEGs which displayed higher expression in 'Frantoio' control, nonmanipulated roots. Remarkably, just 204 were common to both groups of DEGs. Therefore, 'Picual' plants respond to *V. dahliae* infection in a completely different way to 'Frantoio' plants. Moreover, the genes induced in 'Picual' roots upon pathogen infection are basically different from those differentially expressed in 'Frantoio' control, nonmanipulated roots. Besides, these 204 coincidental DEGs corresponded to GO terms not closely related to plant defence response (Fig. S4; Table S3).

## 'Frantoio' and 'Picual' roots show highly divergent DEG patterns during the early root infection process by *Verticillium dahliae*

Transcriptome changes occurring in roots in the early stages of the *V. dahliae* colonization and infection process are potentially crucial to explain the tolerance/resistance to VWO displayed by olive cultivars. Thus, transcriptome changes taking place in roots of the tolerant cv Frantoio were analysed and compared with those in the susceptible cv Picual. DEGs were selected (8FC and 95% significance) by comparing infected roots sampled at 7 DAI with control manipulated roots at 7 d post-manipulation and control untreated roots. Genes were then clustered in four groups according to the expression pattern observed at 2 and 7 DAI in 'Frantoio' and 'Picual' roots (Table S4).

Group 1 included DEGs that were induced at 7 DAI in 'Frantoio' but not in 'Picual'. This is the largest group by far, including 1310 DEGs (Fig. 4a; Table S4). The BP most specific GO-term-enriched analysis ($P < 0.025$) comprised, among others, defence response to fungus, incompatible interaction, defence response signalling pathway, resistance gene-independent, and several biosynthetic and catabolic processes (Fig. 4b).

Group 2 consisted of 598 DEGs up-regulated at 7 d postinfection in both cultivars (Fig. 5a). The BP most specific GO-term-enriched analysis ($P < 0.025$) included processes essentially related to growth and cell function rather than resistance to fungus, except for the process of systemic acquired

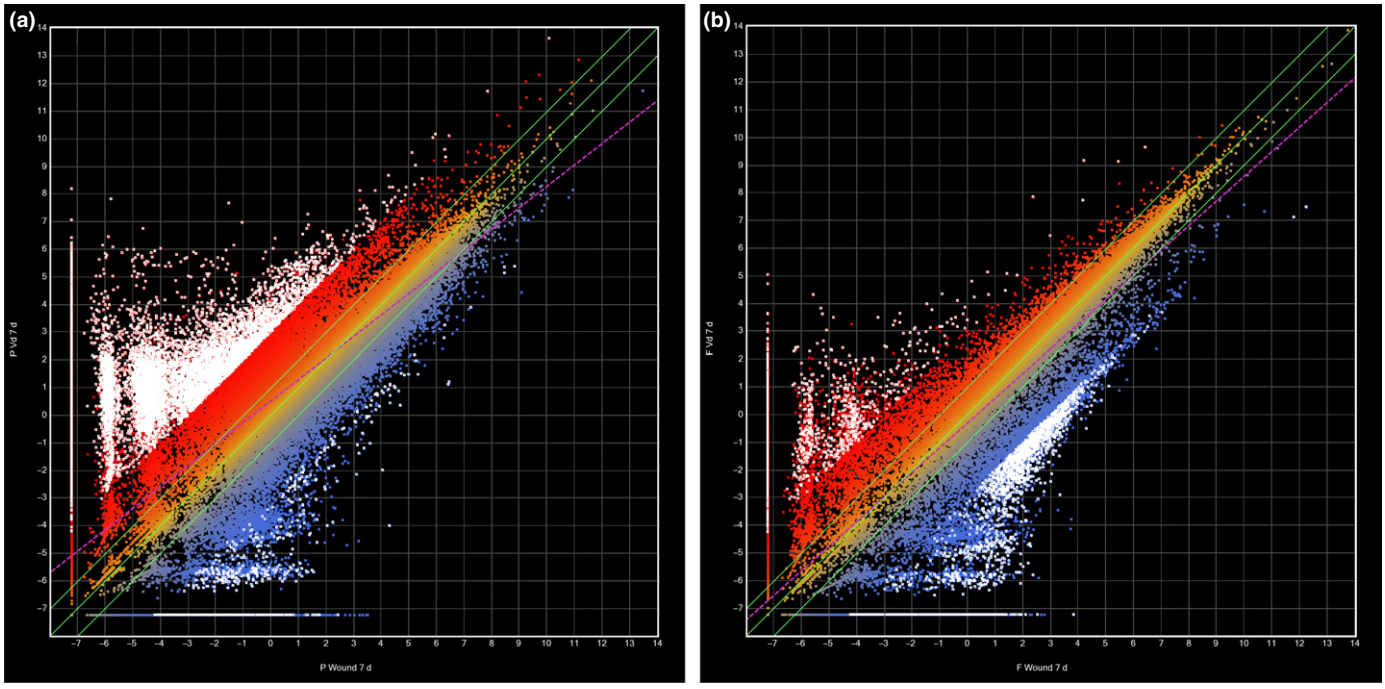


**Fig. 2** Scatter plots of unigenes that modify their expression in olive roots after 7 d of *Verticillium dahliae* inoculation. (a) 'Picual' roots; (b) 'Frantoio' roots.

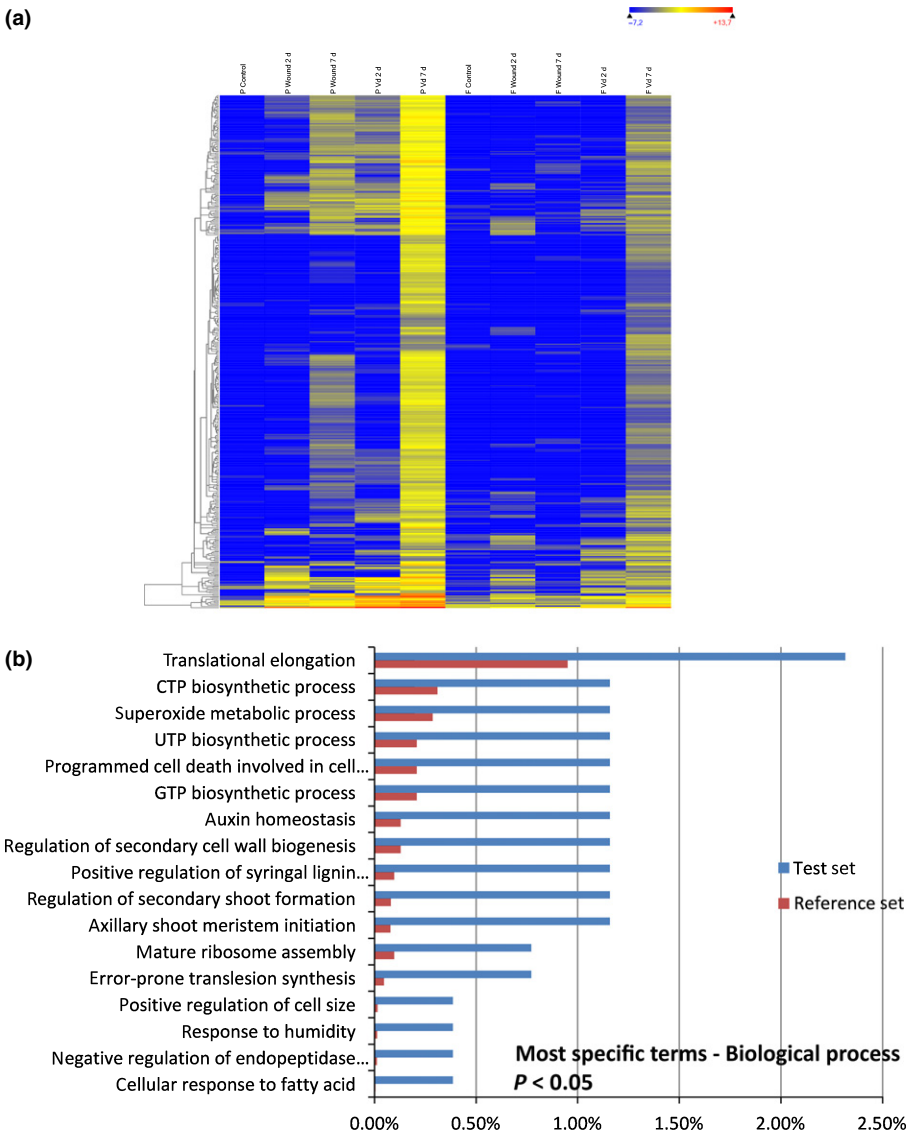


**Fig. 3** Heat map (a) and most specific gene ontology (GO) enrichment terms (b) of the 420 unigenes that are induced in both 'Picual' and 'Frantoio' olive roots 7 d after inoculation with *Verticillium dahliae*.

resistance, salicylic acid-mediated signalling pathway (Fig. 5b; Table S4).

Group 3 contained just 28 DEGs showing a transitory induction in 'Picual' (at 2 DAI) in contrast to 'Frantoio' roots, in which an increased expression over time (from 2 to 7 DAI) was observed (Fig. 6a). This was a small group of genes, and the BP most specific GO-term-enriched analysis ($P < 0.025$) showed transport processes (lactate transmembrane, arsenite, hydrogen peroxide and phosphate ion), response to arsenic-containing substance, and cell–cell junction assembly processes. These processes were represented by just one single DEG each (Fig. 6b; Table S4).

Finally, group 4 comprised 47 DEGs showing up-regulation in infected 'Frantoio' roots at 7 DAI (Fig 7a). Interestingly, these unigenes were already induced in control 'Picual' roots, but not in control 'Frantoio' roots. The BP most specific GO-term-enriched analysis ($P < 0.025$) showed processes represented by just one DEG, except for DNA integration. This might indicate that transposition events are promoted in stressed 'Frantoio' roots, but are already present in 'Picual' control roots (Fig. 7b; Table S4). This result was consistent with data shown in Fig. 1. The pattern of expression of 10 randomly selected DEGs was confirmed in 'Frantoio' and 'Picual' by real-time qPCR experiments (Fig. S5), thus validating the results obtained by the RNA-seq analysis.

## Targeting tolerance/resistance-related olive genes and their differential expression patterns in cultivars differing in susceptibility to *Verticillium dahliae*

Unigenes related to resistance to biotic stress have been identified (Table S5). Differences in expression patterns that could correlate with VWO tolerance level of olive cultivars were examined. First, we focused on a set of genes previously identified in plant–*Verticillium* interactions. Five putative *Ve1* (coding for Verticillium wilt disease resistance protein) unigenes were found in the olive transcriptome (Table S5; cells in green colour). Only two of them showed 8FC differential expression values among

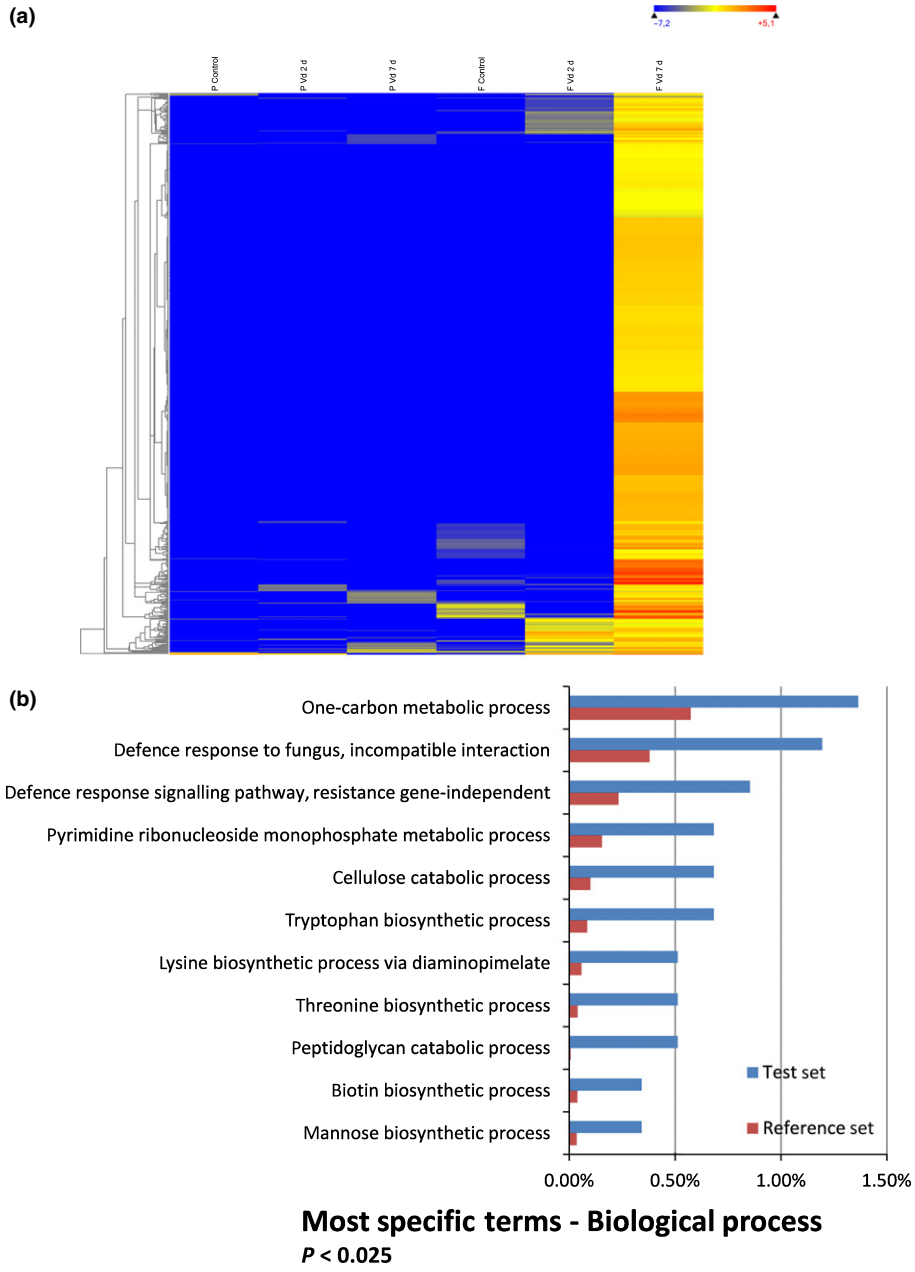


**Fig. 4** Heat map (a) and most specific gene ontology (GO) terms (b) of group 1 of clustered genes according to its pattern of expression at 2 and 7 d after *Verticillium dahliae* infection in olive 'Frantoio' and 'Picual' roots (see the Results section).

'Frantoio' and 'Picual' plants (Fig. 8). Twenty-four unigenes coding for putative NAC domain (for NAM (No Apical Meristem), ATAF1/2 (Arabidopsis Transcription Activation Factor), and CUC2 (Cup-Shaped Cotyledon)) transcription factors were also found (Table S5; cells in orange colour), but only five showed relevant differences between cultivars (Fig. 8). Seventeen *BAK1* (Brassinosteroid insensitive 1-Associated receptor Kinase) unigenes (Table S5; cells in pink colour) were also present in the olive transcriptome, but only three showed noticeable differences (i.e. down-regulation in 'Picual' but not in 'Frantoio' upon pathogen inoculation) (Fig. 8). A similar behaviour was observed in four out of nine NHL1 (=NDR1/HIN1-like) (Harpin-INduced protein-like) detected unigenes (Table S5; cells in grey colour) (Fig. 8). In addition, one *EDS1* (coding for a putative Enhanced Disease Susceptibility 1 protein) (Table S5; cell in dark blue colour), three *NDR1* (Nonrace specific Disease Resistance Protein 1) (Table S5; cells in yellow colour), and two *MEK2* (Mitogen-activated protein kinase kinase) unigenes (Table S5; cells in red colour), as well as one putative unigene coding for a Violaxanthin de-epoxidase (Table S5; cell in light blue colour) were also found. However, no significant differences were observed between the two olive cultivars.

Second, 167 unigenes related to disease resistance were also identified (Table S5). Differential expression patterns between cultivars were found for only a few of them, thereby enabling their classification into the following categories: nine unigenes with higher expression in 'Picual' plants regardless of whether or not *V. dahliae* was inoculated; 11 unigenes that were repressed

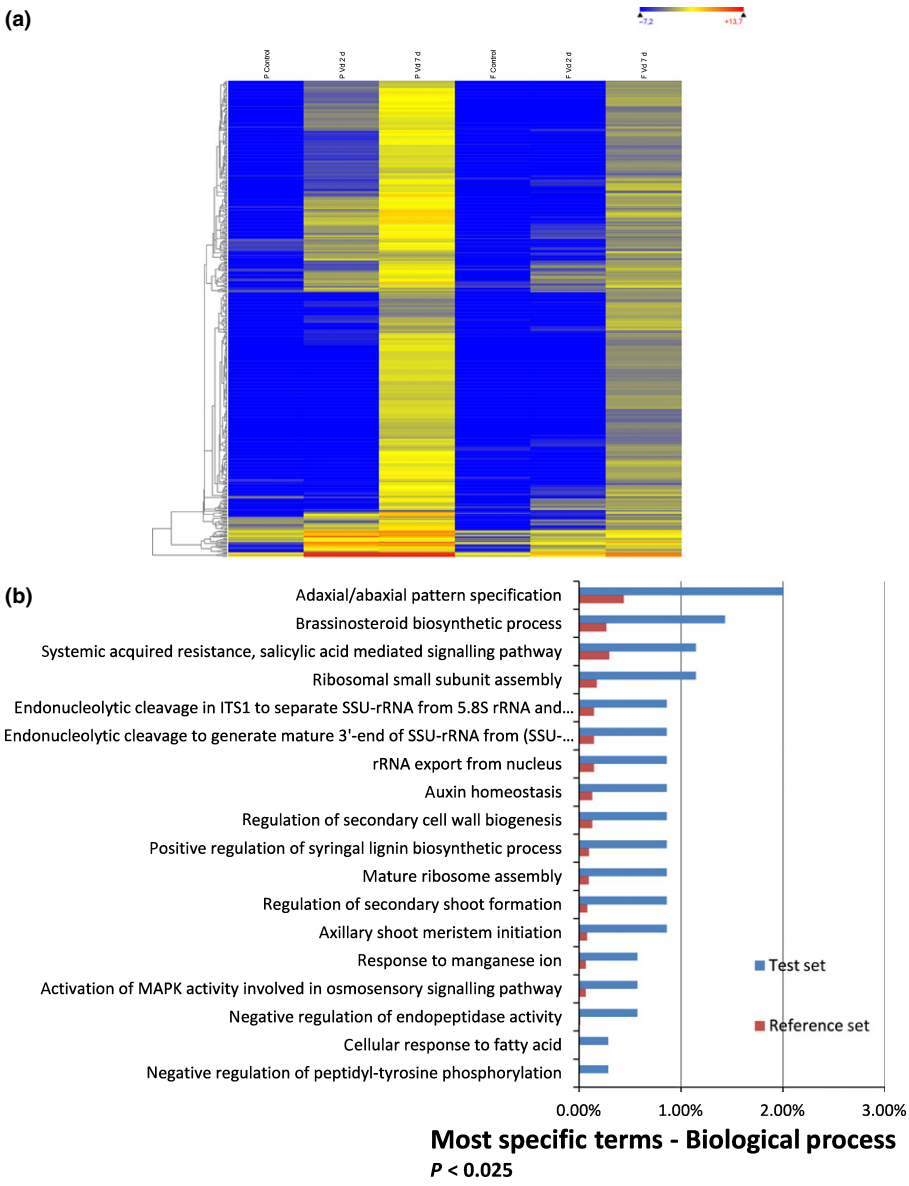


**Fig. 5** Heat map (a) and most specific gene ontology (GO) terms (b) of group 2 of clustered genes according to its pattern of expression at 2 and 7 d after *Verticillium dahliae* infection in 'Frantoio' and 'Picual' olive roots (see the Results section).

(sometimes only transiently at 2 DAI) in 'Picual' but not in 'Frantoio'; seven unigenes with higher expression in 'Frantoio' than in 'Picual', regardless of whether or not the pathogen was present; two unigenes that were induced in 'Frantoio' but not in 'Picual'; nine unigenes that were induced in 'Picual' (sometimes only at 7 DAI) but not in 'Frantoio'; and three unigenes that were repressed in 'Frantoio' (one of them induced in 'Picual). These unigenes mostly coded for putative Dirigent-like, nucleotide-binding site leucine-rich repeat (NBS-LRR) type disease resistance, Disease resistance response, coiled-coil-NBS-LRR (CC-NBS-LRR) kinases, or BED finger-nbs-lrr resistance proteins (Table S5). Among them, eight unigenes are worth mentioning: four NBS-LRR resistance unigenes showing an overall higher expression in 'Frantoio' than in 'Picual' (Fig. 8); one Dirigent unigene, potentially involved in lignification (Table S5), showing a remarkable difference in its expression pattern between the two cultivars (Fig. 8); one Bet v I root-specific unigene, potentially involved in pathogen defence response, down-regulated in 'Picual' during the infection process but remaining up-regulated in 'Frantoio' (Fig. 8); and three RGH disease resistance unigenes that showed higher expression (or a clear up-regulation trend over time upon *V. dahliae* inoculation) in 'Frantoio' compared with the pattern observed in 'Picual' (Fig. 8).

Finally, we analysed and compared the expression patterns of two groups of genes previously studied as per their involvement in response to *V. dahliae* infection: the β-amylase (*BAM*) and the reactive oxygen species (ROS) protective response genes. We found that the 'PicFra' transcriptome contains 18 *BAM* unigenes (Table S6). Although expression patterns differed between 'Picual' and 'Frantoio' plants depending on the specific unigene analysed, an overall up-regulation over time was observed in 'Picual', whereas in 'Frantoio', expression remained relatively constant after *V. dahliae* inoculation. Thus, total $\log_2$(reads per kilobase per million) values in nonmanipulated, noninoculated (control) plants were very similar in 'Picual' and 'Frantoio' roots (10.8354 and 15.8116, respectively) (Table S6). While these

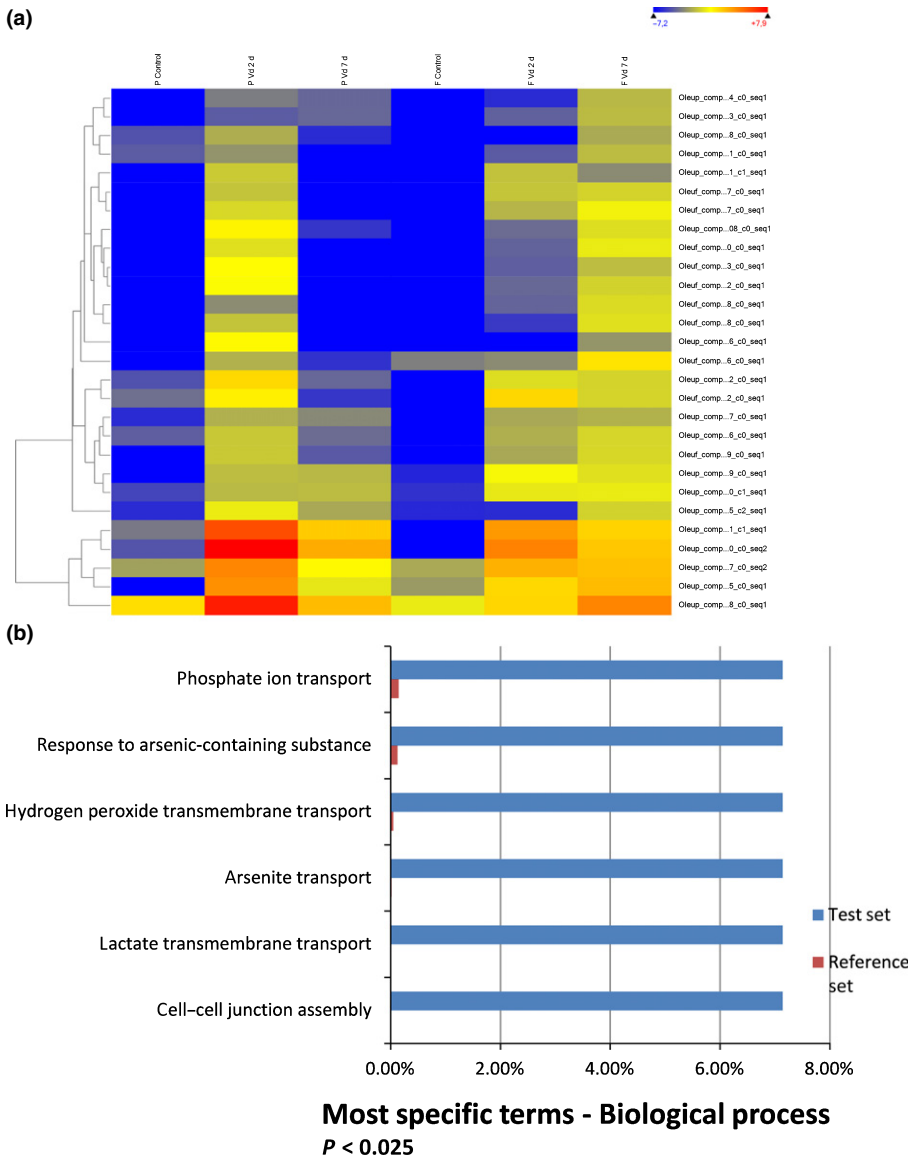


**Fig. 6** Heat map (a) and most specific gene ontology (GO) terms (b) of group 3 of clustered genes according to its pattern of expression at 2 and 7 d after *Verticillium dahliae* infection in 'Frantoio' and 'Picual' olive roots (see the Results section).

values remained unaltered after pathogen inoculation in 'Frantoio' (18.96416 at 2 DAI and 18.97053 at 7 DAI), an overall increase over time was evident in 'Picual' (20.26586 at 2 DAI and 52.68985 at 7 DAI). However, our previous results (Jiménez-Ruiz *et al.*, 2017) showed that an ROS stress-defence response was induced in 'Picual' plants upon *V. dahliae* infection. In this study, none of the six highly up-regulated unigenes in 'Picual' responded in 'Frantoio'; eight ROS protective response-related unigenes showed a trend to be slightly up-regulated after pathogen infection in 'Frantoio' but not in 'Picual' at 7 DAI (Table S7); and, finally, six unigenes showed a trend to be induced after *V. dahliae* infection in both cultivars, although to a higher degree in 'Picual' than in 'Frantoio' (Table S7).

## Discussion

Understanding the mechanisms underlying olive tolerance/resistance to *V. dahliae* is instrumental to improving integrated management strategies of VWO by, for instance, breeding for tolerance or developing novel diagnostic tools based on specific *V. dahliae* tolerance-associated markers. In this study we aimed to unravel differences, at the full olive root transcriptome level, between two olive cultivars showing differential degrees of susceptibility to the pathogen.

The first relevant finding of this study was the remarkable difference found between 'Frantoio' and 'Picual' basal root transcriptomes (Figs 1, S1). Moreover, an outstanding difference in root system architecture between both cultivars was also observed (Fig. S2). Global gene expression profiling studies in which differential responses between susceptible and tolerant cultivars were compared upon phytopathogen infection are available for a number of pathosystems (i.e. Goyer *et al.*, 2015; Kwenda *et al.*, 2016; Matić *et al.*, 2016), including olive (Giampetruzzi *et al.*, 2016). However, comparative analysis of basal gene expression level between host cultivars displaying different disease susceptibility prior to the challenge with the pathogen is frequently overlooked.

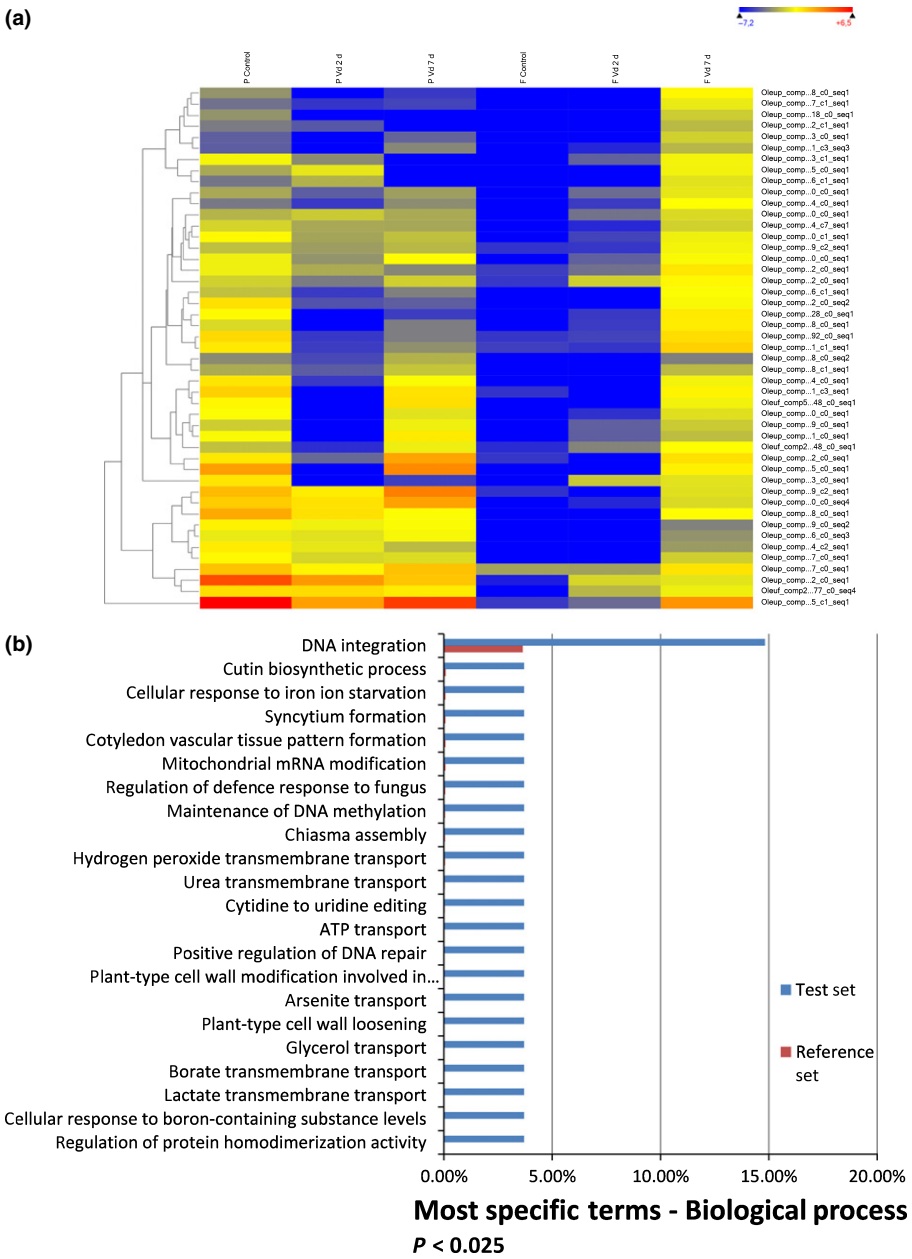


**Fig. 7** Heat map (a) and most specific gene ontology (GO) terms (b) of group 4 of clustered genes according to its pattern of expression at 2 and 7 d after *Verticillium dahliae* infection in 'Frantoio' and 'Picual' olive roots (see the Results section).

This information could be of the upmost importance, as constitutive differential expression levels in unchallenged plants can already help to explain tolerance/susceptibility to a given pathogen, thereby 'preconditioning' the plant towards the disease (Postnikova *et al.*, 2015). In a recent study, the xylem tissue basal transcriptomes of two olive cultivars, one tolerant (Leccino) and another susceptible (Ogliarola salentina) to *Xylella fastidiosa* ssp. *pauca*, causing the olive quick decline syndrome, were similar. In our study, however, differences in whole root tissue basal transcriptomes between 'Frantoio' and 'Picual' plants were remarkable. However, as roots are the first point of contact for soilborne pathogens, it is plausible that differences in root architecture, among other things, may condition plant performance against colonization and invasion by *V. dahliae*. While the genetic and hormonal control of root anatomy and architecture has been studied for some species under different nutritional, environmental and stress conditions (Jung & McCouch, 2013; Wachsman *et al.*, 2015), information regarding olive root architecture and its relationship with tolerance/susceptibility to *V. dahliae* is, to the best of our knowledge, nonexistent. However, it is worth mentioning that plants of the olive cv Koroneiki show roots with little branching (Chatzistathis *et al.*, 2013), similar to the overall appearance of 'Frantoio' roots reported here. Interestingly, 'Koroneiki' is also qualified as tolerant to VWO (Markakis *et al.*, 2010). Higher lignin content in roots of the VWO-resistant cv Sayali has recently been reported, as compared with that observed in the susceptible cv Chemlali (Gharbi *et al.*, 2017b). Related to this, we identified a unigene potentially coding for a Dirigent-like protein involved in lignification that showed a significantly higher basal up-regulation in 'Frantoio' than in 'Picual'.

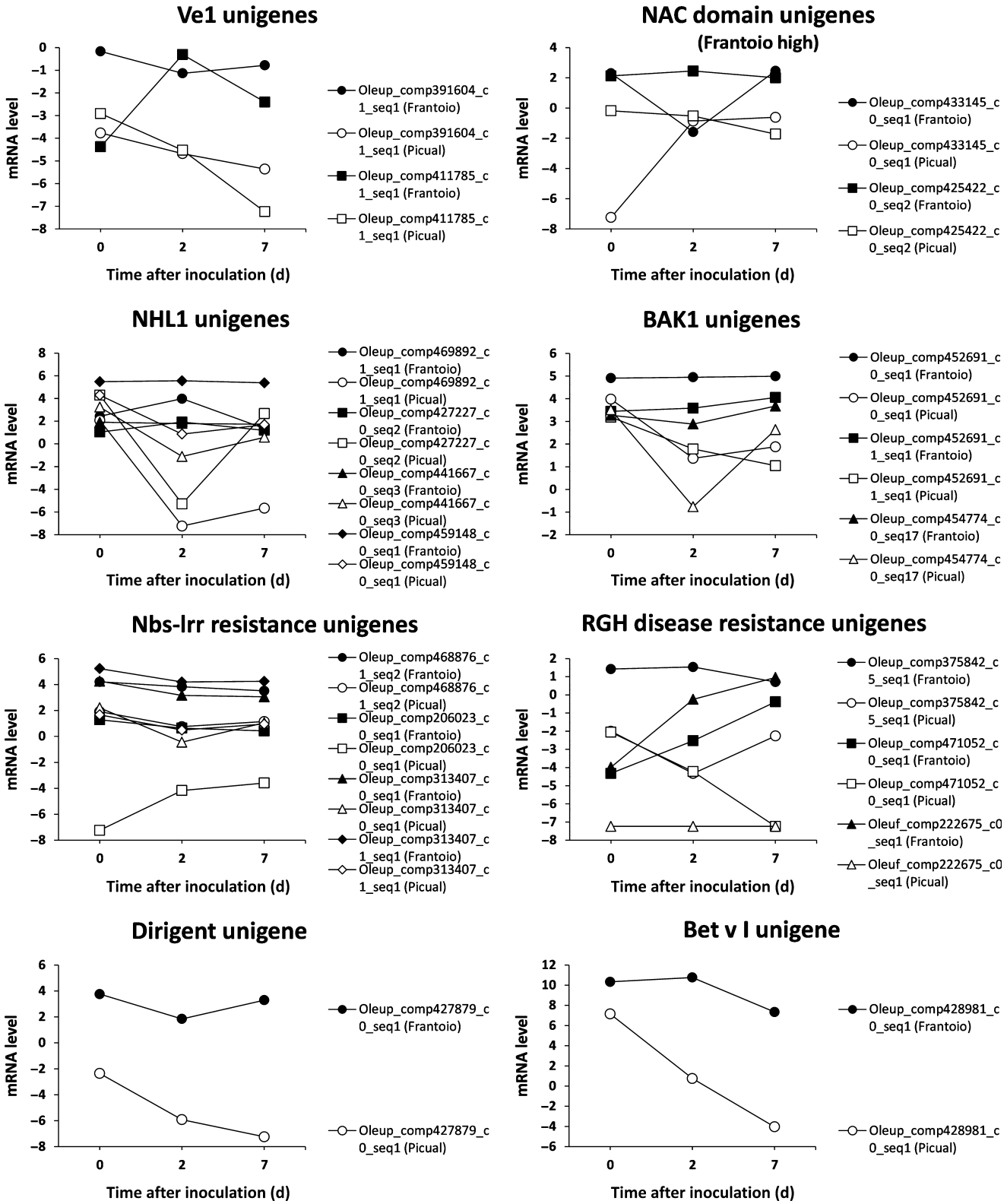


**Fig. 8** Defence differentially expressed genes (DEGs) between 'Frantoio' and 'Picual' olive roots during the *Verticillium dahliae* early infection process.

Overexpression of the *DIRIGENT1* gene in cotton, leading to an enhanced lignification process and hampered invasion by *V. dahliae*, has been reported (Shi *et al.*, 2012). Finally, a pathogenesis-related protein of the Bet v I family, probably encoding a major latex protein involved in pathogen defence responses, is a specific root gene in olive (Jiménez-Ruiz *et al.*,

2015). Remarkably, this gene was found to be repressed in 'Picual', but not in 'Frantoio', upon infection by *V. dahliae*.

Previously, a transcriptome analysis of olive roots (cv Picual) during the early interaction with *V. dahliae* was conducted, thereby obtaining the so-called olive root transcriptome 'Oleup' (Jiménez-Ruiz *et al.*, 2017). In this present work, we improved the available transcriptome by adding unigenes assembled from RNA-seq data from 'Frantoio' roots that were absent in 'Oleup'. The new unigenes correspond to transcripts not previously detected in 'Picual' roots because the corresponding genes are transcribed at very low level, or not at all, in this cultivar but are transcribed at higher level in 'Frantoio'. Thus, a more complete olive transcriptome ('PicFra') was generated to comprehensively compare 'Frantoio' and 'Picual' roots. Interestingly, most of the genes previously described as involved in *V. dahliae* resistance in other hosts were not overexpressed in 'Picual' plants. This outcome was suggested to be a result of the compatible interaction (i.e. olive 'Picual'–*V. dahliae* D pathotype) under study, probably unable to trigger a full resistance response by the host (Jiménez-Ruiz *et al.*, 2017). Results from the present RNA-seq study indicate that transcriptomic responses upon *V. dahliae* infection are quite divergent, depending on the degree of VWO tolerance displayed by the olive cultivar tested. Thus, in the *V. dahliae*–'Picual' interaction, gene up-regulation was clearly predominant over down-regulation, in contrast to the situation observed in the *V. dahliae*–'Frantoio' interaction, in which events of up- and down-regulation were nearly equalized. Besides, most of the induced DEGs in both cultivars were different and only 420 DEGs were shown to be up-regulated in both cultivars. Another relevant outcome was that only a minor fraction (0.9%) of the total number of up-regulated DEGs (i.e. 23,034) was found to be present in both cultivars, and most of them were not closely related to defence responses. Furthermore, 'Frantoio' does not constitutively express defence-related genes that are induced in 'Picual' plants as a consequence of the pathogen attack. As genes induced in 'Picual' roots upon pathogen infection were quite different from those constitutively expressed in nonmanipulated 'Frantoio' roots, the tolerance/susceptibility level observed for each cultivar to *V. dahliae* relies on both complex basal and pathogen-induced differential transcriptomic responses. However, and to reinforce this conclusion, transcriptomic responses in 'Frantoio' and 'Picual' roots were clearly divergent during the early root infection process by *V. dahliae*, with four groups (Figs 4–7) of genes showing differential expression patterns over time. However, the significance and interpretation of these differential patterns will require in-depth analyses beyond the aims of this study.

While genetic resistance against *V. dahliae* has been reported for a number of herbaceous crops (e.g. Lynch *et al.*, 1997; Bae *et al.*, 2008), it is absent or not reported for the vast majority of crops, particularly trees (Fradin & Thomma, 2006). The only locus so far functionally characterized to be involved in resistance to Verticillium wilt is the *Ve* locus in tomato (*Solanum lycopersicum*) (Fradin *et al.*, 2009). *Ve1* homologues seem to be widely distributed in plants (Song *et al.*, 2017). In our study, four olive *Ve1* unigenes have been detected (plus a fifth one with unclear annotation). However, no major differences in their expression patterns related to susceptibility to VWO were found, except for two of them. Indeed, while these two unigenes showed low or very low expression level (both basal and upon infection by the *V. dahliae* D pathotype) in both cultivars, their expression was higher in 'Frantoio' and one of them showed a transient up-regulation upon *V. dahliae* infection (Table S5). In tomato, *Ve1* governs resistance to race 1 isolates of *V. dahliae*. This pathogenic race is defined by the presence of the gene *Ave1* (for Avirulence on Ve1) which activates *Ve1*-mediated resistance and contributes to the greater virulence of this *V. dahliae* race in tomato and *A. thaliana* (de Jonge *et al.*, 2012). The presence of the effector gene *Ave1* confers avirulence to tomato cultivars carrying the resistance gene *Ve1* (or its homologue *Vr1* in lettuce) (Kawchuk *et al.*, 2001; Hayes *et al.*, 2011; de Jonge *et al.*, 2012). The lack of *Ave1* is characteristic of Race 2 isolates which are potentially virulent on hosts carrying *Ve1* as a result of their ability to evade recognition (de Jonge *et al.*, 2012; Short *et al.*, 2014). In a recent study, all Race 1 isolates analysed were only present in *V. dahliae* lineage 2A, and all lineage 2A isolates displayed the race 1 phenotype. In the same study, molecular markers and virulence assays confirmed that the D pathotype is only found in lineage 1A. Moreover, pathogenicity tests showed that cotton and olive D isolates were highly virulent on cotton and olive, but moderately virulent on tomato (Jiménez-Díaz *et al.*, 2016). The *V. dahliae* isolate used in this study (V937I) belongs to the D pathotype (lineage 1A), and the presence of the *Ave1* gene could not be confirmed by PCR analysis (data not shown). According to the overall expression data obtained here for olive *Ve1* unigenes, they do not seem to contribute in a decisive way to the tolerance level displayed by 'Frantoio' plants.

In tomato, the *Ve1*-mediated resistance signalling pathway requires *EDS1*, *NDR1*, *BAK1*, *MEK2* and *SOBIR1* (*LRR-RLK Supressor Of BIR1-1*) (Fradin *et al.*, 2009; Liebrand *et al.*, 2014). Putative olive homologues have been detected in the 'PicFra' transcriptome for all of them except *SOBIR1*. Moreover, no homologues to *vdr5* and *VvVe* have being found in the olive transcriptome. It has been reported that, on the one hand, Verticillium wilt resistance mediated by *GbVe1* and *Gbvdr5* genes in cotton (*G. hirsutum*) require GhNDR1 and GhMKK2 (Gao *et al.*, 2011), whereas, on the other, overexpression of *VvVe* (a *Ve1*-like gene from *Vitis vinifera*) in transgenic *Nicotiana benthamiana* conferred resistance to a D isolate of *V. dahliae* (Tang *et al.*, 2016). The lack of relevant differences in the expression pattern of most of these genes, or their absence in the olive transcriptome, supports the idea that the *Ve1*-mediated resistance signalling pathway is not decisively involved in the differential susceptibility shown by the olive cultivars assessed here, at least against the D pathotype. Nevertheless, some noticeable differential expression patterns between cultivars are worth mentioning. These are the cases for three putative *BAK1* and four putative *NHL1*, all of them showing down-regulation in 'Picual', but not in 'Frantoio', upon *V. dahliae* infection (Fig. 8; Table S5). It has been demonstrated that BAK1 (an LRR receptor-like kinase that regulates the brassinosteroid receptor BRI1; Li *et al.*, 2002; Nam & Li, 2002) has a functional role in pattern recognition receptor-

dependent signalling which initiates innate immunity. Thus, *Arabidopsis thaliana BAK1* mutants showed a decreased response to pathogen-associated molecular patterns (PAMPs; Nürnberger *et al.*, 2004), indicating that BAK1 acts as positive regulator of PAMP receptors, thereby influencing innate immunity (Chinchilla *et al.*, 2007). The observed down-regulation of *BAK1* from basal levels in 'Picual' compared with 'Frantoio', which remains unaltered upon *V. dahliae* infection (Fig. 8; Table S5), might thus help to explain the lower capacity of the susceptible olive cultivar to cope with *V. dahliae* infection.

Jiménez-Ruiz *et al.* (2017) reported that some of the olive-induced DEGs in 'Picual' plants upon *V. dahliae* infection were involved in ROS protection response. In the present study we found that some of these genes showed differential expression patterns between cultivars. On the one hand, the six genes with strongest response to ROS stress during early pathogen infection of 'Picual' roots (Jiménez-Ruiz *et al.*, 2017) were not expressed in 'Frantoio' roots at any time during the infection process. On the other hand, some of them were only induced in 'Frantoio' (at 7 DAI), although at a very low level. Another group, however, showed a shift from very low expression to up-regulation in 'Picual', whereas in 'Frantoio' they remained repressed, although they showed a slight trend to be up-regulated at 7 DAI as well. Overall, the response to ROS stress was clearly stronger in 'Picual' than in 'Frantoio', in accordance with the susceptibility/tolerance of the infected cultivar. This result deserves further investigation, not only because of these differences but also because a ROS protective response also takes place in the pathogen, although at an earlier time than in olive roots (Jiménez-Ruiz *et al.*, 2017). It will be interesting to study whether the ROS protective response in *V. dahliae* also shows differences depending on the VWO tolerance level of the infected olive cultivar.

Gkizi *et al.* (2015) have assigned a negative role for *BAM* genes in the partial resistance of *A. thaliana* plants against *V. dahliae*. Our results indicated that 'Picual' plants showed an overall induction of *BAM* unigenes upon infection with *V. dahliae* D pathotype, in contrast to 'Frantoio' plants in which their global expression remained unaltered. Although this outcome should be studied in more detail and expression of each *BAM* gene considered individually (see Table S6), these genes coding for 1,4-alpha-D-glucan maltohydrolase/beta-amylase and implicated in carbohydrate metabolism/polysaccharide degradation seem to play a role in the higher susceptibility displayed by 'Picual' plants. *BAM* genes are involved in the degradation of starch, the major storage metabolite in higher plants, to maltose (Fulton *et al.*, 2008). Reduced carbohydrate availability seems to affect host susceptibility in different ways. Thus, starch-free mutants of *A. thaliana* were reported to be more resistant to the fungal biotroph *Erysiphe cruciferarum* but more susceptible to the hemibiotroph *Colletotrichum higginsianum* (Engelsdorf *et al.*, 2013). Moreover, *A. thaliana* plants infected by the obligate biotrophic protist *Plasmodiophora brassicae* showed overexpression of *BAM3* (Jubault *et al.*, 2013). Finally, a decline of starch concentrations in leaves of *V. dahliae*-infected pepper plants has also been reported (Goicoechea *et al.*, 2000). It can be speculated that the overall induction of genes involved in starch degradation observed in 'Picual' roots, leading to higher availability of free maltose, is a consequence of a higher demand of carbohydrates that are actively consumed by the pathogen during colonization of the host plant. By contrast, this metabolic pathway was not induced in 'Frantoio'. The genetic basis underlying this finding remains to be elucidated.

Infection by vascular pathogens can either induce transdifferentiation of bundle sheath cells to new and functional xylem vessels or augment xylem cells within the vascular bundle by means of prolonged/renewed activity of the vascular cambium (Reusche *et al.*, 2012). In *A. thaliana*, several NAC transcription factors have been identified as involved in transdifferentiation. They belong to the VND (Vascular-related NAC Domain) subfamily. Two members of this subfamily, VND6 (regulating metaxylem) and VND7 (inducing protoxylem) were proposed to have specific roles in *Verticillium*-triggered transdifferentation of bundle sheath cells (Kubo *et al.*, 2005; Yamaguchi *et al.*, 2010; Reusche *et al.*, 2012). Whether a mechanism resulting in an increase in the number of vascular elements exists in tree species upon vascular infection is unknown. In poplar (*Populus trichocarpa*), however, homologues of NAC domain protein genes (*PtVNS/PtrWND*) have been identified and their role in xylem vessel element differentiation has been demonstrated (Hu *et al.*, 2010; Ohtani *et al.*, 2011). In our study, 24 unigenes coding for putative olive NAC domain transcription factors were found, but only five showed relevant differences between cultivars: two displaying higher expression in 'Frantoio' than in 'Picual' plants, and three showing increased up-regulation over time in 'Picual' plants alone (Table S5). Their potential implication in the tolerance level to VWO of the olive cultivars examined here is not clear, as is the case for the rest of the unigenes associated with defence response found in the 'PicFra' transcriptome.

In summary, our results point to the fact that tolerance of 'Frantoio' plants to VWO is a consequence of a complex and multifaceted process which does not rely on a small number of plant traits. The availability of the 'PicFra' transcriptome will be instrumental in understanding the complex network of genetic responses leading to olive tolerance to this relevant vascular pathogen.

## Acknowledgements

This work was supported by grant AGR-5948 from the Junta de Andalucía (Consejería de Economía, Innovación y Ciencia) and grants AGL2009-07275 and AGL2016-75729 from the Ministerio de Economía y Competitividad (MINECO)/Agencia Estatal de Investigación (co-financed by the European Regional Development Fund, ERDF). Technical and human support provided by CICT of the Universidad de Jaén (UJA, MINECO, Junta de Andalucía, FEDER) is gratefully acknowledged.

## Author contributions

M.O.L-P. performed the RNA-seq experiment and some of the Q-PCR analyses. J.J-R. carried out the transcriptome assembly,

bioinformatics analysis and some of the Q-PCRs. C.G-L.C. and A.V-C. performed bioassay and tissue sampling. J.B.B. conceived the experiments and contributed to the manuscript writing. F.L. and J.M-B. conceived the experiments, analysed the results and wrote the manuscript.

## References


**Antoniou PP, Markakis EA, Tjamos SE, Paplomatas EJ, Tjamos EC. 2008.** Novel methodologies in screening and selecting olive varieties and root-stocks for resistance to *Verticillium dahliae*. *European Journal of Plant Pathology* **122**: 549–560.

**Arias-Calderón R, León L, Bejarano-Alcázar J, Belaj A, de la Rosa R, Rodríguez-Jurado D. 2015a.** Resistance to Verticillium wilt in olive progenies from open-pollination. *Scientia Horticulturae* **185**: 34–42.

**Arias-Calderón R, Rodríguez-Jurado D, León L, Bejarano-Alcázar J, de la Rosa R, Belaj A. 2015b.** Pre-breeding for resistance to Verticillium wilt in olive: fishing in the wild relative gene pool. *Crop Protection* **75**: 25–33.

**Aronesty E. 2011.** *ea-utils: 'Command-line tools for processing biological sequencing data'.* [WWW document] URL https://github.com/ExpressionAnalysis/ea-utils [accessed 10 May 2013].

**Bae J, Halterman DA, Jansky SH. 2008.** Development of a molecular marker associated with Verticillium wilt resistance in diploid interspecific potato hybrids. *Molecular Breeding* **22**: 61–69.

**Baídez AG, Gómez P, Del Rio JA, Ortuño A. 2007.** Dysfunctionality of the xylem in *Olea europaea* L. plants associated with the infection process by *Verticillium dahliae* Kleb. Role of phenolic compounds in plant defense mechanism. *Journal Agricultural of Food Chemistry* **55**: 3373–3377.

**Bubici G, Cirulli M. 2012.** Control of Verticillium wilt of olive by resistant rootstocks. *Plant and Soil* **352**: 363–376.

**Chatzistathis T, Orfanoudakis M, Alifragis D, Therios I. 2013.** Colonization of Greek olive cultivars' root system by arbuscular mycorrhiza fungus: root morphology, growth, and mineral nutrition of olive plants. *Scientia Agricola* **70**: 185–194.

**Chen JY, Huang JQ, Li NY, Ma KF, Wang JL, Liu C, Liu YF, Liang Y, Bao YM, Dai XF. 2015.** Genome-wide analysis of the gene families of resistance gene analogues in cotton and their response to Verticillium wilt. *BMC Plant Biology* **15**: 148.

**Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nürnberger T, Jones JD, Felix G, Boller T. 2007.** A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* **448**: 497–500.

**Colella C, Miacola C, Amenduni M, D'Amico M, Bubici G, Cirulli M. 2008.** Sources of Verticillium wilt resistance in wild olive germplasm from the Mediterranean region. *Plant Pathology* **57**: 533–539.

**Collado-Romero M, Mercado-Blanco J, Olivares-García C, Valverde-Corredor A, Jiménez-Díaz RM. 2006.** Molecular variability within and among *Verticillium dahliae* vegetative compatibility groups determined by fluorescent amplified fragment length polymorphism and polymerase chain reaction markers. *Phytopathology* **96**: 485–495.

**Conesa A, Götz S, Garcia-Gomez JM, Terol J, Talon M, Robles M. 2005.** Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**: 3674–3676.

**Daayf F. 2015.** Verticillium wilts in crop plants: pathogen invasion and host defense responses. *Canadian Journal of Plant Pathology* **37**: 8–20.

**Daayf F, Nicole M, Boher B, Pando-Bahuon A, Geiger JP. 1997.** Early vascular defense reactions of cotton roots infected with a defoliating mutant strain of *Verticillium dahliae*. *European Journal of Plant Pathology* **103**: 125–136.

**Derksen H, Badawi M, Henriquez MA, Yao Z, El-Bebany AF, Daayf F. 2013.** Differential expression of potato defence genes associated with the salicylic acid defence signalling pathway in response to weakly and highly aggressive isolates of *Verticillium dahliae*. *Journal of Phytopathology* **161**: 142–153.

**Dervis S, Mercado-Blanco J, Erten L, Valverde-Corredor A, Pérez-Artés E. 2010.** Verticillium wilt of olive in Turkey: a survey on disease importance, pathogen diversity and susceptibility of relevant olive cultivars. *European Journal of Plant Pathology* **127**: 287–301.

**Engelsdorf T, Horst RJ, Pröls R, Pröschel M, Dietz F, Hückelhoven R, Voll LM. 2013.** Reduced carbohydrate availability enhances the susceptibility of *Arabidopsis* toward *Colletotrichum higginsianum*. *Plant Physiology* **162**: 225–238.

**Erten L, Yildiz M. 2011.** Screening for resistance of Turkish olive cultivars and clonal rootstocks to Verticillium wilt. *Phytoparasitica* **39**: 83–92.

**Faino L, de Jonge R, Thomma BPHJ. 2012.** The transcriptome of *Verticillium dahliae*-infected *Nicotiana benthamiana* determined by deep RNA sequencing. *Plant Signaling and Behavior* **7**: 1065–1069.

**Fradin EF, Thomma BPHJ. 2006.** Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. *Molecular Plant Pathology* **7**: 71–86.

**Fradin EF, Zhang Z, Juarez Ayala JC, Castroverde CDM, Nazar RN, Robb J, Liu CM, Thomma BPHJ. 2009.** Genetic dissection of Verticillium wilt resistance mediated by tomato *Ve1*. *Plant Physiology* **150**: 320–332.

**Fulton DC, Stettler M, Mettler T, Vaughan CK, Li J, Francisco P, Gil M, Reinhold H, Eicke S, Messerli G *et al.* 2008.** b-AMYLASE4, a noncatalytic protein required for starch breakdown, acts upstream of three active b-amylases in *Arabidopsis* chloroplasts. *Plant Cell* **20**: 1040–1058.

**Gao X, Wheeler T, Li Z, Kenerley CM, He P, Shan L. 2011.** Silencing *GhNDR1* and *GhMKK2* compromises cotton resistance to Verticillium wilt. *Plant Journal* **66**: 293–305.

**García-López MC, Vidoy I, Jiménez-Ruiz J, Muñoz-Mérida A, Fernández-Ocaña A, de la Rosa R, Barroso JB, Navarro F, Trelles O, Beuzón CR *et al.* 2014.** Genetic changes involved in the juvenile-to-adult transition in the shoot apex of *Olea europaea* L. occur years before the first flowering. *Tree Genetics and Genomes* **10**: 585–603.

**García-Ruiz GM, Trapero C, del Rio C, López-Escudero FJ. 2014.** Evaluation of resistance of Spanish olive cultivars to *Verticillium dahliae* in inoculations conducted in greenhouse. *Phytoparasitica* **42**: 205–212.

**García-Ruiz GM, Trapero C, Varo-Suárez A, Trapero A, López-Escudero FJ. 2015.** Identifying resistance to Verticillium wilt in local Spanish olive cultivars. *Phytopathologia Mediterranea* **54**: 453–460.

**Gayoso C, Pomar F, Novo-Uzal E, Merino F, de Ilarduya OM. 2010.** The Ve-mediated resistance response of the tomato to *Verticillium dahliae* involves $H_2O_2$, peroxidase and lignins and drives PAL gene expression. *BMC Plant Biology* **10**: 232.

**Gharbi Y, Barkallah M, Bouazizi E, Cheffi M, Gdoura R, Triki MA. 2016.** Differential fungal colonization and physiological defense responses of new olive cultivars infected by the necrotrophic fungus *Verticillium dahliae*. *Acta Physiologiae Plantarum* **38**: 242.

**Gharbi Y, Barkallah M, Bouazizi E, Gdoura R, Triki MA. 2017a.** Differential biochemical and physiological responses of two olive cultivars differing by their susceptibility to the hemibiotrophic pathogen *Verticillium dahliae*. *Physiological and Molecular Plant Pathology* **97**: 30–39.

**Gharbi Y, Barkallah M, Bouazizi E, Hibar K, Gdoura R, Triki MA. 2017b.** Lignification, phenols accumulation, induction of PR proteins and antioxidant-related enzymes are key factors in the resistance of *Olea europaea* to Verticillium wilt of olive. *Acta Physiologiae Plantarum* **39**: 43.

**Giampetruzzi A, Morelli M, Saponari M, Loconsole G, Chiumenti M, Boscia D, Savino VN, Martelli GP, Saldarelli P. 2016.** Transcriptome profiling of two olive cultivars in response to infection by the CoDiRO strain of *Xylella fastidiosa* subsp. *pauca*. *BMC Genomics* **17**: 475.

**Gkizi D, Santos-Rufo A, Rodríguez-Jurado D, Paplomatas EJ, Tjamos SE. 2015.** The β-amylase genes: negative regulators of disease resistance for *Verticillium dahliae*. *Plant Pathology* **64**: 1484–1490.

**Goicoechea N, Aguirreolea J, Cenoz S, Garcia-Mina JM. 2000.** *Verticillium dahliae* modifies the concentrations of proline, soluble sugars, starch, soluble protein and abscisic acid in pepper plants. *European Journal of Plant Pathology* **106**: 19–25.

**Gómez-Lama Cabanás C, Schilirò E, Valverde-Corredor A, Mercado-Blanco J. 2015.** Systemic responses in a tolerant olive (*Olea europaea* L.) cultivar upon root colonization by the vascular pathogen *Verticillium dahliae*. *Frontiers in Microbiology* **6**: 928.

**Goyer A, Hamlin L, Crosslin JM, Buchanan A, Chang JH. 2015.** RNA-Seq analysis of resistant and susceptible potato varieties during the early stages of potato virus Y infection. *BMC Genomics* **16**: 472.

**Häffner E, Diederichsen E. 2016.** Belowground defence strategies against Verticillium Pathogens. In: Vos CMF, Kazan K, eds. *Belowground defence strategies in plants, signaling and communication in plants*. Cham, Switzerland: Springer International Publishing, 119–150.

**Hayes RJ, McHale LK, Vallad GE, Truco MJ, Michelmore RW, Klosterman SJ, Maruthachalam K, Subbarao KV. 2011.** The inheritance of resistance to verticillium wilt caused by race 1 isolates of *Verticillium dahliae* in the lettuce cultivar La Brillante. *Theoretical and Applied Genetics* **123**: 509–517.

**Hu R, Qi G, Kong Y, Kong D, Gao Q, Zhou G. 2010.** Comprehensive analysis of NAC domain transcription factor gene family in *Populus trichocarpa*. *BMC Plant Biology* **10**: 145.

**Jiménez-Díaz RM, Olivares-García C, Trapero-Casas JL, Jiménez-Gasco MM, Navas-Cortés JA, Landa BB, Milgroom MG. 2016.** Variation of pathotypes and races and their correlations with clonal lineages in *Verticillium dahliae*. *Plant Pathology* **66**: 651–666.

**Jiménez-Fernández D, Trapero-Casas JL, Landa BB, Navas-Cortés JA, Bubici G, Cirulli M, Jiménez-Díaz RM. 2016.** Characterization of resistance against the olive-defoliating *Verticillium dahliae* pathotype in selected clones of wild olive. *Plant Pathology* **65**: 1279–1291.

**Jiménez-Ruiz J, García-López MC, Vidoy I, Leyva-Pérez M de la O, Fernández-Ocaña A, Barroso JB, Barceló A, Beuzón CR, de la Rosa R, Luque F. 2015.** Transcriptional analysis of adult cutting and juvenile seedling olive roots. *Tree Genetics & Genomes* **11**: 77.

**Jiménez-Ruiz J, Leyva-Pérez M de la O, Schilirò E, Barroso JB, Bombarely A, Mueller L, Mercado-Blanco J, Luque F. 2017.** Transcriptomic analysis of the *Olea europaea* L. roots during the *Verticillium dahliae* early infection process. *Plant Genome* **10**. doi.org/10.3835/plantgenome2016.07.0060.

**de Jonge R, van Esse HP, Maruthachalam K, Bolton MD, Santhanam P, Keykhasaber M, Zhang Z, Usami T, Lievens B, Subbarao KV *et al.* 2012.** Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. *Proceedings of the National Academy of Sciences, USA* **109**: 5110–5115.

**Jubault M, Lariagon C, Taconnat L, Renou JP, Gravot A, Delourme R, Manzanares-Dauleux MJ. 2013.** Partial resistance to clubroot in *Arabidopsis* is based on changes in the host primary metabolism and targeted cell division and expansion capacity. *Functional & Integrative Genomics* **13**: 191–205.

**Jung JKH, McCouch S. 2013.** Getting to the roots of it: genetic and hormonal control of root architecture. *Frontiers in Plant Science* **4**: 186.

**Kawchuk LM, Hachey J, Lynch DR, Kulcsar F, van Rooijen G, Waterer DR, Robertson A, Kokko E, Byers R, Howard RJ *et al.* 2001.** Tomato *Ve* disease resistance genes encode cell surface-like receptors. *Proceedings of the National Academy of Sciences, USA* **98**: 6511–6515.

**Klosterman SJ, Atallah ZK, Vallad GE, Subbarao KV. 2009.** Diversity, pathogenicity and management of *Verticillium* species. *Annual Review of Phytopathology* **47**: 39–62.

**Kubo M, Udagawa M, Nishikubo N, Horiguchi G, Yamaguchi M, Ito J, Mimura T, Fukuda H, Demura T. 2005.** Transcription switches for protoxylem and metaxylem vessel formation. *Genes and Development* **16**: 1855–1860.

**Kwenda S, Motlolometsi TV, Birch PRJ, Moleleki LN. 2016.** RNA-seq profiling reveals defense responses in a tolerant potato cultivar to stem infection by *Pectobacterium carotovorum* ssp. *brasiliense*. *Frontiers. Plant Science* **7**: 1905.

**Leyva-Pérez MO, Valverde-Corredor A, Valderrama R, Jiménez-Ruiz J, Muñoz-Mérida A, Trelles O, Barroso JB, Mercado-Blanco J, Luque F. 2014.** Early and delayed long-term transcriptional changes and short-term transient responses during cold acclimation in olive leaves. *DNA Research* **22**: 1–11.

**Li J, Wen J, Lease KA, Doke JT, Tax FE, Walker JC. 2002.** BAK1, an Arabidopsis LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signalling. *Cell* **110**: 213–222.

**Liebrand TW, van den Burg HA, Joosten MH. 2014.** Two for all: receptor-associated kinases SOBIR1 and BAK1. *Trends in Plant Science* **19**: 123–132.

**López-Escudero FJ, Del Río C, Caballero JM, Blanco-López MA. 2004.** Evaluation of olive cultivars for resistance to *Verticillium dahliae*. *European Journal of Plant Pathology* **110**: 79–85.

**López-Escudero FJ, Mercado-Blanco J, Roca JM, Valverde-Corredor A, Blanco-López MA. 2010.** Verticillium wilt of olive in the Guadalquivir Valley (southern Spain): relations with some agronomical factors and spread of *Verticillium dahliae*. *Phytopathologia Mediterranea* **49**: 370–380.

**López-Escudero FJ, Mercado-Blanco J. 2011.** Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. *Plant and Soil* **344**: 1–50.

**Lynch DR, Kawchuck LM, Hachey J. 1997.** Identification of a gene conferring high levels of resistance to Verticillium wilt in *Solanum chacoense*. *Plant Disease* **81**: 1001–1014.

**Maldonado-González MM, Bakker PAHM, Prieto P, Mercado-Blanco J. 2015.** *Arabidopsis thaliana* as a tool to identify traits involved in *Verticillium dahliae* biocontrol by the olive root endophyte *Pseudomonas fluorescens* PICF7. *Frontiers in Microbiology* **6**: 266.

**Markakis EA, Tjamos SE, Antoniou PP, Roussos PA, Paplomatas EJ, Tjamos EC. 2010.** Phenolic responses of resistant and susceptible olive cultivars induced by defoliating and non-defoliating *Verticillium dahliae* pathotypes. *Plant Disease* **94**: 1156–1162.

**Martin LBB, Fei Z, Giovannonim JJ, Rose JKC. 2013.** Catalyzing plant science research with RNA-seq. *Frontiers in Plant Science* **4**: 66.

**Martos-Moreno C, López-Escudero FJ, Blanco-López MÁ. 2006.** Resistance of olive cultivars to the defoliating pathotype of *Verticillium dahliae*. *HortScience* **41**: 1313–1316.

**Matić S, Bagnaresi P, Biselli C, Orru' L, Carneiro GA, Siciliano I, Valé G, Gullino ML, Spadaro D. 2016.** Comparative transcriptome profiling of resistant and susceptible rice genotypes in response to the seedborne pathogen *Fusarium fujikuroi*. *BMC Genomics* **17**: 608.

**Mercado-Blanco J, Rodríguez-Jurado D, Pérez-Artés E, Jiménez-Díaz RM. 2002.** Detection of the defoliating pathotype of *Verticillium dahliae* in infected olive plants by nested PCR. *European Journal of Plant Pathology* **108**: 1–13.

**Milgroom MG, Jiménez-Gasco MM, Olivares-García C, Jiménez-Díaz RM. 2016.** Clonal expansion and migration of a highly virulent, defoliating lineage of *Verticillium dahliae*. *Phytopathology* **106**: 1036–1046.

**Mueller WC, Morgham AT. 1993.** Ultrastructure of the vascular responses of cotton to *Verticillium dahliae*. *Canadian Journal of Botany* **71**: 32–36.

**Nam KH, Li J. 2002.** BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signalling. *Cell* **110**: 203–212.

**Nürnberger T, Brunner F, Kemmerling B, Piater L. 2004.** Innate immunity in plants and animals: striking similarities and obvious differences. *Immunological Reviews* **198**: 249–266.

**Ohtani M, Nishikubo N, Xu B, Yamaguchi M, Mitsuda N, Goué N, Shi F, Ohme-Tagaki M, Demura T. 2011.** A NAC domain protein family contributing to the regulation of wood formation in poplar. *Plant Journal* **67**: 499–512.

**Okubara PA, Paulitz TC. 2005.** Root defense responses to fungal pathogens: a molecular perspective. *Plant and Soil* **274**: 215–226.

**Pegg GF, Brady BL. 2002.** *Verticillium wilts*. Wallingford, UK: CAB International.

**Postnikova OA, Hult M, Shao J, Skantar A, Nemchinov LG. 2015.** Transcriptome analysis of resistant and susceptible alfalfa cultivars infected with root-knot nematode *Meloidogyne incognita*. *PLoS ONE* **10**: e0118269.

**Prieto P, Mercado-Blanco J. 2008.** Endophytic colonization of olive roots by the biocontrol strain *Pseudomonas fluorescens* PICF7. *FEMS Microbiology Ecology* **64**: 297–306.

**Prieto P, Navarro-Raya C, Valverde-Corredor A, Amyotte SG, Dobinson KF, Mercado-Blanco J. 2009.** Colonization process of olive tissues by *Verticillium dahliae* and its in planta interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7. *Microbial Biotechnology* **2**: 499–511.

**Reusche M, Thole K, Janz D, Truskina J, Rindfleisch S, Drübert C, Polle A, Lipka V, Teichmann T. 2012.** *Verticillium* infection triggers VASCULAR-RELATED NAC DOMAIN7-dependent *de novo* xylem formation and enhances drought tolerance in *Arabidopsis*. *Plant Cell* **24**: 3823–3837.

**Robb J. 2007.** *Verticillium* tolerance: resistance, susceptibility, or mutualism? *Canadian Journal of Botany* **85**: 903–910.

**Schmieder R, Edwards R. 2011.** Quality control and preprocessing of metagenomic datasets. *Bioinformatics* **27**: 863–864.

**Sesli M, Onan E, Oden S, Yener H, Yegenoglu ED. 2010.** Resistance of olive cultivars to *Verticillium dahliae*. *Scientific Research and Essays* **5**: 1561–1565.

**Shi H, Liu Z, Zhu L, Zhang C, Chen Y, Zhou Y, Li F, Li X. 2012.** Overexpression of cotton (*Gossypium hirsutum*) dirigent1 gene enhances lignification that blocks the spread of *Verticillium dahliae*. *Acta Biochimica et Biophysica Sinica* **44**: 555–564.

**Short DPG, Gurung S, Maruthachalam K, Atallah ZK, Subbarao KV. 2014.** *Verticillium dahliae* race 2-specific PCR reveals a high frequency of race 2 strains in commercial spinach seed lots and delineates race structure. *Phytopathology* **104**: 779–785.

**Song Y, Zhang Z, Seidl MF, Majer A, Jakse J, Javornik B, Thomma BPHJ. 2017.** Broad taxonomic characterization of Verticillium wilt resistance genes reveals ancient origin of the tomato Ve1 immune receptor. *Molecular Plant Pathology* **18**: 195–209.

**Strickler SR, Bombarely A, Mueller LA. 2012.** Designing a transcriptome nextgeneration sequencing project for a non-model plant species. *American Journal of Botany* **99**: 257–266.

**Sun Q, Jiang H, Zhu X, Wang W, He X, Shi Y, Yuan Y, Du X, Cai Y. 2013.** Analysis of sea-island cotton and upland cotton in response to *Verticillium dahliae* infection by RNA sequencing. *BMC Genomics* **14**: 852.

**Tan G, Liu K, Kang J, Xu K, Zhang Y, Hu L, Zhang J, Li C. 2015.** Transcriptome analysis of the compatible interaction of tomato with *Verticillium dahliae* using RNA-sequencing. *Frontiers in Plant Science* **6**: 428.

**Tang J, Lin J, Yang Y, Chen T, Ling X, Zhang B, Chang Y. 2016.** Ectopic expression of a *Ve* homolog *VvVe* gene from *Vitis vinifera* enhances defense response to *Verticillium dahliae* infection in tobacco. *Gene* **576**: 492–498.

**Trapero C, Rallo L, López-Escudero FJ, Barranco D, Díez CM. 2015.** Variability and selection of Verticillium wilt resistant genotypes in cultivated olive and in the *Olea* genus. *Plant Pathology* **64**: 890–900.

**Trapero C, Serrano N, Arquero O, Trapero A, López-Escudero FJ. 2013.** Field resistance to Verticillium wilt in selected olive cultivars grown in two naturally infested soils. *Plant Disease* **97**: 668–674.

**Triki MA, Krid S, Hsairi H, Hammemi I, Ioos R, Gdoura R, Rhouma A. 2011.** Occurrence of *Verticillium dahliae* defoliating pathotypes on olive trees in Tunisia. *Phytopathologia Mediterranea* **50**: 267–272.

**Wachsman G, Sparks EE, Benfey PN. 2015.** Genes and networks regulating root anatomy and architecture. *New Phytologist* **208**: 26–38.

**Xu L, Zhu L, Tu L, Liu L, Yuan D, Jin L, Long L, Zhang X. 2011.** Lignin metabolism has a central role in the resistance of cotton to the wilt fungus *Verticillium dahliae* as revealed by RNA-Seq-dependent transcriptional analysis and histochemistry. *Journal of Experimental Botany* **62**: 5607–5621.

**Yadeta KA, Thomma BPHJ. 2013.** The xylem as battleground for plant hosts and vascular wilt pathogens. *Frontiers in Plant Science* **4**: 97.

**Yamaguchi M, Goué N, Igarashi H, Ohtani M, Nakano Y, Mortimer JC, Nishikubo N, Kubo M, Katayama Y, Kakegawa K *et al.* 2010.** VASCULAR-RELATED NACDOMAIN6 and VASCULAR-RELATED NAC-DOMAIN7 effectively induce transdifferentiation into xylem vessel elements under control fo an induction system. *Plant Physiology* **153**: 906–914.

**Zhang Y, Wang XF, Ding ZG, Ma Q, Zhang GR, Zhang SL, Li ZK, Wu LQ, Zhang GY, Ma ZY. 2013.** Transcriptome profiling of *Gossypium barbadense* inoculated with *Verticillium dahliae* provides a resource for cotton improvement. *BMC Genomics* **14**: 637.

## Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** Scatter plot between nonmanipulated 'Frantoio' and 'Picual' roots.

**Fig. S2** Appearance of the radical systems of 3-month-old and 1-yr-old 'Frantoio' and 'Picual' plants.

**Fig. S3** Real-time qPCR experiments on nonmanipulated nursery-produced plants. Red crosses indicate unigenes with discrepancy between RNA-seq and Q-RT-PCR results.

**Fig. S4** Most specific GO term genes induced in 'Picual' roots infected with *V. dahliae* that are already overexpressed in 'Frantoio' control roots.

**Fig. S5** Validation of the RNA-seq analysis by real-time qPCR of 10 randomly selected DEGs in 'Frantoio' and 'Picual'.

**Table S1** Primers used for Q-RT-PCR

**Table S2** Up-regulated DEGs in both 'Frantoio' and 'Picual' roots

**Table S3** Up-regulated DEGs in 'Picual' roots upon *Verticillium dahliae* infection that are already induced in control, nonmanipulated 'Frantoio' roots

**Table S4** Genes clustered in four groups according to the expression pattern observed at 2 and 7 DAI in 'Frantoio' and 'Picual' roots

**Table S5** Unigenes related to resistance to biotic stress

**Table S6** Expression pattern of 18 *BAM* unigenes in 'Picual' and 'Frantoio' plants upon *Verticillium dahliae* infection

**Table S7** Expression pattern of ROS stress-defence response unigenes in 'Picual' plants upon *Verticillium dahliae* infection

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the New Phytologist Central Office.