Biological Control xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Transcriptional analysis of the interaction between the oomycete biocontrol agent, *Pythium oligandrum*, and the roots of *Vitis vinifera* L.

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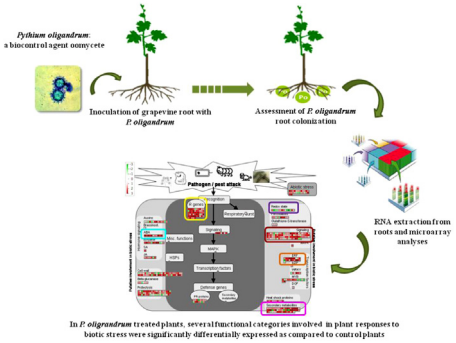
HIGHLIGHTS

- Study on the interaction between grapevine roots and the oomycete *Pythium oligandrum*.
- Global transcriptomic changes in this interaction were investigated by microarrays.
- Root colonization by *P. oligandrum* induced significant changes in root transcriptome.
- This interaction showed some similarities with a symbiotic relationship.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 31 October 2016
Revised 29 January 2017
Accepted 18 February 2017
Available online xxxx

Keywords:
Oomycete
Grapevine
Microarray
Transcriptome
Interaction

ABSTRACT

The oomycete, *Pythium oligandrum*, has received considerable attention as a potential biocontrol agent against various pathogenic fungi of plants, including grapevine. However, much remains underexplored on the interaction between grapevine and *P. oligandrum*. In the present study, following root colonization with *P. oligandrum*, grape-genome microarray analyses showed significant changes in the grapevine root transcriptome. The main changes concerned the genes involved in the biological processes and cellular functions, more particularly those associated with response to stimuli. Several functional categories were differentially expressed in *P. oligandrum*-inoculated plants. The highest inductions concerned the functional categories involved in secondary metabolism (terpenoid and flavonoid pathways), abscisic acid metabolism, resistance genes and some RNA regulation transcription factors. Redox state functional category was, however, significantly repressed. Whereas the expression of several transcripts would suggest that the plant sets up defense systems against the oomycete, certain similarities with symbiotic microorganism/root interactions were also observed, the main one being the stimulation of subtilases. These data provide new insights about the pathways involved in the establishment of the complex relationship between *P. oligandrum*, an oomycete with biocontrol potential, and grapevine.

1. Introduction

Biological control is a useful approach to help reduce chemical pesticide applications in vineyards. Numerous microorganisms have been used to control grapevine diseases (Compant et al.,

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http://dx.doi.org/10.1016/j.biocontrol.2017.02.007
1049-9644/

2013; Zanzotto and Morroni, 2016). One of these, the oomycete *Pythium oligandrum*, whose strains naturally colonize grapevine roots from the vineyards of the Bordeaux region in France (Gerbore et al., 2014b), can induce grapevine defense systems (Mohamed et al., 2007; Yacoub et al., 2016). This phenomenon is associated with the presence of genes encoding for elicitin-like proteins: oligandrin, and cell-wall proteins coded POD-1 and POD-2 (Gerbore et al., 2014b). As shown in the literature, this biocontrol agent (BCA) is able to protect grapevine against grey mold (Mohamed et al., 2007), or Esca, a grapevine trunk disease (Yacoub et al., 2016). *P. oligandrum*, also, displays other beneficial effects, such as increased root development and plant growth promotion (Benhamou et al., 2012; Gerbore et al., 2014a; Rey et al., 2008).

In order to determine the physiological changes induced in plants, following *P. oligandrum* root system colonization, the interaction between the oomycete and roots was studied by Le Floch et al., (2005) and Rey et al., (1998b). This particular relationship has been described as atypical, as it differs from all the interactions described between plants and *Pythium* spp., and plants and biocontrol agents (Benhamou et al., 2012; Le Floch et al., 2005, 2009; Rey et al., 2008). According to Le Floch et al., (2005), *P. oligandrum* intra-root colonization is as rapid as such colonizations with *Pythium* pathogenic species (Rey et al., 1996, 1998a,b). The rapid ingress of *P. oligandrum* within the root tissues *i.e.* epidermal, cortical parenchymal and endodermal tissues is, however, not accompanied by significant disorders in the plant (Le Floch et al., 2005; Rey et al., 1998b). In order to cross the host cell-walls, *P. oligandrum* hyphae exert strong pressure on the wall structures, producing certain targeted enzymes to facilitate their penetration. This occurs, however, without the substantial wall alterations observed with minor or major pathogenic *Pythium* species (Rey et al., 1996, 1998a).

As shown by Le Floch and co-workers (2009), the relationship between *P. oligandrum* and the roots of tomato plants differed from that established with two other BCAs, such as *Trichoderma* spp. and *Fusarium oxysporum* (Fo47). These differences concerned BCAs ingress in root tissues and the host plant responses. While the root colonization of the two fungi was restricted to the surface or upper cortical cell layers, the oomycete succeeded in deeply penetrating the root tissues. Intense host reactions appeared when the fungi (*Trichoderma* spp. and *Fusarium oxysporum* (Fo47) attempted to penetrate inside the root layers. Although, in the case of *P. oligandrum*, host reactions only appeared once the oomycete hyphae had colonized the inner cortical tissues. In cucumber plant roots, Wulff et al., (1998) indicated a similar phenomenon, with *P. oligandrum* hyphae penetration in the root tissues inducing limited host defense responses. They also indicated an increase of the root system, four days after *P. oligandrum* inoculation. In all, this interaction had a positive effect on the growth of young plants, and on the plant's resistance to pathogens.

This last-mentioned property has been abundantly studied in the literature, together with the induction of certain genes in the *P. oligandrum*/plant/pathogen interactions. Many of these studies have focused on plant responses following a pathogen attack, demonstrating that *P. oligandrum* activates signaling pathways such as jasmonic acid (JA) and ethylene, when plant resistance to diseases is induced. The third major signaling pathway, salicylic acid (SA), does not seem to be activated (Hase et al., 2006, 2008; Kawamura et al., 2009; Mohamed et al., 2007; Takahashi et al., 2006; Takenaka et al., 2003).

Previous studies on the relationships between *P. oligandrum* and the host plant were mainly conducted by describing the cytologic changes, using either Transmission Electron Microscopy (TEM) observation or biochemical characterization of antimicrobial molecules (Le Floch et al., 2005; Rey et al., 1998b). Both of these studies have provided particularly useful information about this atypical relationship. It seemed, therefore, interesting to broaden our knowledge of the plant changes induced by *P. oligandrum*, using a global transcriptomic analysis approach. *Vitis vinifera* L. was chosen, because Gerbore et al., (2014b) have shown that *P. oligandrum* naturally colonizes its roots in the vineyards. Accordingly, our study attempts to develop a more comprehensive and detailed understanding of the physiological changes associated with the interaction between a plant-protective oomycete and grapevine roots.

2. Materials and methods

2.1. P. oligandrum strains and inocula

Two different inocula of *P. oligandrum* (Po1 and Po2) were used for all experiments. Each inoculum consisted of two strains: Po1 (strains coded Oth-2 and Oth-3), Po2 (strains coded Sto-1 and Oth-4). One strain (Sto-1) came from the INRA-UMR 1065 SAVE collection (Bordeaux, France), the other three strains (Oth-2, Oth-3 and Oth-4) from the CBS collection (CBS 530.74, CBS 109982 and CBS 118746, respectively). The inocula, composed of an oospore-mycelium homogenate, were prepared by Biovitis (Saint Etienne Chomeil, France), as described by Le Floch et al., (2003), with the concentration for each inoculum being adjusted to 2×10^4 oospores per mL. In short, *P. oligandrum* was cultured in a liquid medium containing mainly cane molasses, for 14 days at 25 °C.

The four different strains of *P. oligandrum* used in this study (Table 1) had already been genetically characterized by sequencing the elicitin-like genes that encode oligandrin (coded Oli-D1 or Oli-S1) and cell-wall proteins (coded POD1-a, POD-1, POS-1) (Gerbore et al., 2014b). In a previous study by Yacoub et al., (2016), the amounts of oligandrin produced by each strain were evaluated, with the mean per inoculum being 72.51 and 80.99 mg L^{-1} for Po1 and Po2, respectively.

2.2. Plant culture and experimental design

Two-node rooted cuttings of a Cabernet Sauvignon cultivar of grapevine, collected from experimental vineyards at the INRA station in Couhins (Gironde, France), were used. The cuttings were rooted in a sandy layer, and then planted in plastic pots filled with Klassman RHP 15 commercial potting mix [fair peat of sphaine (70%), cold black peat (15%), pearlite and Danish clay (15%)]. They were incubated in a greenhouse for six weeks (25 °C, 16 h light/8 h dark). The plants were watered for 2 min per day, via a drip system (2 L/h), and then fertilized twice a week (nutrient solution N/P/K 20/20/20) (Haidar et al., 2016; Laveau et al., 2009; Yacoub et al., 2016).

Two experiments were carried out over two different years (Year-1 and Year-2). The same experimental design was used for both Year-1 and Year-2. For each experiment, a total of 162 plants were used, randomly distributed according to three treatments ((1) control cuttings, (2) root inoculation by Po1 and (3) root inoculation by Po2), with 54 plants per treatment.

2.3. P. oligandrum inoculation and root sampling

At 7–8 leaf stage, rooted cuttings were inoculated twice with *P. oligandrum*, at the collar level of each plant: once with 50 mL of inoculum and, three days later, with additional 40 mL. For each experiment, root samples were collected at six sampling times: 0, 7, 21, 35, 63 and 121 days post inoculation (dpi) with *P. oligandrum*.

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Table 1
Origin, elicitor genes (oligandrin and cell-wall protein genes) and oligandrin production of *P. oligandrum* strains used in this study.

Strains	Inoculum	Niches/Habitats	Origin	Oligandrin (mg/L) (±SE)	Cell wall protein genes	Oligandrin genes
Oth-2	Po1	Grapevine roots	France	61.08 ± 6.68ab	POD1-a	Oli-D1
Oth-3	Po1	Chinese cabbage roots	Denmark	100.07 ± 21.13b	POD-1	Oli-D1
Sto-1	Po2	Zucchini roots	Australia	81.10 ± 5.39ab	POD-1	Oli-D1
Oth-4	Po2	Soil	Netherlands	63.92 ± 6.49ab	POS-1	Oli-S1

Nine plants per treatment were collected at each sampling time. An aliquot of root samples was used on sampling day for the evaluation of *P. oligandrum* root colonization by a cultivable method. The remaining roots were snap-frozen in liquid nitrogen, and then stored at −80 °C for subsequent transcriptomic analyses.

2.4. Assessment of grapevine root colonization by P. oligandrum by cultivable method

P. oligandrum root colonization was assessed during each experiment at six time points: 0, 7, 21, 35, 63 and 121 dpi, as described previously (Gerbore et al., 2014b; Le Floch et al., 2003; Yacoub et al., 2016). The differences between treatments were assessed by ANOVA, after normalization (transformation in log (x + 1) of the data) and validation of variance homogeneity (with Levene test) with SAS software version 9.1, 2004. The Tukey test was used to compare means.

2.5. Total RNA extraction from roots

The root tissues of each plant were ground in liquid nitrogen using a Tissue LyserII (Qiagen), to obtain a fine powder. For each sampling time point and per treatment, 9 plants were collected and combined into 3 "pools" corresponding to three replicates. Each "pool" was composed of three plants (400 mg/ plant), to obtain 1.2 g per replicate. For RNA extractions, 200 mg of pooled samples were used.

The commercial kit Spectrum (Sigma-Aldrich) was used in accordance with the manufacturer's guidelines. A Dnase treatment (On-Column DNase I Digestion Set, Sigma-Aldrich) was added to the extraction protocol to remove DNA contamination from samples. The integrity, purity and yield of the RNA were determined using a Nanodrop (ND-1000, ThermoScientific) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Germany).

2.6. Microarray analyses

In order to analyze grapevine root gene differential expressions in response or not to *P. oligandrum* treatment, the NimbleGen Grape Whole-genome microarray chip (Roche Design name 090918 Vitus exp HX12) was employed. RNA quality control, labeling, hybridizations and imaging were performed at the Get Platform (Toulouse, France), in accordance with the manufacturer's instructions. Microarray data were analyzed using several Bioconductor packages (Gentleman et al., 2004) implemented in R version 2.14.0. The ArrayQuality Metrics package was used to control microarray signal quality (Kauffmann et al., 2009). The Robust Multichip Average (RMA) function of the oligo package was applied for background correction and quantile-normalization of the intensity values (Gautier et al., 2004; Irizarry et al., 2003).

After normalization, the dataset was exported from R as a tabular file, and subsequent statistical analyses performed in the MultiExperiment Viewer software (MeV http://mev.tm4.org/) (Saeed et al., 2003). Within MeV, the Limma package (Smyth, 2005) was used to obtain differentially expressed genes between samples from 7 and 21 dpi. Statistical significance was set at $P < 0.01$, and $\log_2$ of fold changes (FC) were calculated.

2.7. Reverse transcription quantitative real-time PCR analysis of selected genes

In order to validate microarray results, the expression of the most significant genes was analyzed using Reverse Transcriptase quantitative PCR. The same biological material was used for qPCR and microarray analyses. First, cDNA was synthesized from 1.5 μg of RNA using an oligo (dT) primer and the Superscript III kit (Invitrogen Life Technologies, Carlsbad, USA), in accordance with the manufacturer's instructions. Then, qPCR reactions were performed with a Stratagene MX3005P thermocycler (Agilent technologies, France) in a total volume of 14 μL, using the commercial kit MESA BLUE qPCR for SYBR (Eurogentec, Belgium). All samples were run in duplicate under the following conditions: an initial denaturation step (15 min at 95 °C) followed by 45 cycles of denaturation (10 s at 95 °C), annealing (22 s at 55 °C), and extension (30 s at 72 °C). Then, a dissociation curve was performed. The primers used for this analysis were designed on the NCBI (National Center for Biotechnology Information) website using the Primer Blast utility (Ye et al., 2012) and listed in Table S1. Three housekeeping genes were used in our study, and their sequences shown in Table S2. Data were exported from the PCR software, and analyzed using the LinRegPCR software (Ruijter et al., 2009). The comparison of microarray and qPCR expression levels was performed using regression analysis.

3. Results

3.1. Assessment of grapevine root colonization by P. oligandrum

In order to assess the oomycete ability to colonize roots of grapevine, root samples were regularly collected (Table 2) and

Table 2
Evaluation of the percentage of root colonization by *P. oligandrum* in Year-1 and Year-2. Values are the mean (in%) ± standard error of 9 plants sampled by sampling time. Means with the same letter are not significantly different at $P < 0.05$, according to the Student test after ANOVA (mixed model).

Treatment (dpi)		Root colonization	
		Year-1	Year-2
0	Po1	0 ± 0 f	0 ± 0 d
	Po 2	0 ± 0 f	0 ± 0 d
	Control	0 ± 0 f	0 ± 0 d
7	Po1	11.7 ± 5.3 de	36.7 ± 6.3 ab
	Po2	62.2 ± 10.1 a	65.6 ± 10.6 a
	Control	0.60 ± 0.6 f	0 ± 0 d
21	Po1	26.7 ± 9.1 bcd	16.67 ± 6.7 c
	Po2	38.3 ± 8.3 ab	52.8 ± 11.5 ab
	Control	0.60 ± 0 f	0 ± 0 d
35	Po1	13.3 ± 4.2 cde	9.4 ± 5.9 c
	Po2	38.9 ± 9.2 ab	32.2 ± 9.1 b
	Control	0 ± 0 f	0 ± 0 d
63	Po1	7.2 ± 3.3 cde	11.1 ± 3.7 c
	Po2	28.3 ± 9.4 bc	25.6 ± 6.1 b
	Control	0 ± 0 f	0 ± 0 d
121	Po1	8.3 ± 3.9 e	20.6 ± 9.4 c
	Po2	22.8 ± 9.1 e	36.7 ± 7.9 ab
	Control	0 ± 0 f	0 ± 0 d

dpi: days post inoculation.

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deposited on *P. oligandrum* selective medium. Before inoculation (0 dpi), *P. oligandrum* was not detected on the roots of Cabernet Sauvignon cuttings. In Year-1, the highest rate of colonization was observed at 7 dpi with Po2 (67% of root fragments were colonized), and at 21–35 dpi with Po1 (38.3 and 38.9% of colonized root fragments, respectively). Following inoculation with Po2 inoculum, *P. oligandrum* root colonization was significantly higher than with Po1 at 7 and 35 dpi. Four months after inoculation (121 dpi), colonization levels with *P. oligandrum* were not significantly different between Po1 and Po2 treated plants. In experimentation Year-2, the highest *P. oligandrum* root colonization for both inocula was observed at 7 dpi, with 66% and 37% for Po2 and Po1, respectively. After inoculation with Po1 inoculum, root colonization was significantly lower throughout the experiment than it was with Po2. Overall, *P. oligandrum* inocula colonized the root systems of plants throughout the whole experimental period, for each annual experiment.

3.2. Transcriptome analysis of grapevine roots after inoculation with *P. oligandrum*

A transcriptomic analysis using the NimbleGen Grape Whole-genome microarray was performed, in order to assess gene differential expressions induced by *P. oligandrum* at the root level of grapevine plants. As the highest levels of *P. oligandrum* root colonization were observed with Po2 at 7 and 21 dpi, only roots treated with Po2 and collected at those time points were analyzed, using microarrays.

For control and *P. oligandrum* treated plants, our analysis compared the expression of genes at 7 dpi against 21 dpi. The data obtained in Year-1 and Year-2 were similar. The fold changes in gene expressions observed in Year-1 were higher than in the Year-2 experiment, with most of these changes concerning the same functional groups. Accordingly, only Year-1 results are detailed in what follows.

3.2.1. Global transcriptomic shifts induced by *P. oligandrum*

When plant responses, 7 and 21 dpi with *P. oligandrum*, are compared, the number of genes differentially expressed, as shown by the Limma analysis ($P < 0.01$), is 412 and 1916 in control and *P. oligandrum*-inoculated plants, respectively. A Venn analysis performed with these gene lists showed 102 genes specific to the control, 1604 specific to the *P. oligandrum*-inoculated samples, and 312 genes common to both samples (Fig. 1).

To analyze these results in greater detail, transcripts showing significantly differential expressions were annotated according to gene ontology (GO) terms. GO analyses were carried out using the software Agrigo (version 1.2), http://bioinfo.cau.edu.cn/agriGO/

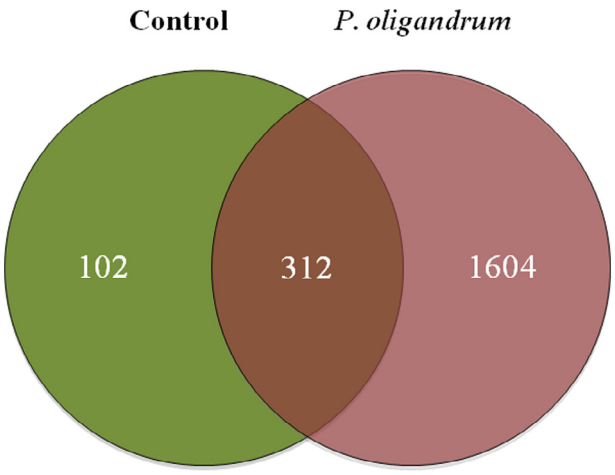

Fig. 1. Venn diagram representing the differentially expressed genes, depending on the treatment ($P < 0.01$ Limma) between 7 and 21 dpi, with *P. oligandrum* and control plants.

(Du et al., 2010). For each treatment, this annotation allows the classification of significantly differentially expressed genes into three GO categories: biological process, molecular function and cellular components (Ashburner et al., 2000).

Results showed that over time (7 *vs* 21 days), five ontologies were significantly modulated in *P. oligandrum* treated plants: these GO grouped genes involved in responses to stimulus, in signal transduction activity, molecular transducer activity, receptor activity and the extracellular region components. In control plants, only the genes involved in cellular components were significantly regulated over time (Table 3).

3.2.2. Functional analyses of transcriptome changes in response to *P. oligandrum* root inoculation

Detailed functional analysis of the differentially expressed grapevine genes was carried out using the Mapman software (version 10). Mapman analysis allows differentially expressed genes to be classified into functional categories (BIN) and sub-categories (Thimm et al., 2004). In control plants, 5 functional categories were differentially expressed over time. Four of them were found to be significantly over-expressed (BIN 16.1.5, BIN 17.8.1, BIN 31.2 and BIN 33.1) and one (BIN 15.2) was down regulated (Table 4). In *P. oligandrum*-inoculated plants different functional categories were specifically modulated (either induced or repressed) (Table 5). Biotic stress category (BIN 20) contained the highest number of modulated genes (with 59 induced and 3 repressed genes). Three

Table 3
Ontologies significantly expressed (induced or repressed) in *P. oligandrum* treated and control plants at 7 vs 21 dpi.

Ontology	Description	DE Gene number	Total gene number	*P*-value
P. oligandrum-treated plants				
Biological process	response to stimulus	301	3959	5.50×10^{-5}
Cell function	signal transducer activity	113	1377	5.90×10^{-4}
Cell function	molecular transducer activity	113	1377	5.90×10^{-4}
Cell function	receptor activity	103	1226	4.50×10^{-4}
Cellular components	extracellular region	122	1245	1.70×10^{-7}
Control plants				
Cellular components	chromosome	18	277	1.30×10^{-6}
Cellular components	intracellular non-membrane-bounded organelle	18	277	1.30×10^{-6}
Cellular components	non-membrane-bounded organelle	18	277	1.30×10^{-6}
Cellular components	extracellular region	40	1245	1.10×10^{-4}
Cellular components	extracellular space	15	292	1.30×10^{-4}
Cellular components	extracellular region part	15	308	2.40×10^{-4}

DE: Differentially Expressed.

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Table 4
Differentially expressed functional categories according to the Wilcoxon rank test ($P < 0.01$) (7 *vs* 21 dpi) in the control plants. The results are extracted from the functional categories of software Mapman showing the number of annotated genes for each process or protein family. The term "BIN" indicates the functional category number used by Mapman.

BIN	Functional category	Number of over-expressed genes	Genes*	*P*-value
16	Secondary metabolism			
16.1	Secondary metabolism.isoprenoids			
16.1.5	Secondary metabolism.isoprenoids.terpenoids	7	85	5.55×10^{-3}
17.8	Hormone metabolism.salicylic acid			
17.8.1	Hormone metabolism.salicylicacid.synthesis-degradation	5	34	8.16×10^{-3}
31	Cell			
31.2	Cell.division	4	49	9.93×10^{-3}
33	Development			
33.1	Development.storage proteins	2	51	3.98×10^{-2}
BIN	Functional category	Number of under-expressed genes	Genes*	*P*-value
15	Metal handling			
15.2	Metal handling.binding, chelation and storage	2	61	2.05×10^{-2}

* Total gene number in the BIN.

Table 5
Differentially expressed functional categories according to the Wilcoxon rank test ($P < 0.01$) (7 *vs* 21 dpi) in *P. oligandrum* treated-plants. The results are extracted from the functional categories of software Mapman showing the number of annotated genes for each process or protein families. The term "BIN" indicates the functional category number used by Mapman.

BIN	Functional category	Number of over-expressed genes	Genes*	*P*-value
20	Stress			
20.1.2	Stress.biotic.receptors	52	667	3.18×10^{-7}
20.1.3	Stress.biotic.signaling	7	33	3.99×10^{-2}
16	Secondary metabolism			
16.1.5	Secondary metabolism.isoprenoids.terpenoids	11	188	4.06×10^{-4}
16.8	Secondary metabolism.flavonoids			
16.8.2	Secondary metabolism.flavonoids.chalcones			
16.8.2.1	Secondary metabolism.flavonoids.chalcones.naringenin-chalcone synthase	7	7	4.82×10^{-4}
21	Redox			
21.1	Redox.thioredoxin	1	56	3.99×10^{-2}
17	Hormone metabolism			
17.1.3	Hormone metabolism.abscisic acid.induced-regulated-responsive-activated	9	40	7.60×10^{-3}
27.3.3	RNA.regulation of transcription.AP2/ERF_Ethylene-responsive element binding protein family	18	119	2.36×10^{-3}
27.3.6	RNA.regulation of transcription.bHLH.Basic Helix-Loop-Helix family	8	89	2.51×10^{-2}
27.3.11	RNA.regulation of transcription.C2H2 zinc finger family	2	79	4.29×10^{-2}
27.3.26	RNA.regulation of transcription.MYB-related transcription factor family	1	36	2.17×10^{-2}
29.5.1	Protein.degradation.subtilases	9	94	1.97×10^{-2}
BIN	Functional category	Number of under- expressed genes	Genes*	*P*-value
20	Stress			
20.1.2	Stress.biotic.receptors	3	667	3.18×10^{-7}
16	Secondary metabolism			
16.8	Secondary metabolism.flavonoids			
16.8.1.21	Secondary metabolism.flavonoids.anthocyanin.anthocy-nin 5-aromatic acyltransferase	2	8	1.54×10^{-2}
21	Redox			
21.1	Redox.thioredoxin	4	56	3.99×10^{-2}
21.3	Redox.heme	3	11	4.78×10^{-3}
27.3.1	RNA.regulation of transcription.ABI3_VP1-related B3-domain-containing transcription factor family	2	5	4.14×10^{-2}
27.3.3	RNA.regulation of transcription.AP2/ERF_Ethylene-responsive element binding protein family	3	119	2.36×10^{-3}
27.3.11	RNA.regulation of transcription.C2H2 zinc finger family	3	79	4.29×10^{-2}
27.3.20	RNA.regulation of transcription.G2-like transcription factor family, GARP	4	31	1.78×10^{-2}
27.3.26	RNA.regulation of transcription.MYB-related transcription factor family	2	36	2.17×10^{-2}

* Total gene number in the BIN.

sub-functional categories involved in secondary metabolism were significantly regulated. Two of them (BIN 16.1.5 and 16.8.2.1), corresponding to terpenoid and chalcone synthase pathways, respectively, were over-expressed. The anthocyanin pathway (BIN 16.8.1.21) was repressed. In the case of hormone metabolism, over-expression of the sub-functional category (BIN 17.1.3) implicated in ABA metabolism was measured. The over-expression of protein degradation subtilase functional category (BIN 29.5.1) was also shown. Most of the genes involved in redox functional category (BIN 21) were repressed. *P. oligandrum*-root inoculation induced a significant modulation of several genes encoding for transcription factors, such as APETALA2/Ethylene Responsive Factor (AP2/ERF), basic Helix-Loop-Helix (bHLH) and MYB. These genes are known to be involved in induced systemic resistance (ISR).

As shown in Fig. 2 a and b, many genes assigned to biotic stress functional categories were differentially expressed in *P. oligandrum*-inoculated plants.

3.2.2.1. P. oligandrum-root colonization and biotic stress gene over-expression. Following root colonization by the oomycete, many Resistance genes (R genes) were significantly differentially

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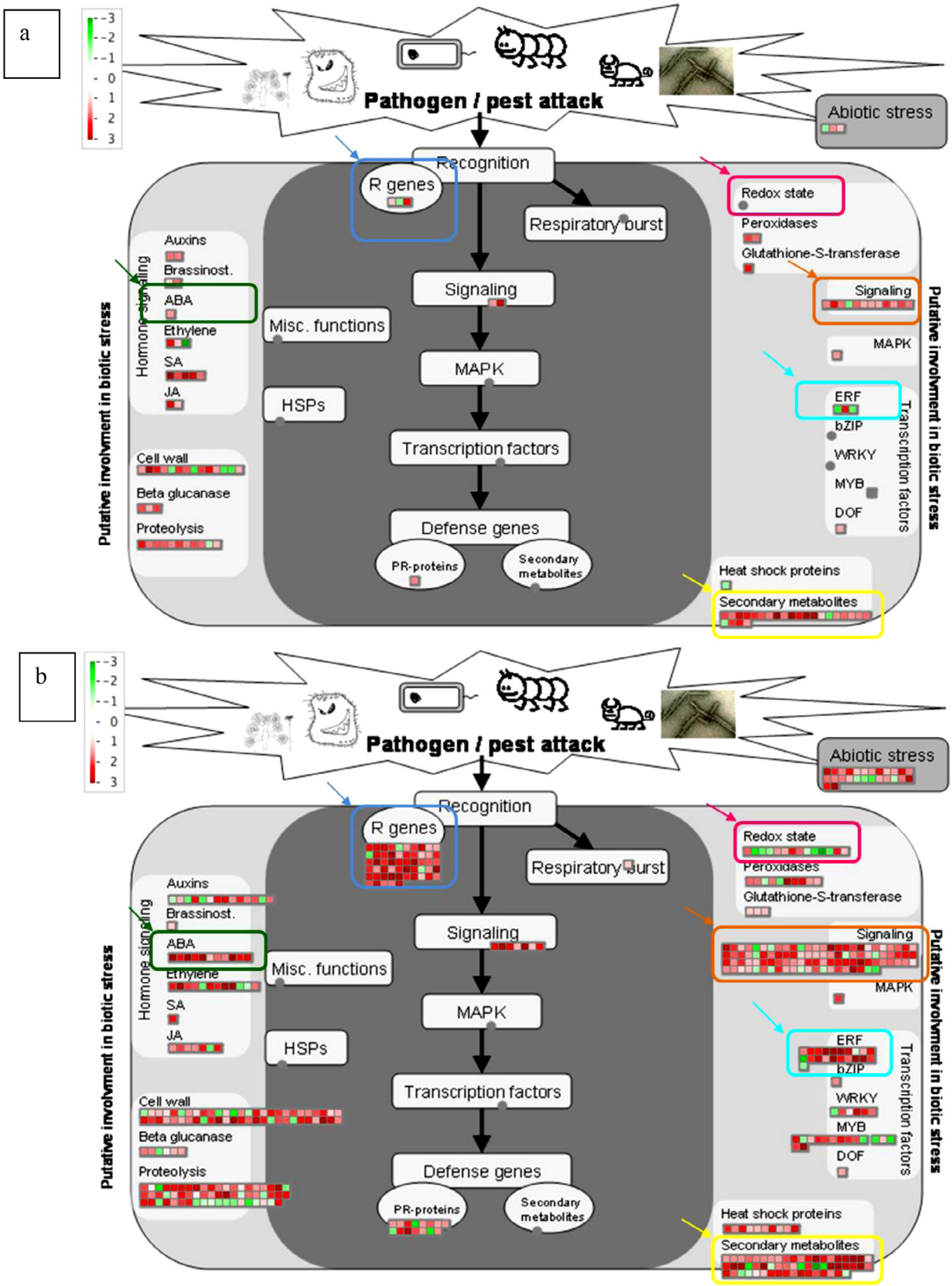

Fig. 2. Mapman visualization of genes assigned to functional categories of biotic stress and differentially expressed (7 *vs* 21 dpi), for control plants (a) and plants treated with *P. oligandrum* (b). Each square represents a gene. Overexpression is presented in red, repression in green. The intensity of the color denotes the fold change level. Significantly expressed functional categories are enclosed within different colored boxes. For each functional category, the same color was used for the two treatments.

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expressed in comparison to those of control plants (Fig. 2 a and b). All of these genes were induced, except for one gene encoding for "disease resistance-like protein" which was repressed (FC = −1.85) (Table S3). Among induced genes, 24 had their FC higher than 2. The most abundant ones were: "resistance protein" (6 transcripts), "resistance gene analog NB S9" (5 transcripts), and "disease resistance protein-like protein MsR1" (4 transcripts).

Depending on the treatment, the genes involved in secondary metabolism were expressed in very different ways (Fig. 2 a and b). In *P. oligandrum*-inoculated plants, 17 genes involved in terpenoid and flavonoid pathways were induced (Table S4). Among these genes, 6 and 7 transcripts encoded for terpene synthase (terpenoid pathway) and chalcone synthase (flavonoid pathway), respectively. One gene was repressed (FC = −2.62), which encoded for a protein similar to malonyl CoA (anthocyanin pathway).

In the case of the biotic "stress signaling sub-category", *P. oligandrum* treated plants showed induction of 3 transcripts, identified as "enhanced disease susceptibility 1" (EDS1), and 3 transcripts encoding for "MLO like protein" (Table S5).

With regard to hormone metabolism, the ABA metabolism was the most significantly regulated functional category ($P = 7.6 \times 10^{-3}$) (Fig. 2 a and b). *P. oligandrum* treatment induced the over-expression of 9 transcripts involved in ABA metabolism. The transcripts encoding for a gene similar to AtHVA22a (genes known for being ABA- and stress-inducible) were the most numerous. Among the 9 statistically significant genes expressed in the category, 6 genes corresponded to a homolog AtHVA22a (Table S6).

The protein degradation sub-category associated to the subtilase family was significantly regulated, following plant root inoculation with the oomycete ($P = 1.97 \times 10^{-2}$). All subtilase genes differentially expressed in our analysis were up regulated (Table S7).

Several genes were statistically differentially expressed (Limma $P < 0.01$), even though the category or sub-category to which they belong was not statistically different according to the Mapman statistical analysis. These genes were involved in regulation processes with, in particular, a relatively large number of transcripts being involved in calcium regulation (38 transcripts) and receptor kinases (68 transcripts). Regarding genes involved in calcium regulation, 25 transcripts were overexpressed, 2 were weakly repressed (FC > −1.5) and 11 were strongly induced (FC > 2). For genes encoding receptor kinase, 44 transcripts were induced, 3 were weakly repressed (FC = −1.5) and 19 highly expressed (FC > 2). The most significant genes (FC > 3) were those encoding for receptor kinases type LRR, XI and III (data not shown).

Root colonization by *P. oligandrum* induced transcription factor gene expressions (Fig. 2 a and b). These genes were involved in RNA transcription regulation process. *P. oligandrum*-root colonization modulated significantly the expression of 26 genes encoding different transcription factors. Fourteen genes belonging to the AP2/ERF sub-category had the highest significance regulation ($P = 2.36 \times 10^{-3}$). These genes are listed in Table S8. Another gene family was highly induced: bHLH ($P = 2.51 \times 10^{-2}$). Among eight significantly induced genes, four showed FC higher than 3 (Table S8).

3.2.2.2. P. oligandrum- root colonization specific biotic stress gene down-regulation. Fig. 2 a and b showed that, in *P. oligandrum*-inoculated plants, genes involved in redox state regulation were significantly more expressed than in control plants. Among all differentially expressed genes involved in this functional category, 83% were repressed. The most repressed genes were "thioredoxin-like" (FC = −2.97), and "non-symbiotic hemoglobin class 1" (FC = −2.97). Only one redox state regulation gene, annotated as "Thioredoxin H", was over-expressed (FC = 1.82) (Table S9).

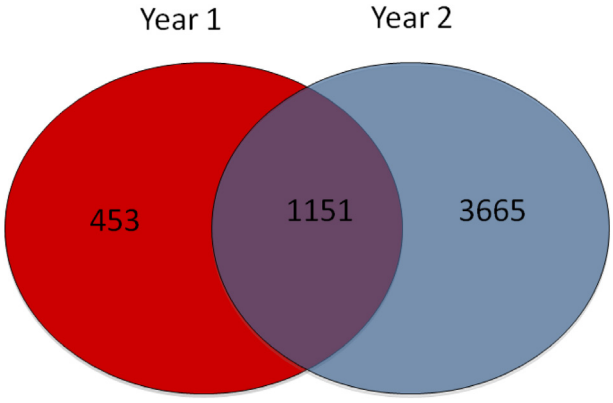

Fig. 3. Venn diagram representing the differentially expressed genes at 7 *vs* 21 dpi, in *P. oligandrum* treated plants, for the two experiments (Year-1 and Year-2).

3.3. Validation of microarray results using qRT-PCR

In order to validate microarray results, a set of 9 genes was selected, representing the most significant functional categories in our transcriptome analysis during *P. oligandrum*-plant interaction. Expression levels of these genes were measured by RT-qPCR at the same time points as those used in the microarray analysis. Fig. S1 shows that, in plants treated with *P. oligandrum*, the resulting qPCR values are in correlation with normalized microarray data ($r^2 = 0.59$).

3.4. Comparison of microarray results obtained in Year-1 and Year-2

Microarray experiments were performed with samples from Year-1 and Year-2. After subtraction of the genes differentially expressed in control samples for each respective year, common genes were identified by a Venn analysis (Fig. 3). A total of 1151 genes were common for the two years, with mostly similar FC, at least in the expression change (over- or under-expressed). Genes with high FC in Year-1 usually had high values in Year-2, but overall FC values were greater for genes in Year-1 than in Year-2. We had observed such differences when analysing these same samples by RT-qPCR, with specific pathogen-related genes (data not shown). The functional categories showing the highest statistical significance according to the Mapman analysis for both years were biotic stress (receptors), secondary metabolism (chalcones, anthocyanin), redox (thioredoxin). Some functional categories were specific to the year of sampling: hormone metabolism (ABA), biotic stress (signaling) were specific to Year-1, and protein degradation (ubiquitin) and calcium signaling for Year-2.

Although the Year-1 and Year-2 results showed overall similarities, they were not perfectly identical. Whereas such important conditions as watering and temperature could be controlled for the most part, this was not the case for plant cuttings coming from the vineyards, or for exterior conditions affecting greenhouse (*e.g.* temperature peaks).

4. Discussion

As reported in the literature (Le Floch et al., 2005, 2009; Rey et al., 1998b), *P. oligandrum* is an oomycte biocontrol agent that has established a unique relationship with plants. Following root colonization by *P. oligandrum*, such beneficial effects as growth promotion and/or induction of disease resistance, have been observed (Benhamou et al., 2012; Gerbore et al., 2014a; Rey et al., 2008; Takenaka, 2015). Although cytologic and biochemical studies have been undertaken to describe the interaction between

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P. oligandrum and its host plant (Le Floch et al., 2005; Rey et al., 1998b), no global transcriptomic analyses have, to the best of our knowledge, been used. In the present study, a transcriptomic analysis was used to study the range of molecular changes induced in plant roots after the oomycete colonization. As the whole grapevine genome has already been sequenced, a grapevine microarray was employed to assess plant responses.

After *P. oligandrum* inocula application, the oomycete was always isolated on grapevine roots during the 4-month experimental period. This ability to first colonize grapevine roots and then persist on them is consistent with previous recent studies (Gerbore et al., 2014b; Yacoub et al., 2016). This is also in line with Gerbore et al., (2014b) who reported that, in the Bordeaux vineyard, *P. oligandrum* strains naturally colonized the grapevine roots.

Grapevine root colonization with *P. oligandrum* was associated, in our study, with significant metabolic pathway changes within plant root cells. Numerous genes differentially expressed over time were identified and their functional categories assigned. These genes were involved in different biological processes, specifically those associated with response to stimulus, and also in cellular functions. Whereas a few of these genes were repressed, most were up-regulated. This result confirms that BCA can induce plant transcriptomic changes after being inoculated either on roots (Brotman et al., 2013) or on aerial parts (Alfano et al., 2007; Mathys et al., 2012; Morán-Diez et al., 2012).

In response to pathogenic or symbiotic microorganism colonization, the flow of calcium, Ca^{2+} is one of the first plant defense responses triggered by microbe-associated molecular patterns (MAMPs) (Dodd et al., 2010; Manzoor et al., 2012; Tena et al., 2011). One example of this is cryptogein, a glycoprotein elicitor from the elicitin family which activates the protein kinases that trigger Ca^{2+} inflow (Lecourieux-Ouaked et al., 2000; Lecourieux et al., 2002, 2005). Our results showed that several protein kinases, LRR III, XI, XII, and a calcium signaling pathway, were both activated after *P. oligandrum* root inoculation. It can be assumed, therefore, that *P. oligandrum* protein elicitors such as oligandrin, the elicitin-like protein or POD-1 and POD-2, cell-wall glycoproteins, are strongly associated with protein kinases and calcium pathway activation.

Thirty resistance genes of the R gene functional category family, including genes encoding the NBS-LRR receptor family, were significantly induced in grapevine roots, in response to *P. oligandrum* inoculation. This is the first time, to the best of our knowledge, that this result has been obtained with a non-pathogenic plant oomycete. Our study also showed that host genes encoding for chalcone synthase, a key enzyme of the flavonoid pathway, were induced in *P. oligandrum* inoculated plants. This is in agreement with Le Floch et al., (2005) who showed that, in the *P. oligandrum*/tomato root interaction, following 14 h post-inoculation with the oomycete, plant cells produced a phytoalexin called rishitin.

One characteristic of the *P. oligandrum*/*V. vinifera* interaction is the repression of genes involved in the redox status. As an example, several gene encoding proteins, annotated as "non-symbiotic hemoglobin", can detoxify reactive molecules of nitrogen, such as nitric oxide (NO) (Dordas et al., 2003; Perazzolli et al., 2004). NO is a key plant signaling molecule, involved in many processes, such as stomatal closure, cell death programation and resistance to pathogens (Thiel et al., 2011; Neill et al., 2003).

Among plant hormones, ABA metabolism functional category was induced in *P. oligandrum*-inoculated plants. Previous studies have shown that ABA has a suppressive effect on the SA signaling pathway (Hayes et al., 2010), and is also involved in the deposition of callose, the production of active oxygen molecules and in the regulation of gene expression (Bari and Jones, 2009; Jalloul et al., 2009). In *P. oligandrum*-colonized plants, a callose deposition at the pathogen infection site was observed (Benhamou et al., 1997). Moreover, according to other authors (Hase et al., 2008; Takahashi et al., 2006; Kawamura et al., 2009), the SA pathway was not activated. Accordingly, the ABA metabolism activation observed here in *P. oligandrum*-inoculated vines, is in line with these observations.

The main transcription factors over-expressed in *P. oligandrum*-inoculated vines belong to the Ethylene-Responsive Factor (ERF) family, AP2/ERF. These transcription factors are mainly associated with resistance to biotic stress (Agarwal and Jha, 2010). The up-regulation of AP2/ERF in transgenic tobacco increased plant resistance to pathogen infections by *Alternaria*, *Ralstonia solanacearum* or tobacco mosaic virus (TMV) (Zhang et al., 2009). In the present study, *P. oligandrum* inoculation also induced plant transcripts encoding for proteins annotated as "Enhanced Disease Susceptibility 1" (EDS1), and "phytoalexin deficient 4" (PAD4). These two genes have a central role in resisting biotrophic pathogens (Wiermer et al., 2005). For instance, on the leaves of downy-mildew grapevine resistant cultivar (Norton), EDS1 showed a higher expression level than in a grapevine sensitive cultivar (Cabernet Sauvignon), in response to *Plasmopara viticola* attack (Gao et al., 2010). The induction of EDS1 we observed indicated that certain plant defenses were triggered in Cabernet Sauvignon plants, even though a non-pathogenic oomycete, *P. oligandrum*, colonized the cultivar's root system. Consequently, in the *P. oligandrum*/grapevine interaction, the expression of these genes (EDS1 and PAD4) could suggest that the plant sets up defense systems, even against the *P. oligandrum* oomycete.

Certain similarities with symbiotic microorganism/root interactions, especially concerning the stimulation of subtilases, were observed in *P. oligandrum*-inoculated plants. The main such similarity was the stimulation of subtilases. Stimulation of subtilases is one of the most typical processes involved when plants interact with arbuscular mycorrhizae (AM) or nitrogen-fixing rhizobia (Takeda et al., 2007). Cangahuala-Inocente et al. (2011) reported that two proteins similar to "subtilisin-like protease" are strongly accumulated in the rootstock of vines colonized by mycorrhizae arbuscules. Interestingly, Kawamura et al. (2009) showed that genes encoding for subtilases are also strongly overexpressed, following application of a foliar treatment of *A. thaliana* with two *P. oligandrum* cell wall-elicitor proteins (POD1 and POD2). These results suggest that certain aspects of symbiotic interaction are also observed when *P. oligandrum* colonized the grapevine roots.

A detailed analysis of selected genes can also provide valuable insights into particularly important genes, even though these are not part of a highly significant functional category. For example, a GRAS-domain transcription factor, called MIG1, present in our data (Table S8), is involved in the symbiotic interactions between *Medicago truncatula* and *Rhizophagus irregularis* (Heck et al., 2016). For those authors, one gene encoding for these transcription factors has to be induced in the plant to ensure a correct symbiotic interaction. Clearly, this kind of observation would require more in-depth investigation, but it does provide an important first step in the characterization of the events taking place during the interaction between *P. oligandrum* and plants.

To conclude, the grapevine root colonization by *P. oligandrum* induced significant changes in the plant root transcriptome. The results indicate that a complex plant response was set up. Whereas, the expression of several transcripts suggested that the plant induced defense systems against *P. oligandrum*, other responses indicated the occurence of strong similarities with other symbiotic microorganism/root interactions. The present study, to the best of our knowledge, provides the first dataset on the transcriptional changes induced by a BCA in the root tissue of perennial plant species. In future work, transcriptomic data from other BCA/-plant interactions could be compared with the present results. This would provide new insights into the pathways involved in the

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establishment of the BCA/plant relationship, with respect to *P. oligandrum*, other specific *P. oligandrum*/plant interaction studies will undoubtedly appear, now that the genome of *P. oligandrum* has recently been sequenced (Berger et al., 2016).

Acknowledgments

We would like to thank the French Ministry of Agriculture (V1302 Casdar research project on Grapevine Trunk Diseases), the Poupelain Foundation and Biovitis for their financial support. We also appreciate the technical support provided by the Toulouse Get Biopuce Platform for microarray experiments (Dr V. LeBerre, Ms L. Trouilh, A. Jouanillou and S. Lamarre).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2017.02.007.

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Please cite this article in press as: Yacoub, A., et al. Transcriptional analysis of the interaction between the oomycete biocontrol agent, *Pythium oligandrum*, and the roots of *Vitis vinifera* L.. Biological Control (2017), http://dx.doi.org/10.1016/j.biocontrol.2017.02.007