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Transcriptome analysis of wild olive (*Olea Europaea* L. subsp. *europaea* var. *sylvestris*) clone AC18 provides insight into the role of lignin as a constitutive defense mechanism underlying resistance to Verticillium wilt

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Abstract

Host resistance is the most effective and practical control method for the management of Verticillium wilt in olive caused by *Verticillium dahliae*, which remains as one of the major current threats to this crop. Regrettably, most olive cultivars of agronomic and commercial interest are susceptible to *V. dahliae*. We previously demonstrated that wild olive (*Olea europaea* L. subsp. *europaea* var. *sylvestris*) clone AC18 harbours resistance to the highly virulent defoliating (D) *V. dahliae* pathotype, which may be valuable as rootstock and for breeding new, resistant olive cultivars. Mechanisms underlying disease resistance may be of constitutive or induced nature. In this work we aim to unravel constitutive defences that may be involved in AC18 resistance, by comparing the transcriptome from uninfected stems, of AC18 with that of the highly susceptible wild olive clone AC15, GO-term enrichment analysis revealed terms related to systemic acquired resistance, plant cell wall biogenesis and assembly, and phenylpropanoid and lignin metabolism. qRT-PCR analysis of phenylpropanoid and lignin metabolism-related genes showed differences in their expression between the two wild olive clones. Phenolic content of stem cell walls was higher in the resistant AC18. The total lignin content was similar in resistant and susceptible clones, but they differed in monolignol composition. Results from this work identifies putative key genes in wild olive that could aid in breeding olive cultivars resistant, to D. *V. dahliae*. The research highlights the constitutive defence mechanisms that are effective in protecting against pathogens and our findings may contribute to the deciphering the molecular basis of VW resistance in olive and the conservation and utilization of wild olive genetic resources to tackle future agricultural challenges towards.

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Introduction

Verticillium wilt (WV) caused by the vascular-colonizing soil-borne mitosporic ascomycete *Verticillium dahliae* [1, 2] is the main disease affecting olive (*Olea europaea* subsp. *europaea* var. *europaea*) in Spain and worldwide [3, 4]. Loses by the disease span from defoliation and necrosis of branches to severe reduction of fruit yield as well as tree death [3, 5]. Careful assessment of yield loss in affected trees estimated 75–89% yield reduction in olive cv. Picual in the third to fifth years after planting in an infested soil [5].

The management of WV is challenging due to the broad host range of the pathogen, its ability to survive in soil and root debris by long-lasting microsclerotia, and the lack of success of fungicide treatments in infected trees [6, 7]. Consequently, an integrated management strategy is necessary for the control of the disease, for which resistant olive cultivars are a key element [3, 4]. The presence of resistance to *V. dahliae* has been evaluated in a World Olive Germplasm Collection and olive breeding programs at Córdoba, Spain, both under controlled conditions and in the field [8–13]. The outcomes demonstrate that no complete resistance is observed; rather, certain cultivars exhibit incomplete resistance that appears to be quantitatively inherited [13]. However, valuable levels of incomplete resistance are overcome by a highly virulent defoliating pathotype (D) [8, 9], the severity of the resistance suppression increasing with increased inoculum density of the pathogen in soil [10].

The prevalence of the D pathotype in Andalusia has been demonstrated to compromise the efficacy of the low resistance level exhibited by certain valuable olive cultivars, despite their grafting onto moderately resistant cultivars such as Changlot Real and Frantoio, which were shown to exhibit effectiveness in a monocycle of infection under controlled conditions [14, 15]. This prompted the search for higher levels of resistance to D *V. dahliae* in wild olive (*O. europaea* subsp. *europaea* var. *sylvestris*) germplasm that could be used as resistant rootstocks as suggested by Wilhelm [16–19]. In a previous search for high resistance to D *V. dahliae*, we collected and rooted stem branches from wild olives growing naturally in non-cultivated areas at southern Andalusia and identified wild olive clone AC18 that remained asymptomatic in a monocycle of infection with a conidial suspension the D pathotype [3]. Further research demonstrated that the asymptomatic phenotype exhibited a high degree of resistance to the pathogen, as indicated by a significant reduction in the growth and amount of *V. dahliae* in stem in acropetal progression. This is the key feature of resistance and is of paramount importance in selecting resistant rootstocks [20, 21]. Moreover, the symptomless reaction and sharp reduction of AC18 stem colonization by D *V. dahliae* hold under recurrent infections in infested soil that may occur under natural conditions, as well as under coinfections with the root knot nematode *Meloidoyne javanica* and the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (previously known as *Glomus intraradices*) [22]. Furthermore, the AC18 wild olive showed effectiveness in the protection of susceptible cvs. Arbequina and Picual grafted onto AC18 as rootstock, which led to the development of grafted susceptible olive cultivars/ AC18 combinations (under the commercial name Vertirés®), jointly by the University of Córdoba and Plantas Continental S.A [23].

Despite the high resistance to D. *V. dahliae* displayed by AC18 wild olive, no molecular information was available on the underlying defense mechanisms. The plant defense strategies to counteract invasion by pathogens rely on constitutive (preformed) and induced mechanisms. Induced defense involve the detection of the pathogen that triggers the activation of induced responses, which then transmit signals and coordinate cellular activities to eliminate the pathogen and block its transport and growth through the vessel elements [24]. Resistance against *V. dahliae* may comprise defense mechanisms that lessen root penetration and lateral spread of the fungus in extravascular root tissues, as well as those operating within the xylem that reduce systemic colonization by the pathogen. These mechanisms involve pattern recognition receptors, transcription factors and salicylic acid (SA)/jasmonic acid (JA)/ethylene (ET)-related signalling pathways [25, 26], and the underlying physiological and molecular nature of resistance to *V. dahliae* have been studied during the last decade [27].

Information on defense mechanisms underlying resistance against *V. dahliae* in olive is still limited and derives mainly from initial defense responses against *V. dahliae* in the root system of cv. Frantoio, which displays a lower level of resistance to the D pathotype compared with AC18 wild olives [10, 14, 20, 28–30]. The objective of this present research was to identify the differential expression pattern of genes that might underly constitutive defense mechanisms in the resistance of AC18 wild olive to D *V. dahliae* in the stem of the plant, as the pathogen is able to invade the root system but its progression, and consequently the progression of the illness, appear to be arrested in this organ [20]. The study of the stem of a resistant rootstock in relation to fungal infection is of crucial importance for the advancement of knowledge

regarding the mechanisms of disease resistance in plants. The stem plays a vital role in limiting fungal proliferation and preventing the spread of pathogens to the scion [31]. This knowledge is pivotal for the development of more robust rootstocks and the refinement of grafting techniques, with the objective of enhancing crop protection against soil-borne diseases. Furthermore, a comprehensive understanding of stem resistance can facilitate the identification of genes and pathways associated with disease resistance. This, in turn, can be employed in breeding programs to generate more resilient crop varieties. To that aim, we used RNA-seq to compare the stem transcriptome from non-infected plants of highly resistant AC18 (23), with that of highly susceptible AC15 wild olive that had been collected from the same geographical site as AC18 (3, 34). Ultimately, our goal is to find molecular markers that can be of use as biotechnological targets for genetic improvement, or as tools for breeding olive cultivars for resistance against *V. dahliae* or other pathogens, as well as for tolerance to abiotic stresses.

Results

AC15 and AC18 show different basal transcriptomes

RNA-seq derived stem transcriptomes from healthy plants led to the identification of 5,131 differentially expressed genes (DEGs) in D *V. dahliae*-susceptible AC15 and -resistant AC18, at ± 1 Log2FC and padj-value ≤ 0.05 (Table S1). In order to increase the stringency and reduce the number of false positive hits, we performed further analysis by selecting DEGs using the median of the baseMean value as a cut-off threshold [32, 33]. Result showed that 2,566 genes were DEGs with this threshold of stringency. Indeed, 1,394 DEGs showed significant higher expression while 1,172 DEGs appeared downregulated in AC18 stems samples compared to AC15. Thereafter, we performed Gene Ontology enrichment analysis (Fig. 1A-B; Supplementary Tables S2-S6) using the webtool ShinyGO [32]. Results obtained after the biological process (BP) most specific GO-term-enriched analysis (FDR cutoff ≤ 0.05) for the upregulated genes in the resistant AC18 wild olive were mainly: "Regulation of immune response" and other co-ocurring terms terms related to "plant-type cell wall organization" and "Phenylpropanoid catabolic process", including "Lignin catabolic process" and "Lignin metabolic process" (Fig. 1A; Supplementary Table S2). The most enriched terms for the downregulated genes were mainly related to "Cellular chemical homeostasis", "Response to oxygen-containing compound" and "Cell wall biogenesis" (Fig. 1B; Supplementary Table S5).

To get a deeper insight in the analysis of the RNA-seq comparison, we performed a Gene Family based classification and enrichment analysis, using the webtool GenFam [34]. As described above, we analysed the upregulated and downregulated DEGs separately (Fig. 2A-B). The main enriched gene families (P-value < 0.05) in the list of upregulated genes were: NB-LRR, HSP20, Glycoside hydrolase (GH), WRKY transcription factor family, Sodium/Calcium exchanger (NCX), Cellulose synthase (CS) and Fasciclin-like arabinogalactan (FLA) gene family (Fig. 2A; Supplementary Table S7). The main enriched gene families in the list of the downregulated genes were: UDP-glycosyltransferases (UGT), Cytochrome P450, light-harvesting chlorophyll a/b-binding (LHC) and AP2/EREBP transcription factor family (Fig. 2B; Supplementary Table S8). Taken together, the Gene Ontology of Biological Processes and Gene Family Enrichment analyses suggest that the differences found in the transcriptomes of the two clones may be associated with a different behaviour against pathogen attack.

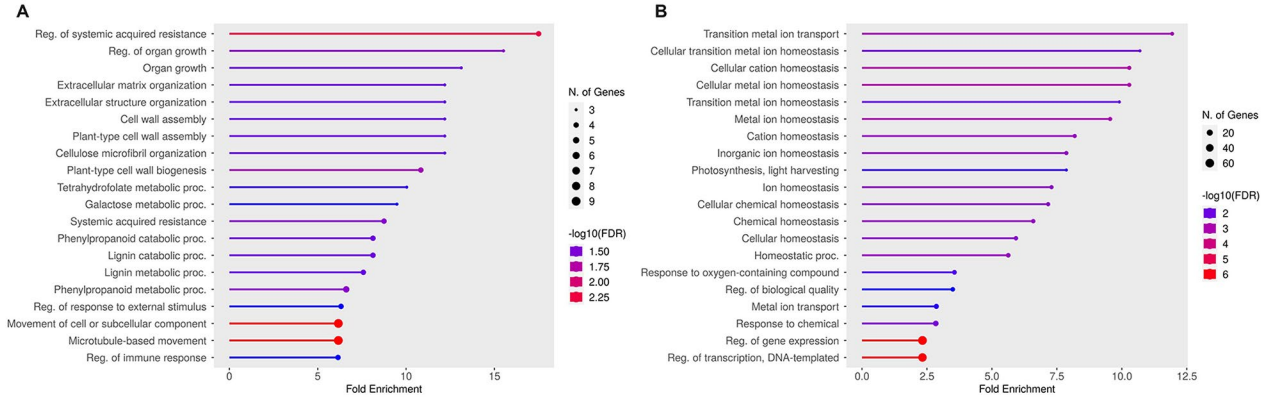

Fig. 1 Bar chart highlighting statistically significant pathways of biological processes, alongside its fold enrichment levels of DEGs and median of the basemean values ≥ 38,75 with (**A**) padj ≤ 0,05; Log2FC of + 1 (**B**) padj ≤ 0,05; Log2FC of– 1. FDR cut-off 0.05. FDR is calculated based on nominal P-value from the hypergeometric test. Fold Enrichment is defined as the percentage of genes in your list belonging to a pathway, divided by the corresponding percentage in the background

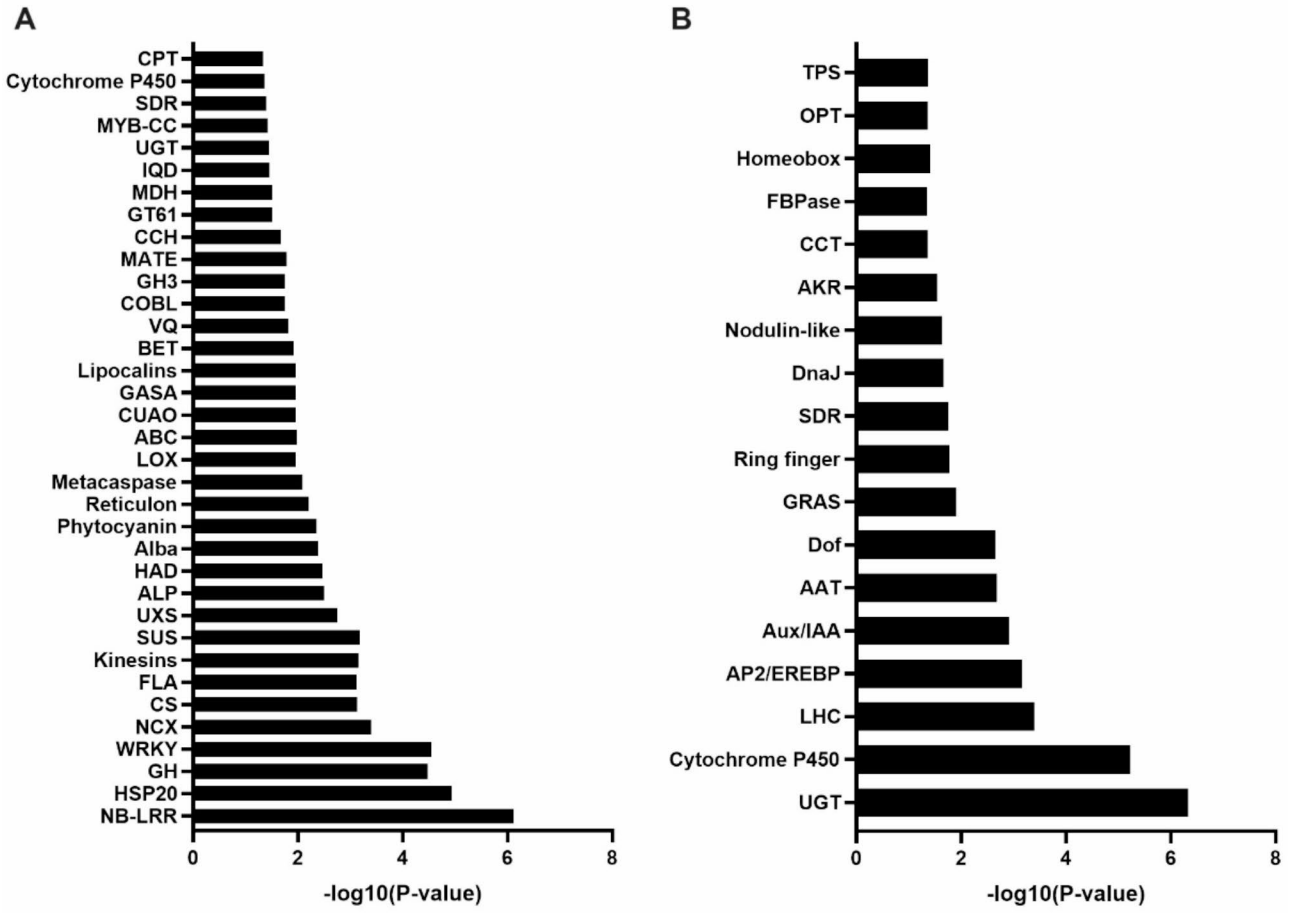

Fig. 2 Summary of GenFam enrichment analysis of DEGs and median of the basemean values ≥ 38,75 with (**A**) padj ≤ 0,05; Log2FC + 1 (**B**) padj ≤ 0,05; Log2FC– 1. The bar charts were created using -log10(*p*-value) scores to illustrate the results. A higher -log10(*p*-value) score indicates greater confidence in gene family enrichment

Expression profile of key genes for lignin synthesis

As GO-term-enrichment analysis highlighted the phenylpropanoid and lignin biosynthetic pathway and its relationship with WV resistance is well demonstrated [30, 35–38], we performed real-time qPCR to confirm the differences found between stems transcriptomes of healthy AC15 and AC18 wild olives. We conducted tests on the expression levels of 12 genes identified as putatively involved in the phenylpropanoid metabolic pathway and the lignin-specific synthetic pathway (Table S9) by using KEGG [39], including *OeuPAL*, *OeuC4H*, *OeuCL*, *OeuCHS*, *OeuCHI*, *OeuHCT*, *OeuC3'H*, *OeuCOMT1*, *OeuCOMT2*, *OeuCCR*, *OeuCAD* and *OeuF5H*. Results indicated that the relative expression levels of *OeuC4H*, *OeuCHS*, *OeuCAD*, and *OeuF5H* were significantly higher in the resistant AC18 than in the susceptible AC15. By contrast, *OeuHCT*, *OeuCOMT1*, and *OeuCOMT2* displayed higher expression in AC15 than in AC18 (Fig. 3). Interestingly, all of them appear to be repressed upon infection of both clones with *V. dahliae* (Supplementary Fig. 1A), reinforcing the idea that these enzymes play a role in the constitutive defence mechanisms that confer resistance in AC18.

It has already been proven that peroxidases and laccases are involved in plant cell wall lignin polymerization [40, 41]. Actually, four peroxidases (*OeuPOX1*,*OeuPOX2 OeuPOX3*, *OeuPOX4*) and five laccases (*OeuLac1*, *OeuLac2*, *OeuLac3*, *OeuLac4 and OeuLac5*) were differential expressed between AC18 and AC15 and identified as putatively involved in the lignin polymerization in the *O. europaea* genome by using KEGG [39]. All of them showed different expression profiles between AC18 and AC15 wild olives. *OeuPOX1* and *OeuPOX2* were highly expressed in AC15 compared with AC18; whilst *OeuPOX3* and *OeuPOX4* showed the opposite expression profile (Fig. 4). The *OeuPOX1* and *OeuPOX2* genes exhibit induction by *V. dahliae*. It is noteworthy that *OeuPOX3* appears to be repressed upon infection in both clones, while *OeuPOX4* did not change in this treatment (Supplementary Fig. 1B). In the case of laccases, four out of the five analysed showed higher amounts of transcripts in AC18 (*OeuLac1*, *OeuLac3*, *OeuLac4 and OeuLac5*),

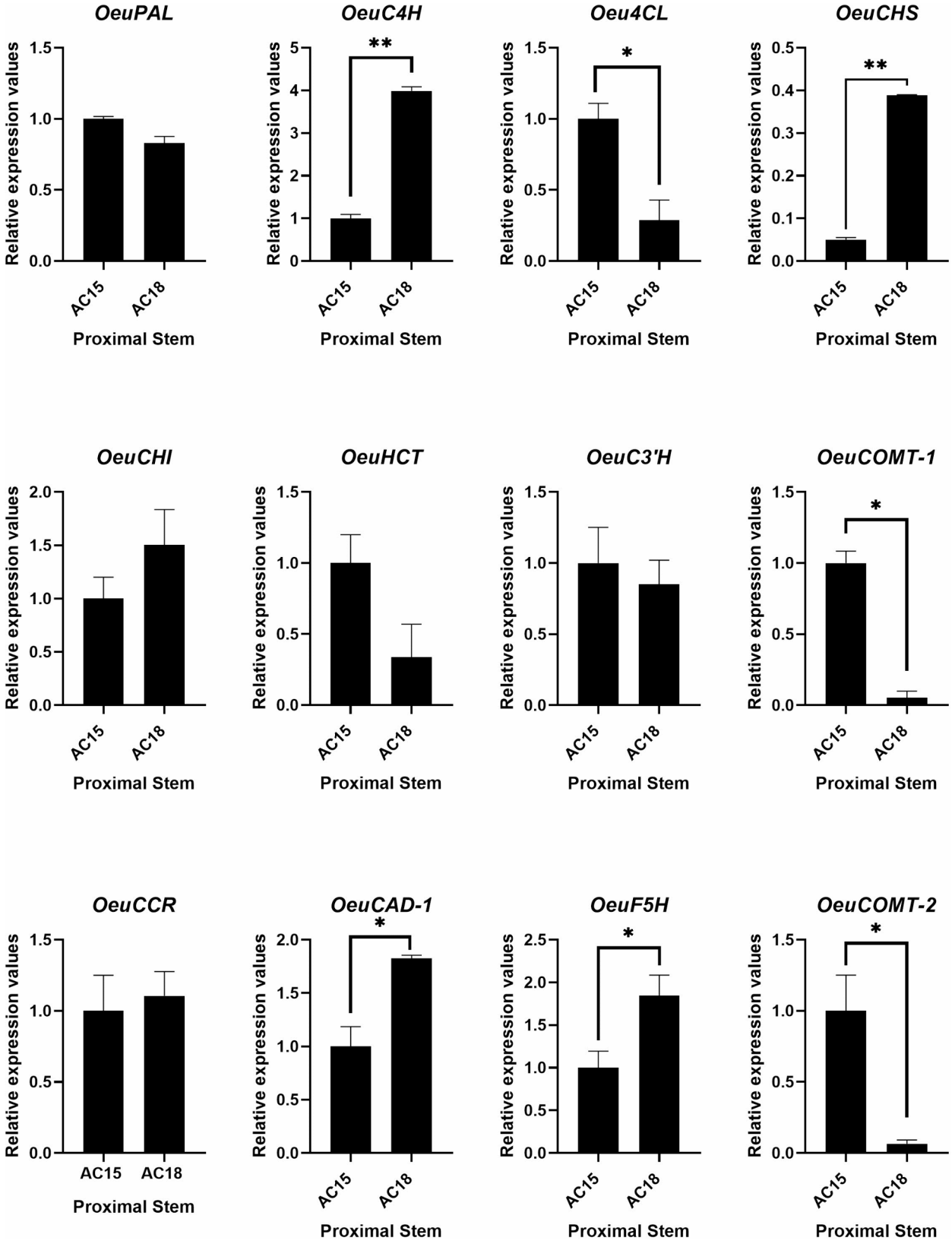

Fig. 3 (See legend on next page.)

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Fig. 3 qRT-PCR analysis of expression of selected genes corresponding to flavonol/phenylpropanoid pathway in wild olive stems. qRT-PCR results were obtained by using specific primers for each gene. Quantification was based on Ct values. mRNA levels were normalized to the variety AC15 value in all experiments. Mean values ± SD are shown. PAL, phenyl ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaryl-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; HCT, quinate o-hydroxycinnamoyltransferase shikimate; C3'H, coumaryl-3'-hydroxylase; COMT (1 y 2), caffeoyl-CoA O-methyltransferase; CCR, (hydroxy)cinnamoyl CoA reductase; CAD, cinnamyl alcohol dehydrogenase; F5H, ferulate hydroxylase. Statistical significance with respect to reference sample was determined by the Student's t-test in all experiments. (*) p-value < 0.05; (**) p-value < 0.01

while *OeuLac2* was strongly expressed in AC15 compared with AC18 (Fig. 5). *OeuLac1*, *OeuLac3*, *OeuLac4* and *OeuLac5*, which are highly expressed in AC18, there were also repressed in the infected samples at 30 days post-infection (dpi) (Supplementary Fig. 1C).

The same groups of genes were analysed in the root tissue to ascertain the preservation of the gene expression pattern in the two more lignified organs. We observed that certain genes exhibited a similar trend in the two types of tissues, but a substantial group of genes did not show any change between AC15 and AC18 wild olives (Fig. 6, Supplementary Fig. 2A-C). Thus, remarkable differences occur in the regulation of lignin biosynthesis in root and stem tissues.

Lignin determination

Following the previously described transcriptomic comparison, we investigated the amount and composition of lignin present in the stem samples of AC15 and AC18 healthy plants. Raman spectroscopy was used to characterize the secondary cell walls. In order to compare the spectra, the averaged spectrums were normalized to the highest peak, corresponding with 1,096 cm^{-1} assigned to C-O and C-C bond stretches specific for the cellulose polymer [42]. According to the relative values, the spectrums of AC15 and AC18 wild olives showed similar profiles except for several peaks associated with phenolics and cellulose, which showed higher intensity in the resistant AC18 clone than in the susceptible AC15 one (Fig. 7). According to Agarwal [42], peaks at 1,270, 1,600, and 1,654 cm^{-1} in the Raman spectrum, defined by resonance of the guaiacyl ring, the phenyl ring deformation, and the stretching of the coniferyl alcohol ring, respectively, are specifically associated with lignin,. The signals at 1,270 and 1,600 cm^{-1} were clearly higher in the AC18 clone compared with AC15. Also, there were differences in other peaks related to lignin and cellulose, such as 1,460 and 1,330 cm^{-1}, which are defined by specific bending deformations of HCH, HOC, HCC, and HCO [43]. Finally, the higher intensity of the peak at 370 cm^{-1} could be related to differences in cellulose crystallinity [42].

The content of phenolics and lignin was also measured in isolated stems cell walls from AC15 and AC18 wild olives (Fig. 8A-C). Both phenolic compounds soluble in methanol (Fig. 8A) and bound to the cell wall extracted with ethyl acetate (Fig. 8B), were significantly higher in the resistant AC18 clone compared with susceptible AC15. However, there were no significant differences between the two clones in the lignin content derivatized with thioglycolic acid (Fig. 8C).

In summary, we found an accumulation of cell wall phenolic compounds and differences in the lignin structure in the resistant clone compared with those in the susceptible one. However, no differences were found between the two clones in the total amount of lignin in the AIR extract of stems samples, when this polymer was extracted with thioglycolic acid. As results from Raman spectroscopy suggest a different monolignol composition and/or lignin amount in AC18 when compared with AC15, further studies need to be performed to confirm this result.

Discussion

In the present study, we conducted a transcriptomic comparison between uninoculated plants of highly D *V. dahliae*-resistant and -susceptible wild olive clones AC18 and AC15, respectively [3, 20, 44], focused on mining differences in gene expression between them, which could be correlated with resistance against the pathogen.

Constitutive transcriptome may determine the resistance of AC18 wild olive against D *Verticillium dahliae*

We identified 5,131 DEGs (Table S1). To enhance the reliability of the relevant differences, we focused on those with higher levels of expression as described above. This led to 1,394 upregulated genes in AC18 stems compared with AC15, while 1,172 appeared downregulated. Among the GO terms retrieved after the enrichment analysis we found terms related to "systemic acquired resistance" (SAR), which is a mechanism of induced defense that confers long-lasting protection against a broad spectrum of microorganisms. Recently, it has been published that *V. dahliae* secreted protein VdSP8 activates hyper-sensitive response (HR) and SAR [45]. In our analysis, this term include DEGs that putatively codify NIM1-INTERACTING (NIMIN) proteins (*Oeu006381.1*,*Oeu009100.1*, *Oeu024972.1* and *OEU038060.1*) that interact with NPR1/NIM1, key regulator of SAR in plants [46]. Other important GO term "plant cell wall biogenesis and assembly", with genes that putatively codify a cellulose synthase (*Oeu39890*) or COBRA-like proteins (*Oeu004421.1*, *Oeu014543.1* and *Oeu051027.1*) that has been associated with determining the orientation of cell expansion by playing an important role in cellulose

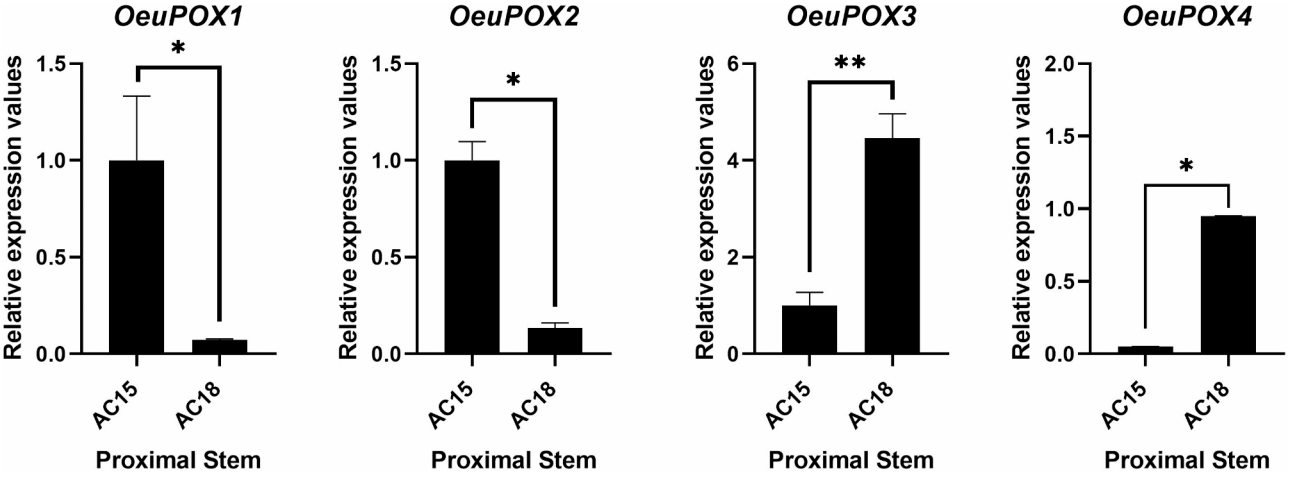

Fig. 4 qRT-PCR analysis of expression of selected peroxidases genes in wild olive stems. qRT-PCR results were obtained by using specific primers for each gene. Quantification was based on Ct values. mRNA levels were normalized to the variety AC15 value in all experiments. Mean values ± SD are shown. Statistical significance with respect to reference sample was determined by the Student's t-test in all experiments. (*) *p*-value < 0.05; (**) *p*-value < 0.01

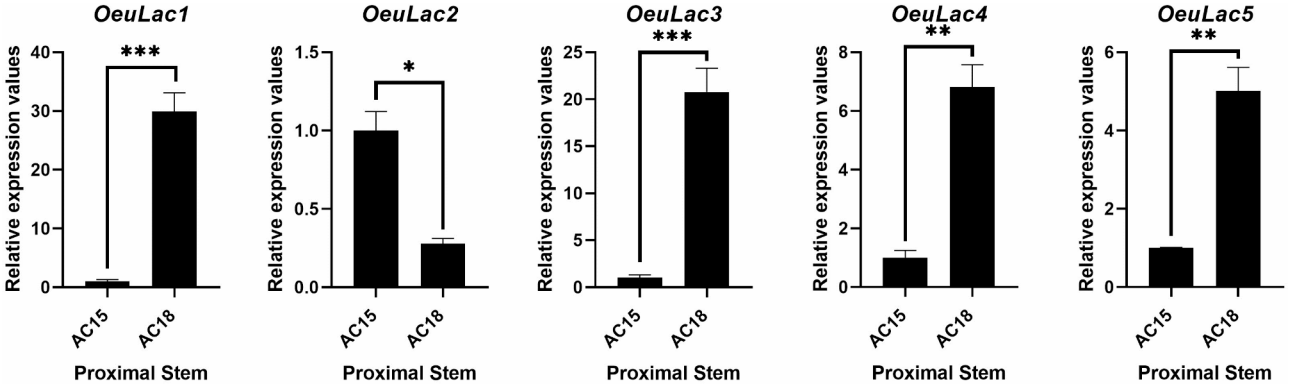

Fig. 5 qRT-PCR analysis of expression of selected laccases genes in wild olive stems. qRT-PCR results were obtained by using specific primers for each gene. Quantification was based on Ct values. mRNA levels were normalized to the variety AC15 value in all experiments. Mean values ± SD are shown. Statistical significance with respect to reference sample was determined by the Student's t-test in all experiments. (*) *p*-value < 0.05; (**) *p*-value < 0.01; (***) *p*-value < 0.001

deposition [47], functions that could be mainly attributed to the up-regulated genes in AC18 (Fig. 1A). Same and similar terms were found in cotton plants when they were inoculated with the endophytic fungus *G. nigrescens* CEF08111 isolated from healthy and this inoculation significantly improved the resistance of cotton to Verticillium wilt [48]. Most of the genes here mentioned either did not significantly see their expression affected by the infection with *V. dahliae* or were repressed (Supplementary Figs. 3–5). Within the terms related to the phenylpropanoid biosynthesis pathway and lignin metabolism, we include Phenylalanine ammonia-lyase (*Oeu36623.1*) or laccases (*Oeu001859.1*, *Oeu006654.1*, *Oeu058596.1*, *Oeu027627.1* and *Oeu061462.1*) genes. Similar terms have been found in roots of moderately D *V. dahliae* resistant "Frantoio" olive as compared to susceptible cv. Picual, where the GO-term enriched analysis also showed terms related to several types of stress response as well as cell wall biogenesis [28]. In this later study, the two cultivars also exhibited distinct transcriptomic behaviors following infection by D *V. dahliae*, which support the hypothesis that constitutive genetic mechanisms determined defense mechanisms that contribute to resistance, highlighting that four NB-LRR resistance unigenes showed higher expression in 'Frantoio' than in 'Picual'. In our study, we have demonstrated that the NB-LRR is indeed the most enriched family among the upregulated DEG.

The classification of gene families and their enrichment analysis revealed that some of them were extensively related to plant response to biotic and abiotic stresses (Fig. 2). Below we describe some of the most relevant among those gene families. As mentioned before, the largest group of DEGs appeared annotated as Nucleotide binding and leucine rich-repeat (*NB-LRR*, *NB-ARC-LRR* or *NLR*) genes. The NLR gene family plays a significant

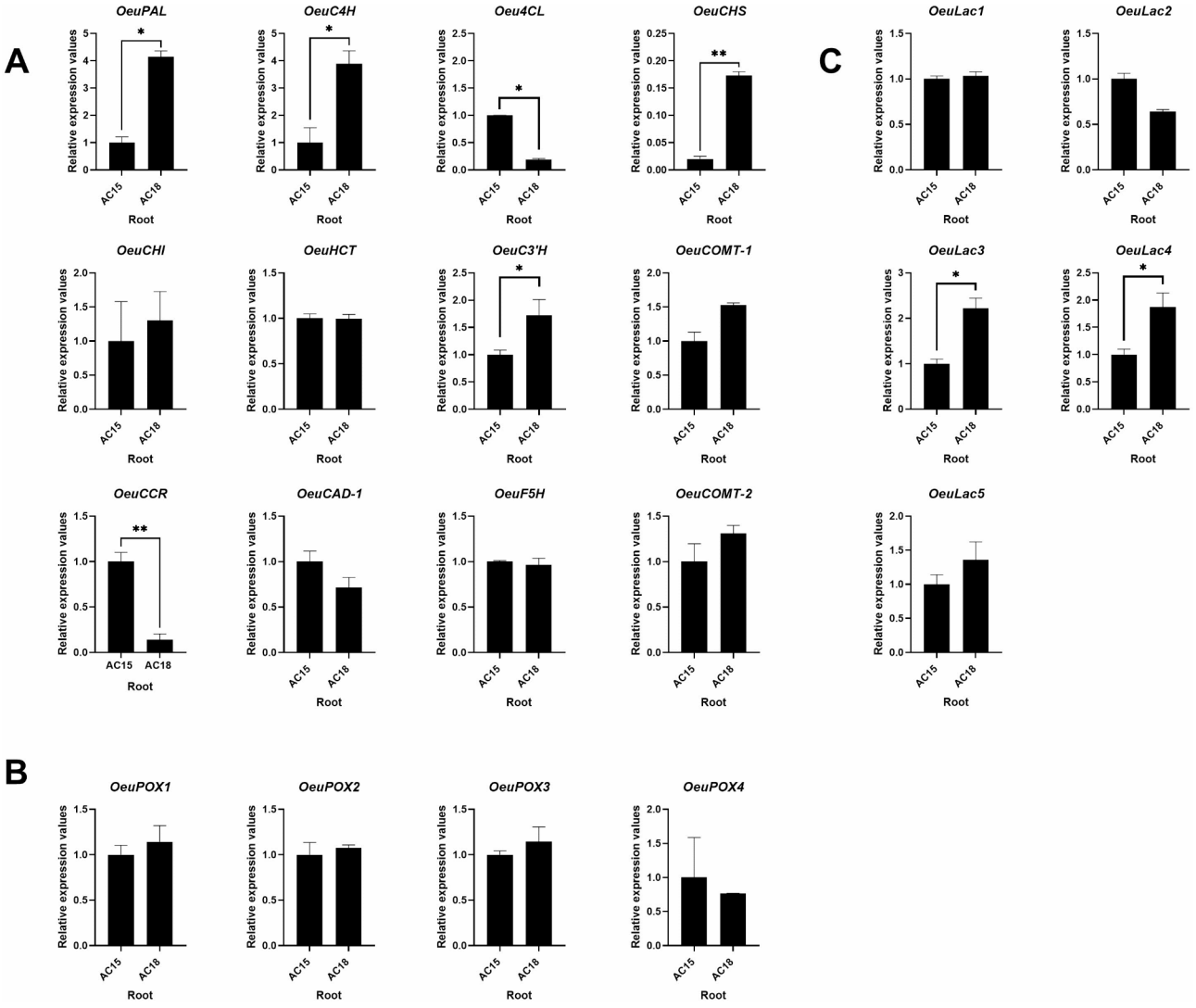

Fig. 6 qRT-PCR analysis of expression of selected genes corresponding to flavonol/phenylpropanoid pathway (**A**), laccases (**B**) and peroxidases (**C**) in wild olive roots. qRT-PCR results were obtained by using specific primers for each gene. Quantification was based on Ct values. mRNA levels were normalized to the variety AC15 value in all experiments. Mean values ± SD are shown. PAL, phenyl ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaryl-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; HCT, quinate o-hydroxycinnamoyltransferase shikimate; C3'H, coumaryl-3'-hydroxylase; COMT (1 y 2), caffeoyl-CoA O-methyltransferase; CCR, (hydroxy)cinnamoyl CoA reductase; CAD, cinnamyl alcohol dehydrogenase; F5H, ferulate hydroxylase. Statistical significance with respect to reference sample was determined by the Student's t-test in all experiments. (*) p-value < 0.05; (**) p-value < 0.01

role in pathogen detection and activation of effector-triggered immunity [49]. This gene family constitutes the largest subclass of resistant (R) proteins. NB domains function as molecular switches, determining whether the protein is in an active or inactive state. This molecular switch is controlled by ADP and ATP binding to activate NLRs following recognition of avirulent effector proteins (Avr). The LRR domain, however, is variable in length and shows large sequence variations since this domain is responsible for Avr recognition [50, 51]. Some members of the NLR gene family have been related to host plant resistance against *V. dahliae*. In *V. dahliae*-resistant cotton (*Gossypium barbadense*) plants, the silencing of *GbRVd* expression, a NLR gene, through virus-induced gene silencing (VIGS) resulted in greater susceptibility to *V. dahliae*, suggesting the important role of *GbRVd* in protecting cotton against infection by *V. dahliae* [52]. In a similar way, GbaNA1, another NLR protein from cotton, has been shown to confer resistance to *V. dahliae* [53] and the heterologous expression of the GbaNA1 enhanced WV resistance in *Arabidopsis* [54].

Other large group of DEGs belongs to the UDP-glycosyltransferase (UGT) gene family. In plants, UGTs glycosylate various phytohormones and metabolites in response to biotic and abiotic stresses [55]. Glycosylation is one of the final steps involved in the triterpenoid

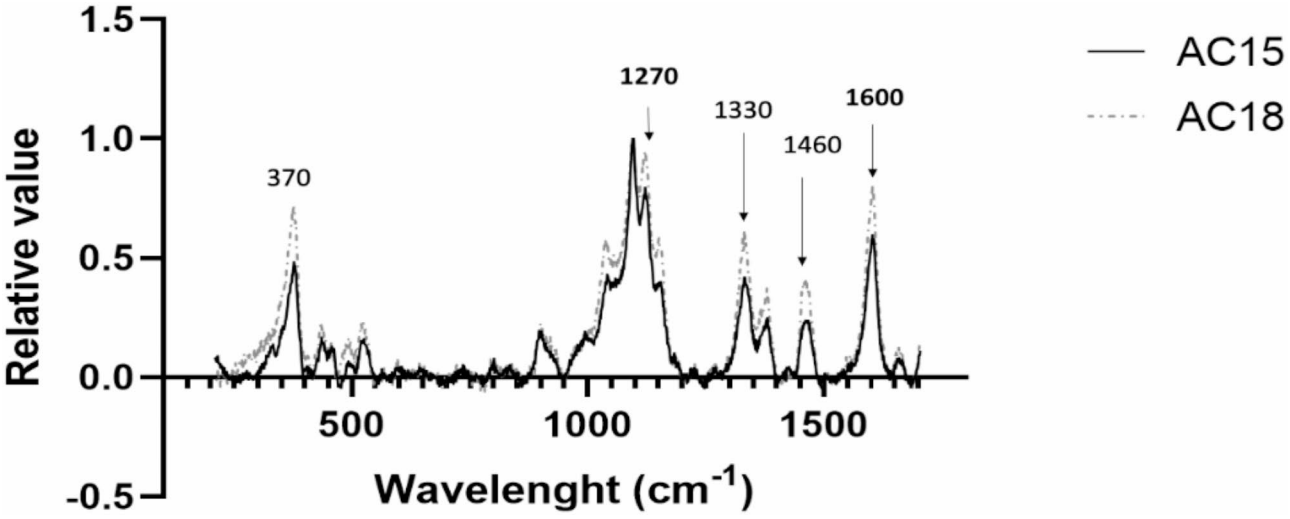

Fig. 7 Raman spectroscopy of the isolated cell walls from the stems of AC15 (black line) and AC18 (grey dashed line) wild olive clones. The spectra were recorded over the range from 100 to 1700 cm^{-1}. Each spectrum is the average of at least two different measures of the cell wall pool. Spectrums were normalized at the highest peak (1090 cm^{-1}). From left to right the five differential peaks specifically associated with lignin structure in the spectrum are: 1270, 1330, 1460 and1600 cm^{-1}

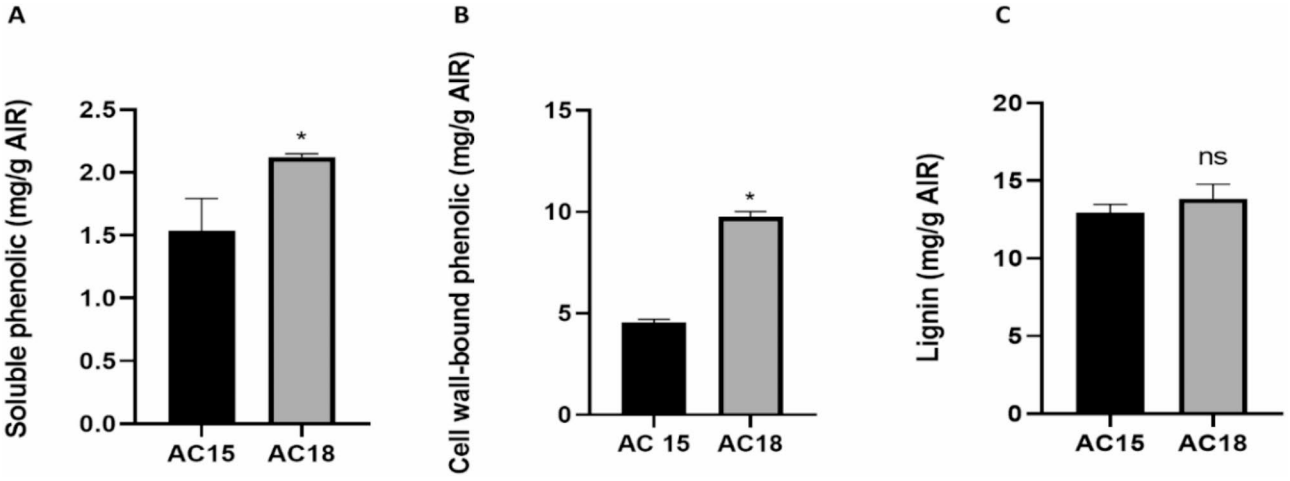

Fig. 8 Colorimetric method to quantify phenolic compounds at the plant cell wall. Bar graphs show the first fraction of soluble phenolic compounds (**A**), second fraction of cell wall-bound phenolic compounds (**B**) and lignin (**C**) from isolated cell wall from the stems of AC15 (black) and AC18 (grey) wild olive clones. Quantification was done using alkaline lignin as a standard. The bars correspond to mean ± SD. Asterisk represents significant difference with unpaired t test at $p < 0.0001$

biosynthesis pathway for many plants defense compounds, such as phenolics, glucosinolates, salicylates, and anthocyanins [56, 57]. In *Arabidopsis*, transgenic plants overexpressing *UGT72E2*, which is involved in the synthesis of coniferin/syringin from coniferyl alcohol/sinapyl alcohol in leaves, were less susceptible to *Verticillium longisporum* [58] another soil-borne fungus which causes vascular infections primarily in Brassicaceae [25].

Another important family that appeared enriched in this present study was the WRKY transcription factors (TFs), which play an important role in regulating the mechanism of plant self-defense [59]. In this study, we examine the expression of the most representative members of this transcription factor family, which are overrepresented in the AC18 transcriptome. Our findings indicate that these members are induced upon infection with *V. dahliae* after 30 dpi in both wild olive clones and in both analysed tissues (Supplementary Fig. 6). Several WRKY TFs have been identified in cotton (*Gossypium hirsutum*) and related with response to infection by *V. dahliae*. Silencing *GhWRKY53* by means of VIGS showed that *GhWRKY53* could change response to *V. dahliae* by activating the JA signaling pathway and repressing the SA signaling one [60]. Also, it has been demonstrated that *GhWRKY70* is considerably upregulated when infected with *V. dahliae*. Transgenic

Arabidopsis plants overexpressing *GhWRKY70* showed improved growth and higher lignin content, antioxidant enzyme activities, and JA levels compared with wild-type plants upon infection with the pathogen. This implies that the *GhWRKY70* transcription factor is a positive regulator in enhancing WV resistance in cotton and may stimulate production of JA [61]. Similarly, *GhWRKY1-like* was identified as a positive regulator of resistance to *V. dahliae* via directly manipulating lignin biosynthesis. *GhWRKY1-like* activates the expression of *GhPAL6* and *GhCOMT1*, which led to enhanced total lignin, especially S monomers biosynthesis [37]. Playing and opposite role, *GhWRKY70D13*, whose knock-down improved resistance to *V. dahliae* in both resistant and susceptible cotton cultivars, showed a negative regulation of the ethylene (ET) and JA biosynthesis and signaling related genes [62]. It has been reported that WRKY proteins can interact with proteins containing a conserved FxxxVQx-LTG or VQ motif [63]. This protein family that we found enriched in the resistant AC18 wild olive. The VQ motif-containing proteins represent a group of plant-specific proteins that control a wide range of developmental processes, such as response to biotic/abiotic stresses, seed development and photomorphogenesis [64]. As example, the complex formed by MdVQ37-MdWRKY100 modulates resistance to Glomerella leaf spot, caused by the fungus *Colletotrichum fructicola*, in apple by regulating salicylic acid levels and MdRPM1 expression [65]. In wheat, TaVQ22 interacts with TaWRKY19-2B to to regulate ROS homeostasis and negatively regulate the defense response to sheath blight, caused by the necrotic fungal pathogen *Rhizoctonia cerealis* [66].

Phenylpropanoid biosynthesis genes are differentially expressed in healthy D *Verticillium dahliae*-susceptible AC15 and–resistant AC18 wild olive clones

Plant resistance to pathogens is determined by a combination of defense mechanisms that are constitutive in healthy plants or induced following infection [24]. Regarding VW of olive, it was suggested that the initial defense response occurs in the root system [28]. Constitutive plant defenses passively contribute as the first line of defense against the pathogen early during the infection process. These passive defenses include morphological and structural barriers in the root cell walls, such as callose, lignin, and suberin reinforcement [67, 68].

Since the GO enrichment analysis in the present study highlighted terms related to lignin metabolism, we analyzed by qRT-PCR the expression pattern of genes that can putatively codify enzymes involved in the phenylpropanoid and lignin pathways in D *V. dahliae*-susceptible AC15 and–resistant AC18 wild olive clones. We found that some important genes, such as *OeuC4H*, *OeuCHS*, *OeuCAD*, and *OeuF5H* are upregulated in the highly resistant AC18; while *OeuHCT*, *OeuCOMT1*, and *OeuCOMT2* genes were downregulated (Fig. 3). Recently, it was demonstrated that over-expression of two genes from the desert moss *Syntrichia caninervis*, which encode transcription factors *ScAPD1-like* and *AP2/ERF*, regulates lignin biosynthesis pathway that enhance *V. dahliae*-resistance in *Arabidopsis* by directly binding to *PAL* and *C4H* genes promoters and thus activating the phenylpropanoid metabolism pathway [69]. In cotton, *GhC4H* and *Gh4CL* expression are directly activated by a homodimer of GhWRKY41, thus modulating the accumulation of lignin and flavonoids that increase cotton defence response against *V. dahliae* [70]. We also found that *OeuC4H* was expressed in AC18 resistant genotype, a finding that is consistent with the results reported in the existing literature. CAD is a key enzyme in lignin biosynthesis, and heterogeneous overexpression of *GhCAD35*, *GhCAD45*, or *GhCAD43* from cotton in *Arabidopsis* enhanced both defense-related lignin biosynthesis and resistance against *V. dahlia* [71]. Moreover, in the same study authors showed that silencing the three *GhCADs* affected the biosynthesis of S-lignin, leading to a decrease of the syringyl/guaiacyl (S/G) ratio. In our study, a putative CAD codifying gene, *OeuCAD*, was found significantly overexpressed in the AC18 clone. Caffeic acid O-methyltransferases (COMTs) also play an important role in lignin synthesis. However, in our study we found that the two *COMTs* analysed were less expressed in the resistant wild olive clone (Fig. 3). Contrary to the aforementioned situation wherein production of lignin correlated with resistance to *V. dahliae*, a number of studies have shown that a reduction in lignin, as a result of down-regulation of several genes encoding metabolic enzymes, increases resistance to other vascular pathogens. For example, silencing *HCT* and *CCoAOMT* led to decreased levels of lignin. As a result, alfalfa (*Medicago sativa*) exhibited significantly activated defence responses that led to increased resistance against *Fusarium. Oxysporum* f. sp. *medicaginis* [72, 73].

We also investigated the pattern of four peroxidases and five laccase enzymes involved in cell wall lignin polymerisation [40, 41]. Our results showed that two out of the four peroxidases analysed, *OeuPOX3* and *OeuPOX4*, were upregulated in the resistant AC18 clone and the other two in the susceptible AC15 one (Fig. 4). Similarly, four out of the five laccases analysed, *OeuLac1*, *OeuLac3*, *OeuLac4* and *OeuLac5*, were overrepresented in the AC18 transcriptome while one of them, *OeuLAC2*, did in the transcriptome of the susceptible AC15 (Fig. 5). Up-regulation of peroxidase activity in a resistant oilseed rape (*Brassica napus*) cultivar correlated with basal resistance to *V. longisporum* [35]. In pepper (*Capsicum annuum*) cultivars with incomplete resistance to *V. dahliae*, stem lignins are composed mainly of p-hydroxyphenyl

units, while levels of β-O-4-linked coniferyl and sinapyl alcohols are significantly lower [74]. Authors of this later study related that composition with peroxidase activity, and concluded that monomer composition and cross-linking in the stem lignin slowed down penetration of host tissues by the pathogen hyphae [74].

Previous research indicated that plant laccases are needed for lignin polymerisation, and that these enzymes are non-redundant with peroxidase [41]. In cotton, modulation of *GhLac1* expression leads to a redirection of metabolic flux in the phenylpropanoid pathway. Thus, overexpression of *GhLac1* gives rise to enhanced resistance to *V. dahliae* and cotton aphids, the up-regulation of GhLac1 leading to increased lignin content [75]. Similarly, heterologous expression of *GhLAC15* in transgenic Arabidopsis improved cell wall lignification, leading to increased total lignin, G monolignol and G/S ratio, as well as enhanced resistance to WV. Additionally, suppression of *GhLAC15* expression level increased the susceptibility of cotton to WV [36].

The observation that the majority of genes examined via qRT-PCR exhibit repression or no discernible alteration in response to *V. dahliae* infection lends further support to the idea that, at least in the case of the genes under consideration, they may play a role in the passive constitutive defence mechanisms that confer resistance in AC18. In summary, the results of the present study described above point out that both the amount and composition of lignin may play an important role in the constitutive defence mechanisms underlying the resistance of AC18 wild olive clone against D *V. dahliae*.

AC18 and AC15 wild olives present dissimilar lignin composition

In the performed transcriptomic comparison between D *V. dahliae*-resistant AC18 and–susceptible AC15 wild olive clones, we found several DEGs related to lignin and cell wall composition and metabolism (Table S10). Lignin reinforces the secondary cell wall in vascular plants, conferring strength and resilience to them [68]. Gharbi et al. [76] reported that olive cultivars which exhibit resistance to *V. dahliae* have higher levels of constitutive lignin in their roots and stems even in the absence of infection when compared to susceptible cultivars. Lignin can be additionally deposited on cell walls in case that constitutive defenses become compromised upon infection: the pathogen's elicitors induce expression of additional defense responses in the plant cell, which include thickening of the cell wall, with additional deposits of lignin and suberin, as well as the production of SA that stimulates systemic acquired resistance (SAR) [77]. This defense process also involves acceleration of host cell death (hypersensitive response), production of reactive oxygen species (ROS), generation of phenolic compounds, as well as pathogenesis related (PR) and other defense-related proteins [24, 76, 78, 79].

Phenolics and lignin contents were analyzed in the cell walls of stems from wild olive clones AC15 and AC18. The difference between these two clones in total lignin content derivatized with thioglycolic acid was not significantly different (Fig. 8C). Although this procedure for lignin quantification is widely used, it is known that it may underestimate the real content due to the high specificity of the reaction with ether bonds of lignin [80]. However, the Raman analysis indicated a different monolignol composition (Fig. 7). These variations could be attributed to a dissimilar expression pattern of the phenylpropanoid related genes (Fig. 3). In fact, knockdown of *Gh4CL30* had different effects on the production of the G and S lignin monomers in cotton [38], and it was postulated that both the levels of G and S monomers and the G/S ratio may be correlated with disease resistance since this ratio increased and decreased in *V. dahliae*-resistant and -susceptible cotton cultivars, respectively [81, 82]. This would suggest that the G/S ratio rather than the total lignin content could determine resistance to *V. dahliae* and would explain why susceptible cultivars may exhibit an amount of total lignin similar to that in cultivars with enhanced resistance to this pathogen.

It is widely recognized that phenolics wall deposition serves as a significant physical and biochemical barrier that contributes to defense of plants against vascular pathogens [76, 82]. In our study, the amount of methanol-soluble phenolic compounds as well as of that cell wall-bound extracted with ethyl acetate were significantly larger in resistant AC18 wild olive clone compared with those in the susceptible AC15 one (Fig. 8A-B). This accumulation of soluble phenolic compounds can be toxic to some pathogens. For instance, the concentration of soluble phenolics and lignin was markedly higher in tomato cultivars resistant to xylem-colonizing bacterium *Ralstonia solanacearum* compared with that in susceptible ones [83, 84]. The enhanced accumulation of mono- and diferulates in cell walls of oat and wheat upon infection with *Puccinia coronata* f. sp. *avenae* and *Agrobacterium* sp., respectively, has been associated with resistance to these pathogens [85, 86]. In pepper, both cell wall reinforcement and the bioactive effect of some phenylpropanoid derivatives seem to play a role in the impairment of colonization by *V. dahliae*. The accumulation of the hydroxycinnamic acid amide N-feruloyltyraminemay act directly by inhibiting growth of the pathogen or indirectly by sealing plant cell walls and delaying fungal invasion [87]. Generally, a positive correlation between the quantity of lignin and resistance to the pathogen has been noted, particularly in plant-pathogen interactions involving vascular fungi like *Fusarium oxysporum* ff. spp. and *Verticilium* sp. that commonly propagate via

the secondary-thickened xylem. However, it still remains unclear whether the role of lignin in resistance is passive or active in regulating specific immune responses [88].

Conclusions

Most olive cultivars of agronomic and commercial interest are vulnerable or show incomplete resistance to *V. dahliae*, and particularly to the highly virulent D pathotype. Thus, the development of new cultivars with enhanced resistance and/or use of available wild olive rootstocks highly resistant to D *V. dahliae* are key for attaining an effective management of Verticillium wilt in olive. The differential gene expression profile in stems of D *V. dahliae*-resistant AC18 and–susceptible AC15 uninfected plants found in this present study, may underline constitutive defense mechanisms for the effective protection against the pathogen in tissues were the scion of susceptible or moderately resistant olive cultivars would be grafted. These differences are evident in the lignin composition of stem cell walls. Moreover, these data elucidate the function of genes and proteins that could be utilized as targets for molecular markers in 'marker-assisted selection' in plant breeding programs. Overall, this study has shed light on the wild olive gene pool and is a first step towards deciphering the molecular basis of VW resistance in olive tree.

Experimental procedures

Plant material

Wild olive clones AC15 and AC18 were selected for this study, AC18 was demonstrated highly resistant to the highly virulent D *V. dahliae* pathotype [20, 22, 23] whereas AC15 is highly susceptible [44]. Certified, self-rooted plants, 6–10-month-old and 40–52 cm tall, were propagated by "Plantas Continental, S.A.", a commercial olive nursery located in the province of Córdoba (southern Spain). Leafy stem cuttings were rooted in a pasteurised potting mix (peat: sand, 2:1, v/v) under mist conditions in plastic tunnels. Before starting the experiments, plants were acclimated for 2 months in a greenhouse under natural lighting at 23 ± 4 °C and a relative humidity ranging from 40% (day) to 80% (night).

Verticillium dahliae infection assay

The disease reactions to the D pathotype of *V. dahliae* in the two wild olive clones, AC15 and AC18, were assessed in accordance with the methodology previously described [20]. Monosporic isolates of *V. dahliae* from cotton (V-138: D pathotype, race 2) were used [89]. Inoculation was performed on 5-month-old plants. The inoculation procedures and disease reactions in the plants were assessed as described by Narváez et al. [77]. Additionally, plants of the Picual olive variety were inoculated as a means of monitoring the development of symptoms. For each plant genotype, 5 to 10 plants were used. Samples were harvested and frozen in liquid nitrogen after 30 days following inoculation, when the Picual plants exhibited the initial symptoms of defoliation.

Bioinformatic tools

Bioinformatic analyses were performed using resources from the European Bioinformatics Institute server and the National Centre for Biotechnology Information (BlastP, BlastX). Wild olive sequences were obtained from the Phytozome database [90], using the *Olea europaea* var. *sylvestris* genome (v1.0) and their involvement in the metabolic pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG) [39]. Gene Family based classification and enrichment analysis was performed by using the web tool GenFam [34] setting the options of Fisher exact test for statistical enrichment, and the Benjamini-Hochberg method [91] to control false discovery rate (FDR).

RNA extraction

Total RNA was isolated and purified from wild olive stems samples using three independent biological replicates. The automated extraction system Maxwell® 16 Lev Plant RSC Instrument (manufactured by Promega Corporation, Wisconsin, USA) and Maxwell® RSC RNA FFPE kit (AS1440, Promega, Wisconsin, USA) were used. The process was conducted according to the manufacturer's instructions. In the final step, RNA was eluted in 50 µL RNase-free water. The RNA quality and integrity were checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Deutschland). Only samples with a RIN value ≥ 8 were used for subsequent transcriptomic analyses.

RNA-seq analysis of differentially expressed genes (DEGs) and gene ontology (GO) enrichment

Library preparation and transcriptome sequencing were carried out by the Beijing Novogene Technology Corporation (Beijing, China). Briefly, Illumina's TruSeq Stranded mRNA Library Prep Kit was used to prepare the libraries following the manufacturer's instructions. Briefly, each sample was enriched in mRNA by selection 3' poly(A) tails using poly(T) magnetic beads. Captured mRNAs were then converted into cDNA, and sequencing adaptors were added to their ends to make the molecules ready for sequencing. Samples were dual-indexed for post-sequencing demultiplexing. The fragment size distribution and concentration of the libraries were checked in an Agilent 2100 Bioanalyzer using the Agilent DNA 1000 kit. These were sequenced into a HiSeq-4000 Illumina platform as 2 × 100b paired-end sequences. A total of 50 ± 10 million of reads were obtained for each one of the libraries, which corresponded to approximately

40-50X coverage of the total haploid genome size. These were analyzed with FastQC and trimmed with Trimmomatic when required to get rid of low-quality bases and/or adapters. Reads were then mapped to the fasta coding sequences corresponding to *Olea europaea* var. *sylvestris* genome (v1.0) using Kallisto v0.46.1. Differential expression was then analyzed using the R package DESeq2 after importing the data with the tximport package. A threshold cut-off was set for genes differentially expressed using a log2FoldChange ≥ 1 or ≤ – 1, and an adjusted p-adjusted value ≤ 0.05. The fasta sequences corresponding to the coding sequences for each of the genes were downloaded from the Phytozome web page. Sequences and mapped data have been deposited in the public GEO database GSE245934. Table S1 depict the total RNA-seq results.

Enrichment analyses of DEGs were conducted using the web tool ShinyGO [32], that is an application developed based on several R/Bioconductor packages, and a large annotation and pathway database compiled from many sources. Only GO terms showing significant enrichment among DEGs, as indicated by a false discovery rate (FDR) cut-off of ≤ 0.05, were considered.

Expression analysis by quantitative real-time PCR

At least three separate pools of each tissue type were used to isolate and purify total RNA, following the methodology outlined in Molina-Hidalgo et al. [92]. The ensuing cDNA was generated by means of the Bio-Rad iScript kit and used in the qRT-PCR reaction in an iCycler system from the same company. Relative differential expression was determined by using the $2^{-\Delta\Delta Ct}$ method [93], which compared Ct values of the target gene to those of the *OeuEF1α* [87] and *OeuH2b* [94]. Each reaction was performed at least three times, and the presence of a single amplicon was confirmed by the melting temperatures. Primers used are listed in Table S10.

Cell wall extraction

Stem segments, 12 cm-long, were sampled from the apex of actively growing plants of AC15 and AC18 wild olives. At least three segments per clone were frozen in liquid nitrogen and ground with a mortar. Cell walls were extracted following the procedure described in Barnes et al. [95] with several modifications. Six grams of powder material was extracted using 80% ethanol and homogenized with an ultra-TURRAX (T18 Digital ULTRA-TURRAXTM, IKATM) with a speed of 25,000 rpm. After the homogenisation, samples were washed with 80% ethanol during 2 h (X3), acetone 2 h (x 3), chloroform: methanol 2 h (x 3) and acetone 1.5 h (x 1). The incubation of each step was done in an orbital shaker followed by centrifugation at 5,000 rpm and 4°C for 5'. The alcohol insoluble residues (AIR) or cell wall extracts were filtered with Miracloth (Merk Millipore) and dried overnight under a fume hood.

Raman microscopy

Raman microscopy was carried out using Invia Qontor Raman Confocal (RENISHAW) with a 785 nm HPNIR diode laser. A Leica microscope with 50 X objective, and numeric aperture of 0.75 was coupled to a Raman. Raman acquisition used a 1,200 l/mm grating, slit opening or pinhole of 65 µm, 10% laser power (14mW at sample plane), 5 s of acquisition time and 5 accumulations per scan. The spectra were recorded over the range of 100 to 1,700 cm^{-1}. Baseline were corrected with an 11th -order polynomial fit, and the spectrums were normalized at the highest peak. At least two replicates were done for each cell wall extract.

Lignin extraction and quantification

Lignin, cell wall-bound and methanol soluble phenolic compounds were extracted according to the protocol previously described [96]. Briefly, 10 mg of alcohol insoluble residue (AIR) was incubated with 1 ml of 80% aqueous methanol for 1 h at room temperature with shaking. The sample was centrifuged at 13,000 g for 10 min. Supernatants were considered as the first fraction of soluble phenolic compounds. The remaining pellets were subsequently washed with 1 ml of 80% aqueous methanol, distilled water and acetone. Dried pellets were incubated in 1 M of NaOH for 1 h at 80 °C and kept overnight at room temperature for alkaline hydrolysis. Cell wall-bound phenolic compounds were extracted by incubating the sample with 500 µl of ethyl acetated during 30 min at room temperature on a rotatory shaker. Phenolic compounds were recovered after centrifugation of the sample at 13,000 g for 10 min.

For lignin extraction, the pellets of the previous step were derivatized with thioglycolic acid. Specifically, samples were incubated with 0.3 ml of thioglycolic acid and 1.5 mL of HCL for 4 h at 95 °C. After the derivatization, pellets were washed twice with distilled water, centrifuged at 13,000 g (10 min) at room temperature, and incubated with 1 mL of 0.5 M NaOH overnight on a shaker. After the overnight incubation, samples were centrifuged at 13,000 g (5 min) at room temperature and were acidified with 32% HCl, for the ligno-thioglycolic acid complex precipitation. The resultant pellet was dissolved in 0.5 M of NaOH, and the absorbance at 350 nm was measured. A standard curve was performed using lignin alkali (Sigma-Aldrich, catalog number: 370959). Four replicates were done for each cell wall extract in two independent experiments.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions
BMS and FJMH performed the experiments, analyzed the data and wrote the manuscript. VGS performed RNA-seq analysis. JCE, CP, FPA and JAM performed the cell wall analysis. RMJD characterized the plant material, contributed to the working hypothesis, designed the infection assay and contributed to the writing of the manuscript. JLTC performed the infection assay. RBP and JLC provided technical assistance to BMS and FJMH. JMB conceived and co-directed the project with FJMH and helped to write the manuscript with valuable suggestions from all other authors.

Data availability
The authors confirm that the data in this study are available within the article and its supplementary materials. RNA-seq data have been deposited in the public GEO database GSE245934.

Declarations

Ethics and consent to publish declarations
Not applicable.

Competing interests
The authors declare no competing interests.

Statistical analysis of data
All statistical analyses were performed with the GraphPad Instat program.

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