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Innate Immunity of *Phlomis purpurea* against *Phytophthora cinnamomi*: A Transcriptomic Analysis

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Abstract: *Phlomis purpurea* L. grows spontaneously in dry and stony habitats from the south of Iberian Peninsula and in cork oak (*Quercus suber* L.) and holm oak (*Q. ilex* ssp. *rotundifolia*, Lam.) plantations infested with *Phytophthora cinnamomi* (Rands). The aim of this study is to understand the genetic basis of *P. purpurea* innate immunity to this pathogen. The transcriptome analysis of *P. purpurea* upon challenging with *P. cinnamomi* revealed a set of up-regulated genes, related to signaling, transcription factors and response to stress. Transcripts involved in the synthesis of a number of proteins, namely: *ANKYRIN*, *AP2*, *AQUAPORIN*, *ARMADILLO*, *At1G69870-LIKE*, *BHLH*, *BON1*, *CALMODULIN*, *CALNEXIN*, *CALRETICULINE*, *CC-NBS-LRR*, *CHAPERONE*, *CYTOCHROME*, *DUF*, *GH3*, *GMP*, *G-TYPE*, *LIPOXYGENASE*, *MLO-LIKE*, *MYB*, *NAC*, *NBS-LRR*, *PENTATRICOPEPTIDE*, *SUBTILISIN*, *WAK*, *bZIP* and hormones such as *BRASSINOSTEROID*, *JASMONATE*, *SALICYLATE*, *ETHYLENE-RESPONSIVE* were identified. *P. purpurea* ability to cope with *P. cinnamomi* attack is based on the expression of a set of transcription factors and signaling molecules targeted by the pathogen. The information gathered contributes to the elucidation of the overall response of *P. purpurea* to *P. cinnamomi* attempted infection which can be helpful for improving woody species resistance to pathogenic oomycetes.

Key words: *Phlomis purpurea*, transcriptomics, *Phytophthora cinnamomi*, plant immune response, stress regulatory network.

1. Introduction

Plants, being non-moving organisms had to develop special strategies to defend themselves from the challenges they face, in particular biotic threats. Biotic and abiotic stresses are responsible for a wide range of plant responses, including alteration in gene expression and cellular metabolism. After infection with oomycetes or fungi, following the release of elicitors by the pathogen, plants upon receptor-mediated perception, activate the so called "surveillance system" by recognizing pathogen-associated molecular patterns (PAMPs) and react by inducing a wide range of defence related proteins, commonly referred to as pathogenesis-related proteins (PRs). PAMPs, microbe-associated molecular patterns (MAMPs), herbivore-associated molecular patterns (HAMPs), and damage-associated molecular patterns (DAMPs) are molecules produced by microorganisms and insects in the sequence of infection, microbial priming and insect predation [1]. These molecules are then recognized by the plant receptor molecules, which activate defence signaling pathways, resulting often in plant's ability to overcome pathogenic invasion or induce systemic resistance [1]. Plants use extracellular leucine-rich repeat (LRR) receptors for elicitor recognition, the downstream processing being mediated by serine/threonine kinases [2], and intracellular immune receptors (nucleotide-binding leucine-rich repeat (NLR) proteins) that are key initiators of plant defence responses [3].

Resistant hosts have in their genomes, resistance

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R-genes coding for specific proteins able to recognize avirulence factors and trigger defence response [4]. The underlying mechanism consists in the interaction of the product of a dominant or semi-dominant plant resistance (R) gene with a product derived from the corresponding dominant pathogena virulence (Avr) gene (gene-for-gene interactions), leading to subsequent signal transduction events that coordinate the activation of an array of defense responses [5].

In the past decade, significant progress has been achieved in defining the molecular mechanisms of innate immune responses in plants, in particular pathogen recognition by the host, signaling events, signaling pathways and their involvement in activating defence responses [6, 7]. PAMP-induced signaling cascades lead to the transcriptional activation of genes that trigger innate immune responses further leading to the production of antimicrobial compounds. For instance, in *Arabidopsis*, Najafi *et al*. [8] have demonstrated the role of PAMP-induced secreted peptides (PIP1, 2) in plant immunity and showed that plants overexpressing prePIP1 and prePIP2 present increased resistance against *Pseudomonas syringae* and *Fusarium oxysporum*.

Transcription regulation of gene expression may influence many biological processes including biotic stress response and cell signaling. Transcription factors (TFs) and *cis*-elements function in the promoter region of different stress-related genes, and the over-expression or suppression of these genes may improve the plant's tolerance to both biotic and abiotic stresses [9]. Lin *et al*. [10] have reported the role of NAC genes in molecular mechanisms underlying signaling pathways and their involvement in activation of defence responses in rice, in particular in rice innate immune responses, recognition of pathogens by the host, and recognition-triggered early signaling events. Many other TFs as well as a number of genes have been recognized to be involved in plant immunity. It is the case of mildew resistance locus (MLO) gene, a plant specific gene family, which has been reported to play an important role in *Citrus sinensis* resistance to mildew infection [11]. Such a similar role has previously been demonstrated for grapevine *VvMLO7*, *11* and *13* genes which are up-regulated following powdery mildew infection [12].

Plant defence, in response to microbial attack is further regulated through a complex network of signaling pathways that involve three signaling molecules: salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) [13] and their role in basal resistance against pathogens has been emphasized. Increased levels of Ca^{2+} are important for signaling and response to stress tolerance. Signal transduction uses discrete Ca^{2+} fluxes to connect stimuli with adaptative responses [14]. Other signaling components are cysteine-rich receptor-like kinases (CRK36) that seem to play a role in signal amplification by reactive oxygen species (ROS) generated in plants by specific PAMPs [15]. CRK36 is activated by sensing ROS through redox modification of Cys residues in the Domain of Unknown Function 26 (DUF26). These domains are relevant for the functionality of plant CRKs.

Interaction of a pathogen with its host is a very complex process, many aspects having been discussed for a number of plant species. The variability of processes undergone by the different pathogens is responsible for the great amount of research that has insistently been performed in the last decade. Despite the existence of very good models used to understand the mechanisms of plant immunity, there is still a very hard way to go on.

In previous papers, attention has been devoted to the reaction of woody species from the fagaceae family to *Phytophthora cinnamomi* infection. Duclos *et al*. [16] identified four elicitin genes from *P. cinnamomi*, one of them named α-cinnamomin. A comprehensive study of the role of cinnamomins in the induction of defence responses against the

pathogen invasion and restriction of its proliferation in cork and holm oaks roots was described by Ebadzad *et al*. [17]. Due to the need of improving health of Fagaceae plants, several efforts have been developed trying to find a solution to cope with this important disease whose causal agent is *P. cinnamomi*. An approach to cope with infestation of cork and holm oaks has demonstrated that co-existence of oak species with *Phlomis purpurea* L. (purple phlomis, a perennial species of the Lamiaceae family) protects them from *P. cinnamomi* [18]. Mateus *et al*. [19] have reported on the production of the nortriterpenoid (17S)-2α,3α,11α,23,24-pentahydroxy-19(18→17)-*abeo*-28-norolean-12-en-18-one (phlomispurpentaolone) by *P. purpurea*, and its exudation from the roots to the rhizosphere. This compound has been shown to present anti-*Phytophthora* effect. *Phlomis purpurea*, growing spontaneously in highly contaminated *Phytophthora* oak stands is not affected by *P. cinnamomi*. On the contrary, it presents immunity to this pathogenic oomycete. Taking these considerations into account, Baldé *et al*. [20] have performed a transcriptome analysis of *P. purpurea* challenged with *P. cinnamomi*. These authors described important innate anatomic characteristics that may contribute to difficult hyphae penetration in the host cells, and revealed a set of up-regulated genes responsible for the maintenance/increase of the physical barriers the pathogen encounters when contacting with *P. purpurea* roots.

The comparative transcriptome analysis presented in this paper was based on the transcriptome data performed using *P. purpurea* plants challenged with *P. cinnamomi*. A set of genes up-regulated after attack by the pathogen is discussed as well as the interplay of events occurring in *P. purpurea* as a response to the challenge by the oomycete *P. cinnamomi*. These findings contribute to increasing knowledge on the genetic machinery underlying the complex phenomenon of *P. purpurea* innate immunity. This knowledge will be of great importance for a global understanding of the complexity of stress regulatory network responsible for plant immunity against infection by pathogens.

2. Materials and Methods

2.1 Phytophthora cinnamomi *Isolates*

Pure stock cultures of the *P. cinnamomi* isolates—PA37 and PA45—both mating type A2, were tested for pathogenicity. They were isolated in the Algarve region (southern Portugal) from *Quercus suber* roots at Lagos and from soil associated with declining *Q. suber* stands at S. Brás de Alportel, respectively.

2.2 Zoospore Production

Zoospores were produced following a modification of the procedure reported by Byrt & Grant [21]. Briefly, a *P. cinnamomi* culture plug was transferred onto 10% V8 juice agar medium (V8A) and incubated for 3 days, at 24 ℃. V8A plugs from the growing colony in Petri dishes were transferred to Miracloth membranes. The cultures were incubated for 15 days at 24 ℃. The Miracloth support and mycelia were transferred to 100 mL 5% V8 broth (V8B) and the culture shaken overnight (16 h) at 90 rpm at 24 ℃. The nutrient medium was replaced with a solution (MSS) consisting of 0.01 M $Ca(NO_3)_2 \cdot 4H_2O$, 0.005 M KNO_3 and 0.004 M $MgSO_4 \cdot 7H_2O$ dissolved in 1 L of distilled water, autoclaved and subsequently supplemented with 1 mL 0.1 M $C_{10}H_{12}N_2NaFeO_8$ solution. The culture was then shaken for 24 h. Sporangia were induced to release zoospores by incubating the Miracloth covered with MSS in Petri dishes at 4 ℃ for 20 min. Then, the Petri dishes were exposed to fluorescent light at room temperature, for 3 h. The zoospore suspension from each Miracloth was transferred into a 15 mL conical tube. The upper 2 mL was transferred to a second tube and shaken for 70 s to have zoospores encysted. Then 10^4-10^5 zoospores per milliliter were routinely produced.

2.3 Plant Material

Phlomis purpurea seedlings were produced from seeds collected in the field, across southern Portugal. Briefly, seeds were surface sterilized and covered with wet absorbent paper in *Petri* dishes until germination occurred (*ca* 7 days). When the radicles were 2-3 cm long (24 to 48 h) they were transferred into cylindrical soft black plastic tubes (25 cm × 3 cm) containing vermiculite.

Details on challenging with zoospores, RNA extraction and cDNA libraries construction, Sequencing and assembly, De novo assembly, and differential expressed genes analysis using DESeq, have been previously described by Baldé *et al.* [20]. The assembled unigenes, with sequence length longer than 200 bp, were deposited in the NCBI Sequence Read Archive (SRA) under the accession number SRP046996.

Using the transcriptome previously described, a randomly selection has been performed taking as criterium the log2FoldChange transcript value higher than 2. A final amount of 82 up-regulated (log2FoldChange > 2) genes were evaluated to the main factors regulating innate immunity of *P. purpurea* to *P. cinnamomi.* The up-regulation levels of the selected transcripts were screened for three time points (12, 24 and 48 h) after challenging of *P. purpurea* with *P. cinnamomi* zoospores.

3. Results and Discussion

Comparison of the transcripts abundance of the up-regulated genes at 12, 24 and 48 h and post-challenge (hpc) are shown in Figs. 1 and 2.

A decrease in the transcript abundance since 12 to 48 hpc was detected for genes coding the proteins presented in Fig. 1.

In Fig. 2, transcripts are shown whose log2FoldChange values decrease from 12 to 24 hpc and increase to 48 hpc (left side of the chart) or increase from 12 to 48 hpc (right side of the chart).

The genes *At1G69870-LIKE*, *AP2*, *BHLH*, *BON1-ASSOCIATED*, *BRASSINOSTEROID*, *CALNEXIN*, *CHAPERONE*, *ETHYLENE-RESPONSIVE*, *FERONIA*,

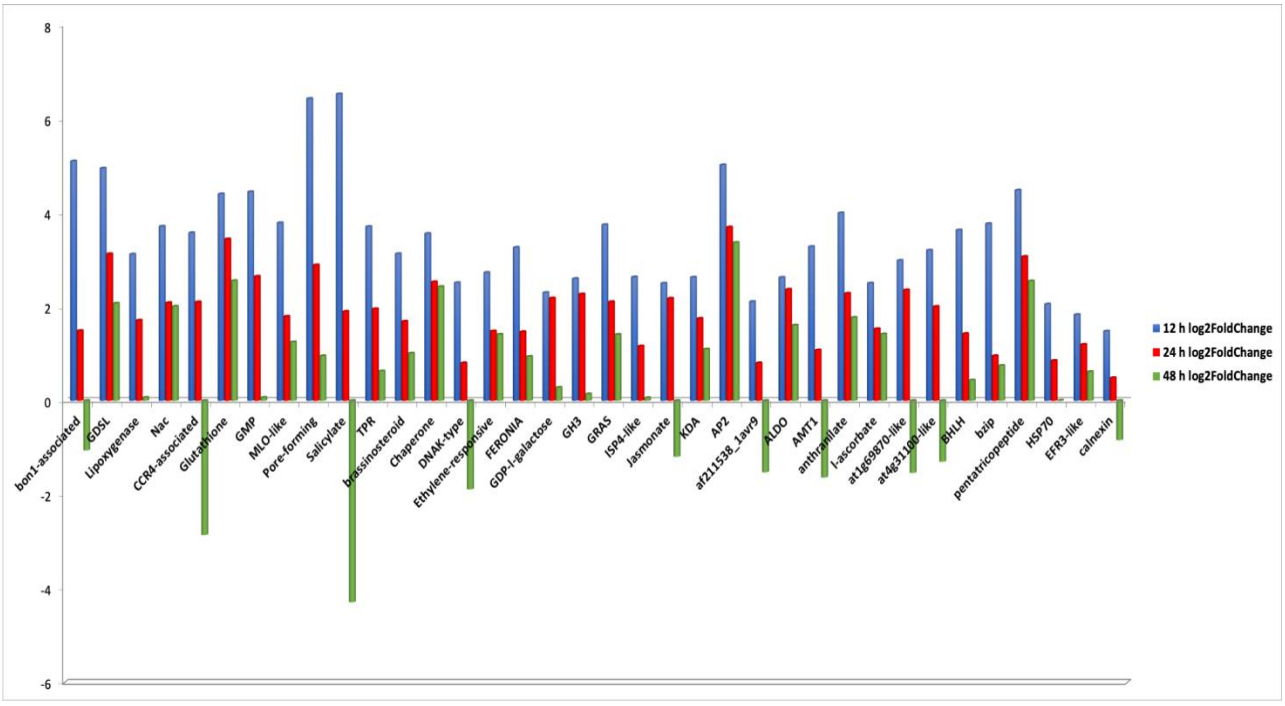

Fig.1 Profile of gene transcript values decreasing along the three timepoints under study.

Ordinate refers to transcripts log2FoldChange values for each timepoint post-challenge (hours post-challenge = hpc). Transcripts from non-challenged plants were used as reference. The abscissa shows the name of the transcripts identified.

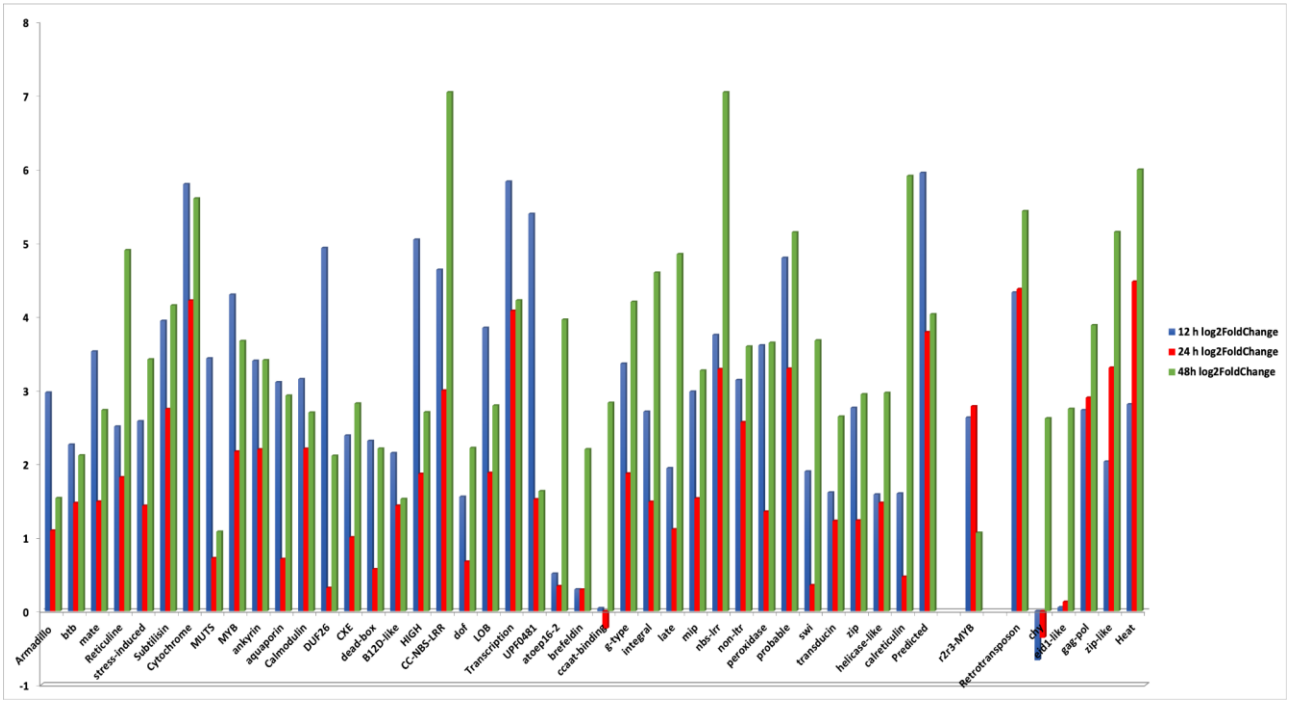

Fig. 2 Profile of gene transcript values along the three timepoints under study.
Ordinate refers to transcripts log2FoldChange values for each timepoint post-challenge. Transcripts from non-challenged plants were used as reference. The abscissa shows the name of the transcripts identified.

GH3, *GMP*, *JASMONATE*, *MLO-like*, *NAC*, *NBS-LRR*, *PENTATRICOPEPTIDE*, *SALICYLATE*, *WAK* (*at4g31100*) and *bZIP* present very high transcript levels at 12 hpc, decreasing thereafter until 48 hpc (Fig. 1). *ANKYRIN*, *AQUAPORINE*, *ARMADILLO*, *CALMODULIN*, *CALRETICULIN*, *CC-NBS LRR*, *CYTOCHROME*, *DUF26*, *G-TYPE*, *MYB*, and *SUBTILISIN* show high transcript levels at 12 or at 48 hpc, presenting a minimum at 24 hpc (Fig. 2). Transcript level profiles of *RETROTRANSPOSON*, *CHY*, *EID1-LIKE*, *GAG-POL*, *ZIP-LIKE* and *HEAT* increase from 24 hpc to 48 hpc while *R2R3-MYB* presents a maximum a 24 hpc (Fig. 2).

*3.1 Sensing the External Stimulus (*Phytophthora cinnamomi *Challenge)*

Plant-pathogen interactions involve communication between two living organisms which require reciprocal recognition. Plant defence system relies on several different mechanisms, including morphological or physical barriers, chemical defence, and innate immunity. Plant innate immunity depends on a set of specialized receptors, called pattern recognition receptors (PRRs) that recognize microbe-associated molecules [22]. They activate signal transduction and respond defensively by developing signaling pathways involving many genes and their products. Early recognition and fast response are important in building a successful defence [23]. Stress sensing is a complex phenomenon, being impossible to establish a single sensing mechanism common to the different types of stresses. In both animals and plants, the extracellular matrix (ECM) separates the cell from the external environment and plays a fundamental role in filtering and interpreting external cues such as pathogen attack [24]. Ebadzad *et al*. [17] showed that elicitins (β and α-cinnamomin) produced by *P. cinnamomi* act as elicitors activating defence responses in *Q. suber* (L.) and *Quercus ilex* (Lam.) when their roots are invaded by this oomycete.

3.1.1 Receptor-Like Kinases (RLK)

RLKs are a group of conserved signalling components that play fundamental roles in the perception of external signals and activate

defence-associated signaling pathways, thereby regulating cellular responses to biotic stress [25]. RLKs have been described as playing key roles in disease resistance. Plant RLKs involved in immunity are so-called pattern-recognition receptors (PRRs) that detect PAMPs and, upon binding of their cognate elicitors, initiate a well-characterized set of defence responses termed PAMP-triggered immunity (PTI) [26]. Eckardt [27] has provided a comprehensive model of the crosstalk between RLKs and ROS. According to this author, plant perception occurs at the symplast via NBS-LRR and at the apoplast via RLKs. Kanzaki *et al*. [28] pointed out that in *Nicotiana benthamiana*, the lectin-like receptor kinase NbLRK1 is a component of the *N. benthamiana* protein complex that recognizes *Phytophthora infestans* INF1 elicitor and mediates INF1-induced cell death. Studies by Singh and Zimmerli [29] have revealed the involvement of lectin receptor kinases in plant innate immunity. LecRKs, in particular LecRK-I.9 and NbLRK1, have been shown to influence the defence response against *Phytophthora* in *Arabidopsis*, tomato and *N. benthamiana* [30]. The same authors have also demonstrated that silencing of several LecRKs in tomato and *N. benthamiana* resulted in increased susceptibility to *Phytophthora capsici* and *P. infestans*, respectively. Wang and Bouwmeester [31] have reported the role of RLKs with LRR ectodomains on the initiation of defence responses. Recently, Muchero *et al*. [32] have demonstrated the role of one putative membrane-bound L-type RLK and two receptor-like proteins in mediating plant pathogen interaction in *Populus trichocarpa*, these receptor-like proteins attaining the peak of expression at 24 h post-infection.

3.1.1.1 WAK

Among the RLKs reported, the wall-associated kinases (WAKs), often transcriptionally regulated during infection, have been shown to positively regulate resistance to fungal diseases of many plant species [33]. According to the same authors, pathogen infection of angiosperms relies on the interaction between the ECM and the invading agent and may be accompanied by signaling between the ECM and cytoplasm. Due to their membrane configuration (the cytoplasmic Ser/Thr kinase signature) and the extracellular domain (ectodomain), WAK1 kinases are able to perceive DAMPs molecules and communicate them, inducing plant defence. Verica and He [34] suggested that WAK1 is required for plant survival to *P. syringae* infection. B. D. Kohom and S. L. Kohom [35] have postulated that WAKs are receptors for both pectin in the cell wall, and for pectin fragments, oligogalacturonic acids (OGs), generated during attacks by some pathogens. *WAK1* is induced by the fungal pathogen *Alternaria brassicicola* and the defence-related signaling molecules methyl JA and ET [36]. According to Delteil *et al*. [37], the recognition of PAMPs like chitin would lead to an increased expression of *Oryza sativaw* wall-associated kinase (OsWAKs). The same authors pointed out that DAMPs produced by the pathogen could be recognized by OsWAK91 receptors at the plasma membrane, triggering an enhanced immune response. These authors suggested that the rice OsWAK genes are part of basal defence response, potentially mediated by chitin from fungal cell walls. According to He *et al*. [33], WAK1 is induced by SA in a non-expresser of pathogenesis-related genes (NPR1-related genes) dependent manner, demonstrating that it is encoded by a pathogenesis-related gene. The transcript profile of *at4g31100* (a WAK gene) in *P. purpurea* challenged with *P. cinnamomi* is similar to that of JA and SA, highly expressed at 12 hpc decreasing thereafter and being down-regulated at 48 hpc (Fig. 1). Transgenic rice lines overexpressing OsWAK1 showed enhanced resistance to *Magnaporthe oryzae* strain P007 [38].

3.1.1.2 DUF26

DUF26 containing proteins constitute one of the largest RLKs. These proteins, also called cysteine-rich receptor-like kinases (CRKs), have been suggested to

play important roles in the regulation of pathogens. Tanveer *et al*. [39] reported an increased number of DUF26 domain-containing proteins at 12 hpc of rice infected with *M. oryzae*, which is in accordance with the results obtained for *P. purpurea* challenged with *P. cinnamomi*. According to Yeh *et al*. [40], cysteine-rich receptor-like kinases (CRKs) possess two copies of the C-X8-C-X2-C (DUF26) motif in their extracellular domains and are thought to be involved in plant stress resistance. According to the same authors, as soon as the plants recognize the pathogen, they activate the pattern-triggered immunity response by over-expression of CRKs which is in agreement with our data showing a huge amount of *DUF 26* (a CRK) transcripts at 12 hpc, drastically decreasing to 24 hpc and increasing again to 48 hpc but reaching only around half of the initial log2FoldChange value (Fig. 2).

3.1.1.3 FERONIA

In challenged *P. purpurea*, the plasma-membrane receptor-like kinase FERONIA (FER) presents a high transcript level at 12 hpc progressively decreasing until 48 hpc (Fig. 1). Feng *et al*. [41] reported the interplay of FER dependent signaling on elicitation of cell-specific calcium transients to maintain cell-wall integrity during salt stress. According to Gronnier *et al*. [42], FER positively regulates immune signaling by controlling the ligand-induced complex formation between FLAGELLIN SENSING2 (FLS2) and its co-receptor BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (BAK1/SERK3). According to the same authors, FER acts as an anchoring point connecting cell wall and plasma membrane nano-environments to enable the nucleation of pre-formed receptor/co-receptor complexes at the cell surface. The high transcript levels of FER at 12 hpc of challenged *P. purpurea* may likely function as a sensor at pectin cell wall layer, further contributing to the maintenance of cell integrity following the oomycete infection.

3.2 G-Type Protein

In challenged *P. purpurea*, G-type protein presents a high value at 12 h, decreasing to a lower one at 24 hpc and attaining the highest transcript level at 48 hpc (Fig. 2). In *Arabidopsis*, it has been suggested that a number of RLKs are associated to G-proteins that are recognized as universal signaling transducers [43]. Other authors have pointed out the important role of G-proteins in plant innate immune response, the Gβγ dimer and two different XLGs having been considered as mediating signaling in the pathogen-associated molecular patterns-triggered immunity (PTI) [44].

3.3 ARMADILLO

In *P. purpurea*, *ARMADILLO* (*ARM*) transcript profile values decrease from 12 hpc to 24 hpc, with a slight increase at 48 hpc (Fig. 2). ARM makes part of a subset of proteins functioning in intra-cellular signaling. According to Phillips *et al*. [45], ARM repeat proteins, in association with RLKs or co-receptors, act in the regulation of defence responses, which is coherent with their high expression in *P. purpurea*, at the first hours after challenge.

3.4 MLO-CALMODULIN Interaction

In *P. purpurea*, *MLO* coding genes present high transcript levels at 12 hpc, decreasing thereafter to 24 and 48 hpc (Fig. 1). *MLO* genes encode a plant-specific integral membrane protein, comprising small to medium size families, that varies among plant species. According to Kim *et al*. [46], the MLO family is believed to be unique to plants and green algae. A meta-analysis of genome-wide barley and *Arabidopsis* expression data revealed that barley *MLO* and *AtMLO2* are coexpressed with other genes recognized to have functions in plant immunity. According to Humphry *et al*. [47], some of the co-expressed genes are required for non-host resistance against powdery mildew. Lewis *et al*. [48] identified *AtMLO2* as a

target of the *P. syringae* type III effector HopZ2, accounting for a direct role in plant defence. According to Lee *et al*. [49], plants exposed to a variety of stresses, increase intracellular Ca^{2+} level through binding to CALMODULIN (CaM) and CALMODULIN-LIKE (CML) (CaM/CML). It is widely accepted that calcium ions play essential roles in cell signaling and that CaM acts as an intermediary protein that sense calcium levels and relays signals to different calcium-sensitive enzymes. Previously, Kim *et al*. [50] reported the interaction of CaM with MLO, stressing its importance for defence responses in plants. These authors suggested the existence of a potential communication between plant abiotic responses and immunity via signaling from MLO to Ca^{2+}-dependent CaM/CML, which stimulates the production of H_2O_2. The same authors demonstrated that the MLO protein functions independently of G proteins and mediates a Ca^{2+}-dependent interaction with calmodulin. Considerable amount of research supports the existence of a connection between MLO proteins and RLKs. In challenged *P. purpurea*, the profile of *CaM* transcripts follows closely that of *MLO* at 12 and 24 hpc (Figs. 1 and 2).

Taken together, our results for *WAK*, *DUF*, *FER*, *CaM* and *MLO* allow the assumption that an interplay among the proteins encoded by these genes may be responsible for the early recognition and signaling of the pathogen by *P. purpurea*. Our results agree with those of Humphry *et al*. [47], according to which a number of RLKs are present in the set of genes co-expressed with *MLO*/*AtMLO2* and that *FERONIA* and *AtMLO2* mutants show a similar level of powdery mildew resistance. FER, RLK and MLO proteins may co-function in the same biochemical pathway. Lalonde *et al*. [51], using yeast-based interaction, suggest a direct physical contact between *At*MLO proteins and various RLKs. Lyngkjær and Carver [52] have demonstrated that *mlo* allele confers pre-invasion resistance by inhibiting fungal pathogenesis before plant cell wall penetration. This is in agreement with the results reported by Baldé *et al*. [20], emphasizing that hyphae of *P. cinnamomi*, although being visible over the cuticle of *P. purpurea*, do not penetrate the cells.

3.5 CHAPERONE, CALRETICULIN, CALNEXIN

The stress imposed by the oomycete attack induces PAMP-associated responses that may be responsible for the very fast increase of *CHAPERONE* transcripts, whose profiles present maximal values at 12 hpc decreasing to 48 hpc (Fig. 1).

CALRETICULIN (CRT) is a calcium binding protein having, among other functions, a role as molecular chaperone. CRT presents very high values at 48 h (Fig. 2). According to Joshi *et al*. [53], CRT3, one of the three CRTs, plays an important role in plant protection against fungi and bacteria infections and in plant innate immunity. The same authors suggest a crosstalk between CRT mediated signaling pathways and biotic, abiotic stress, and phytohormone mediated signaling pathways. Matsukawa *et al*. [54] reported the role of a *Nicotiana tabacum* CRT (NtCRT3a) on resistance reactions of *N. tabacum* against *P. infestans.* According to these authors, CRT encodes an ER quality control chaperone responsible for proper glycoproteins maturation, especially, glycosylated cell-surface receptors. The very high level of CRT at 48 hpc accounts for its role in innate immunity of *P. purpurea*, probably by interfering in glycoproteins maturation as suggested by Matsukawa *et al*. [54].

CALNEXIN is down-regulated at 48 hpc (Fig. 1). CALNEXIN and CRT are two closely related calcium-binding molecular chaperones localized in ER [55]. According to Tuteja and Mahajan [56], calcium functions as a central node in the overall signaling network, playing an important role in stress tolerance in plants. In response to stress, the cytosolic calcium concentration increases, which initiate the stress signal transduction pathways for stress tolerance [56]. According to Gupta and Tuteja [57], the role of chaperones on stress signaling in the endoplasmic

reticulum is very important for proper protein folding under stress conditions. CALNEXIN binds to Ca^{2+} which is a central node in various signaling pathways for maintaining calcium homeostasis [58]. The expression levels of CRT and CALNEXIN in *P. purpurea*, suggest an interplay of these calcium-binding chaperones in proper glycoproteins maturation and in signalling after *P. cinnamomi* attack.

3.6 BON1-ASSOCIATED

The transcript values of *BON1-ASSOCIATED*, are very high at 12 hpc and show down-regulation at 48 hpc (Fig. 1). A similar profile at 12 hpc has been observed for *CALNEXIN*. BON1 is a calcium binding protein, an important regulator of calcium signature. According to Yang *et al*. [59], calcium signaling is essential for environmental responses including immune responses. The same authors pointed out that BON1 protein interaction with *Arabidopsis*-autoinhibited Ca^{2+}-ATPases (ACA10/8) controls pathogen growth as a result of its negative regulation of immune receptor gene expression, possibly associated with calcium homeostasis. Altered steady state of calcium might mimic signals from pathogen invasion and therefore up-regulate or activate *NLR* genes.

The transcript values recorded for this gene account for its important role in the maintenance of correct protein folding and calcium homeostasis following the stress imposed by *P. cinnamomi* challenge.

3.7 NBS-LRR

NUCLEOTIDE-BINDING SITE LEUCINE-RICH REPEAT (NBS-LRR) is a type of resistance proteins identified in a number of plant species following biotic stress. Plants, which have no specialized immune cells, should be able to autonomously recognize effectors. In response to effector proteins released from pathogens, plants maintain a large number of disease resistance (R) genes that, directly or indirectly, recognize effectors and initiate effector triggered immunity. Most of R genes encode NBS-LRR proteins [60]. According to these authors, NBS-LRRs are evolved to recognize effector proteins and mediate a “high impact” defence responses, a type of programmed cell death known as the hypersensitive response (HR).

The transcript levels of *NBS-LRR* in *P. purpurea* are between 3.7 at 12 hpc and 2.8 log2FoldChange at 48 hpc, which represents a high up-regulation (Fig. 1). Xu *et al*. [61] have demonstrated that NBS-LRR domain proteins are immune sensors and play critical roles in plant disease resistance in rice and *Arabidopsis*. According to the same authors, in *Zea mays*, *ZmNBS25* may be involved in ROS signaling pathways for disease resistance by inducing SA accumulation. Plant NBS-LRR proteins act through a network of signaling pathways and induce a series of plant defence responses, such as activation of oxidative burst, calcium and ion fluxes, mitogen-associated protein kinase cascade, induction of pathogenesis-related genes, and HR [62, 63]. Previously, Kunkel and Brooks [13] pointed out that signaling molecules related to plant defence response, such as SA, JA and ET, are involved downstream of NBS-LRR proteins, and that a complicated cross-talk occurs between the different signaling pathways, involving both synergism and mutual antagonism. Recently, several authors have conducted research on different plant pathogen interactions and on the role of NBS-LRRs in plant pathogen interactions (for a review see Wu *et al*. [64]). Sagi *et al*. [65] identified *NBS-LRR* genes potentially involved in response to *Ascochyta* blight infection, based on their co-localization with the known QTLs for *Ascochyta* blight resistance and on their expression profiles. Plant *NBS-LRR* genes interact with pathogen effector proteins to activate signal transduction pathways involved in innate immunity while TIR and CC domains recognize specifically R-Avr complexes and initiate downstream defense signaling [66]. According

to Elmore *et al*. [67], NBS-LRRs are specialized immune proteins that recognize specific pathogen proteins and require a conserved chaperone complex for correct functioning at the nucleus and cytoplasm levels to activate immunity. In challenged *P. purpurea*, the transcript levels of *CHAPERONE* present a profile very similar to that of *NBS-LRR* (Fig. 1).

Many other authors have reported the role of *NBS-LRR* in resistance of different species to different pathogens: in *N. benthamiana* [68], rice and *Arabidopsis* [61]. Goyal *et al*. [69] have reported the isolation of *NBS-LRR* genes responsible for resistance of *Vitis vinifera* to powdery mildew disease. According to Li *et al*. [70], *VaRGA1*, a *TIR-NBS-LRR* gene, enhanced resistance to *Plasmopara viticola*, and its overexpression in *N. benthamiana* conferred enhanced resistance to *Phytophthora parasitica* through the activation of SA signaling. In *P. purpurea* challenged with *P. cinnamomi*, SA transcript value is 6.5 at 12 hpc, decreasing until 48 hpc, a profile similar to that of *NBS-LRR*, which may account for its role in SA signaling as suggested by Li *et al*. [70]. The role of *NBS-LRR* genes in conferring disease resistance in different species and to different pathogens has been emphasized [61, 71].

3.8 CC-NBS-LRR

CC-NBS-LRR genes, a sub-group of *NBS-LRR*, are widely distributed in monocots and dicots. The transcript levels of *CC-NBS-LRR* in *P. purpurea* challenged with *P. cinnamomi* are between 4.6 at 12 hpc and 7 at 48 hpc (Fig. 2). According to Moffett *et al*. [72], CC-NBS-LRR recognizes specific pathogen-derived products and initiates a resistance response. Recent results suggest that these domains play a significant role in the interaction of R protein with effector proteins from pathogens, in activating signal transduction pathways involved in innate immunity. The very high transcript levels of *CC-NBS-LRR*, attaining around 7 at 48 hpc, combined with the high transcript levels of *NBS-LRR* account for their role in innate immunity of *P. purpurea* undergoneafter challenging with *P. cinnamomi.*

3.9 AQUAPORINS

AQUAPORINS (AQP) is a family of membrane integral proteins present in different organisms, including plants. In *P. purpurea* challenged with *P. cinnamomi*, the transcript levels have a log2FoldChange around 3 at 12 and 48 hpc (Fig. 2). After recognition of the biotic agressor, the host rapidly reacts producing hydrogen peroxide at the apoplast level. AQP facilitates H_2O_2 across cell membranes and are involved in transmembrane redox signalling processes. H_2O_2 transport across a biomembrane is mediated by particular AQP isoforms in addition to certain membrane lipids. H_2O_2 trafficking across the plasma membrane is induced but is not constitutive, and occurs only when apoplastic H_2O_2 is generated in response to pathogens, microbial patterns, or environmental signals [73]. According to Zhang *et al*. [74], H_2O_2 generated in the apoplast upon infection translocates quickly into *Arabidopsis* cells, where it interacts with pathways of intracellular immunity to confer plant resistance against diseases (for a review see Li *et al*. [75]). The transcript values of AQP at 12 hpc in challenged *P. purpurea* may account for its role in traficking of H_2O_2 across the plasma membrane as a response to pathogen aggression, while at 48 hpc that may be probably related to traficking across other cell membranes like the tonoplast, facilitating the cell osmo-regulation under fungi infection conditions.

3.10 Transcription Factors

In response to different stresses, plants have developed complex defence systems of signal perception and transduction networks. They have evolved sophisticated stress response strategies, and transcription factors (TFs) that are master regulators of stress-responsive genes. For a revision see Baillo *et al*. [76]. TFs play pivotal roles at transcriptional level

by either suppressing or activating associated genes, thus serving as molecular switches that orchestrate the regulation of plant developmental processes in response to a variety of stresses [77]. In plants, TFs can bind specific DNA sequences and interact with different proteins in transcriptional complexes. They are activated by different pathways of signal transduction and combine, directly or indirectly with local and distal cis-acting elements to regulate and modulate transcription efficiency of target genes in different biological contexts. TFs identified in the transcriptome of challenged *P. purpurea* include:

NAC TFs are one of the largest families of transcriptional regulators in plants. They are central components of many aspects of the plant innate immune system [9]. Members of the *NAC* gene family have been suggested to play important roles in the regulation of transcriptional reprogramming associated with plant stress responses [9]. In *P. purpurea*, *NAC* shows a transcriptional profile decreasing from 12 to 48 hpc although remaining high (Fig. 1). TFs control transcription of their downstream genes by interacting with other proteins and binding to a consensus sequence in promoters, thereby signaling cascades and generating specificity in stress responses [78]. According to Yuan *et al*. [79], NAC TFs play a role in plant immunity through their regulatory impact on signaling of plant hormones SA, JA and ET that, in turn, are key players in plant immune responses. Other authors have demonstrated the induction of *NAC* genes following exogenous application of JA, SA and ET in different species. The research on NAC TFs suggests that some NAC proteins participate in modulating the immune signaling pathways to their critical functions in plant immunity [79].

*My*elo*b*lastosis (MYB) family proteins, one of the best known TFs in plants, have been recognized responsible for many biological functions namely in phenylpropanoid metabolism [80], and in biotic and abiotic stresses [81, 82]. In *P. purpurea*, very high transcript levels were observed at 12 hpc, declining slightly to 24 hpc and increasing to 48 hpc (Fig. 2). The very high transcript levels at 12 and 48 hpc may account for MYB's role in the production of the triterpenoids in the sequence of *Phytophthora* challenge. According to Ambawat *et al*. [81], there are four different classes of MYB proteins, and to the class R2R3-MYB (R2R3) the following functions have been assigned: Primary metabolism, Cell fate and Identity, Secondary metabolism, Developmental processes and Responses to biotic and abiotic stresses. The family of R2R3-MYB-likeTFs has been implicated in JA-dependent defence responses. Mengiste *et al*. [83] have previously considered the interaction of R2R3-MYB-like TFs with JA signaling pathway, mediating the response to signals by reactive oxygen intermediates from biotic as well as abiotic stress. In *P. purpurea* challenged with *P. cinnamomi*, *R2R3-MYB* transcripts are highly up-regulated at 12 and 24 hpc (Fig. 2). According to Javed *et al*. [77], TF families, namely MYB and *AP*ETALA2/*E*THYLENE-*R*ESPONSIVE *F*ACTOR (AP2/ERF), act as regulators of gene expression in plants response to abiotic and biotic stresses. Noman *et al*. [84] have also reported on a MYB TF from *Capsicum annuum* PHL (CaPHL8) as being a positive regulator of pepper defence against *Ralstonia solanacerum* inoculation. MYB and *H*elix-*l*oop-*H*elix (bHLH) TFs are members of two of the largest families of TFs, and they are at the core of the regulation of many plant cellular processes. In challenged *P. purpurea*, the transcript levels profile of bHLH is high at 12 hpc decreasing thereafter (Fig. 1). According to Feller *et al*. [85], bHLH functions in cooperation with MYB domain proteins, forming MBW complexes. The same authors considered that the physical interaction and regulatory synergy between particular sub-classes of MYB and bHLH factors is perhaps one of the best examples of combinatorial plant gene regulation. Members of the MYB and bHLH families also interact with a number of other regulatory proteins, forming complexes that

either activate or repress the expression of sets of target genes [85]. In *P. purpurea*, bHLH present a transcript profile similar to TFs previously discussed (Fig. 1). bHLH regulates gene expression by interaction with specific motif of target gene. bHLH functions as a transcriptional activator that enhances the auto-immunity of *NLR* mutant *snc1* (suppressor of npr1-1, constitutive 1) and confers enhanced immunity in wild-type backgrounds when overexpressed [86]. According to these authors, bHLH84 family TFs, in association with NLRs, activate defence responses, enabling potentially faster and more robust transcriptional reprogramming upon pathogen recognition. The number of TFs over-expressed after pathogen challenge accounts for the existence, in *P. purpurea* challenged by *P. cinnamomi*, of a transcriptional network impacting selective signaling pathways and expression of several target genes underlying plant immunity. Taking this knowledge into account, it is likely that MYB and bHLH may interact at the first hours of challenge of *P. purpurea* with *P. cinnamomi* playing a role in hormone signalling and response to the stress imposed by the oomycete.

In challenged *P. purpure*, bZIP encoding genes present a very high transcript level at 12 hpc, decreasing thereafter to about a fourth, and a fifth of the initial log2FoldChange value. *ZIP* transcripts decrease from 12 to 24 hpc, increasing to a maximum at 48 hpc (Figs. 1 and 2, respectively). Plant bZIP proteins have been reported to be involved in stress signaling. Being one of the largest families of transcriptional regulators, the basic region/leucine zipper motif (bZIP) TFs have been systematically characterized in many higher plants. According to Wei *et al*. [87], the functions of plant bZIP proteins may be more complex than those of many other TFs. Jakoby *et al*. [88] have pointed out that *bZIP* genes from the group D confer resistance against diseases and are involved in transduction of different systemic signals (SA and ET) in response to pathogen infection. Previously, Alves *et al.* [89] have emphasized the role of bZip proteins during pathogen attack and considered them as key components of the signal transduction pathway. In a recent work, Liu *et al*. [90] suggested that *O. sativa* bZIP (OsbZIP81) may positively regulate JA levels and may play a role in pathogen resistance. According to these authors, OsbZIP81 might directly regulate pathogenesis-related (PR) proteins and the enzymes involved in JA synthesis, to positively affect endogenous JA and SA, which may enhance the resistance to pathogens. In challenged *P. purpurea*, the SA transcript levels show a profile similar to that observed for *bZIP*, very high at 12 hpc, decreasing thereafter, to negative values.

ANKYRIN (*ANK*) presents high transcript values at 12 hpc, decreasing the log2FoldChange to nearly half at 24 hpc and increasing at 48 hpc to values identical to those of 12 hpc (Fig. 2). Vo *et al*. [91] have correlated bZIPTFs with the ANK domain that plays a primary role in protein-protein interaction to stabilize the protein complex. According to these authors, ANK domain repeating protein complexes may act at different cellular levels, namely as: effector perception at the plasma membrane level, signal transduction in the cytosol, as well as on transcriptional expression of different defence genes in the nucleus. ANK1 interacting with the bZIPTF bZIP-1 regulates hypersensitive reaction. A vast number of ANK proteins discovered have been considered to play a role in plant defence to pathogens.

The TFs here discussed present higher transcript levels at 12 hpc decreasing thereafter to 48 hpc with exception to MYB and ZIP that decrease to 24 hpc increasing again to 48 hpc (Fig. 2). In what concerns the TFs NAC, AP2 and bZIP, since all of them are implicated in hormone signaling, the results reported for *P. purpurea* suggest that very early after challenge (12 hpc, Fig. 1), they may play a major role in hormone signaling, a step following the perception of the pathogen.

3.11 SALICYLATE, JASMONATE and ETHYLENE

Phytohormones are signaling molecules that regulate major steps of plant immunity [92]. Phytohormones including SA, JA, ET, abscisic acid (ABA), gibberellic acid (GA), auxin, cytokinin and brassinosteroid (BR) have been involved in plant immunity. They form complex interwoven phytohormone signaling networks to coordinate diverse stress responses and growth. According to Pieterse *et al*. [93], SA, JA and ET are core immune phytohormones. Phytohormone biosynthesis is regulated during immunity, and the produced phytohormones are perceived by receptors that transduce signals to transcriptional complexes. These processes are governed by core TFs in each signaling cascade and the hormonal crosstalk is regulated by TFs. Upon microbial attack, plants modify the relative abundance of these hormones as a defence mechanism that further can activate defence responses at the molecular level and enable plant survival. *P. purpurea* plants challenged with *P. cinnamomi*, a hemibiotrophic pathogen [94], present the highest transcript level of JA at 12 hpc, becoming down-regulated at 48 hpc (Fig. 1). The transcript levels at 12 and 24 hpc of JA are closely accompanied by LIPOXYGENASE (Fig. 1). In fact, this enzyme is a key enzyme in the synthetic pathway of oxilipins that are in the base of JA biosynthesis. A similar transcript profile has been observed for SA, although the log2FoldChange values at 12 hpc are around 3 times those of JA (Fig. 1). According to Epple *et al*. [95], JA activates transcription of genes coding for proteases inhibitors (defensins and basic PRs) while SA plays a role in the expression of acidic proteins, acting on systemic acquired resistance (SAR). SA has been involved in the activation of defence responses against biotrophic and hemibiotrophic pathogens, as well as in SAR. Inducible defence against leaf-chewing insects and necrotrophic microbes is mediated by JA-dependent signaling [96]. Resistance to biotrophic fungi, in plants, has been reported to be dependent on SA [97]. SA is known to play an important role in the recognition of the pathogen while JA and ET may act as signaling molecules. The higher values of SA transcripts, compared to JA ones, may be related to the involvement of SA in the regulation of immunity against hemibiotrophic pathogens like *P. cinnamomi*. The lower transcript values of JA and ET could be understood as a lower involvement of these hormones in regulation of immunity to biotrophic fungi. In fact, they are considered regulators of resistance to necrotrophic and insect pests [93].

According to Di *et al*. [98], the SA and ET signaling pathways appear to act synergistically, since a ET pathway is required for the induction of a SA marker gene, and *vice versa*. The transcript values recorded for challenged *P. purpurea* account for a very early over-expression of SA and JA that may function as signaling molecules. Guerreiro *et al*. [99] have demonstrated the involvement of JA and SA in grapevine resistance against the oomycete *P. viticola*. Activation of complex phytohormone signaling networks is a universal defence response employed by plants [36]. According to these authors a network of regulatory interactions may occur among the different defence signaling pathways, in particular between the SA and JA pathways.

Guo *et al*. [100] have demonstrated an interplay between FER, NAC and JA to achieve plant immunity in *Arabidopsis*. Although JA may play a minor role in plant immunity to biotrophic fungi, its interaction with FER and NAC may contribute to achieving immunity. In *P. purpurea*, the transcript levels of FER and NAC are lower than those of SA and higher than those of JA, although the transcript profile is very similar and SA and JA are down-regulated at 48 hpc (Fig. 1).

ETHYLENE-RESPONSIVE FACTOR 8 (ERF8) is a member of one of the largest TF families in plants, the AP2/ERF superfamily that has been considered as an immunity mediator [101]. According to these authors, this AP2/ERF superfamily plays an important role in enhancing resistance against the

hemibiotrophic bacteria *P. syringae*. The AP2 domain transcription factor activates expression of JA regulated defence genes, and enhances resistance of *Arabidopsis* to the fungal pathogen *Fusarium oxysporum* [102]. *P. purpurea* challenged with *P. cinnamomi* presents transcript profiles of *ERF* (high at 12 hpc, decreasing thereafter until 48 hpc) like that of *AP2* (log2FoldChange transcript values around the double of that of *ERF* through the time schedule under study) (Fig. 1). The data reported for *P. purpurea* suggest a role of these proteins in the very early recognition of the oomycete *P. cinnamomi* by the plant, enhancing its immune response. Previously, Huang *et al*. [103] have reported the involvement of ERF in *Arabidopsis thaliana* immunity. Li *et al*. [104] pointed out that ERF TFs are associated with hormone signal transduction of SA, JA, ET and pathogenesis-related (PR) proteins via binding to the GCC box of target genes that positively or negatively regulate transcription of various stress responses. According to Thirugnanasambantham *et al*. [105], the expression of ERFs may be ET dependent or independent and is regulated by a feedback mechanism.

3.12 BTB, NPT, GMP, GH3, BRASSINOSTEROIDS

The transcript levels of SA are followed by those of *BTB* (Figs. 1 and 2) until 24 hpc, although the transcript values of *BTB* are lower than those of SA. Zhang *et al*. [106] have reported the transcriptional up-regulation of BTB/POZ domain in response of soybean to *Phytophthora sojae.* According to these authors, overexpression of Gm*BTB*/*POZ-OE* in soybean plants shows enhanced resistance to *P. sojae*. In *P. purpurea*, the similar expression profiles and the high expression levels of *BTB* and of *PEROXIDASE* transcripts, mainly at 12 hpc (Fig. 2), suggest a coordinated action of both as antioxidants in protecting *P. purpurea* from *P. cinnamomi* invasion, as suggested by Zhang *et al*. [106] for soybean. The same authors have also demonstrated that the resistance to *P. sojae* induced by Gm*BTB*/*POZ* depends on the SA pathway. In accordance with the results of previous authors, the transcript profile of SA in *P. purpurea* presents the highest values at 12 hpc, decreasing thereafter to negative values at 48 hpc, while *BTB* increases from 24 to 48 hpc which suggests its antioxidant role at the first hours of *P. purpurea* challenged by *P. cinnamomi*.

The transcript profile of at1g69870-like (Fig. 1), which corresponds to the *NPF*2.13 gene from *Arabidopsis*, and codes for proteins of the nitrate/peptide transporter (NRT1/PTR) FAMILY (NPT), is very similar to that of JA. Although there is little understanding of the functions of these proteins, some authors have suggested their role as transporters of JA [107]. It is interesting to notice the similarity of the transcript profiles of SAand those of JA and NPT whose values drastically decrease from 12 hpc until 48 hpc (Fig. 1).

According to Isner *et al*. [108], plant hormones, and in particular, JA signaling depends on the activity of cGMP that is involved in JA signal transduction. In *P. purpurea*, the transcript profiles of JA and *GMP* present the same tendency, the highest values at 12 hpc, decreasing to very low values at 48 hpc (Fig. 1).

The transcriptome analysis of challenged *P. purpurea* revealed a high transcript level of acyl acid amido synthetases of the Gretchen Hagen 3 protein family (GH3) at 12 and 24 hpc, decreasing thereafter to 48 hpc, a profile following that of JA, SA and ERF (Fig. 1). Westfall *et al*. [109] have suggested that GH3 plays a role in conjugating several molecules in these phytohormone pathways, probably mediating a crosstalk between auxin production and response to pathogen attack. Recently, Zou *et al.* [110] have demonstrated that GH3 plays an important role in defence of *Citrus sinensis* against *Xanthomonas citri* by down-regulation of the auxin IAA at the same time as they up-regulate biotic stress related functions and pathways. The same authors have pointed out that

levels of SA and ET involved in plant resistance responses markedly increased in the transgenic plants overexpressing *CsGH3.1* and *CsGH3.1L*, reducing plant susceptibility to *Citrus* canker by repressing auxin signaling and inhibiting the accumulation of active auxin, thus enhancing defence responses.

In *P. purpurea*, *BRASSINOSTEROIDS* (*BRs*) transcript level shows at 12 hpc, a log2FoldChange value around 3, decreasing thereafter to present values lower than 1 at 48 hpc (Fig. 1). BRASSINOSTEROIDS (BRs) are known to show modulatory effects upon growth and immunity [111]. Interaction between BRs and SA revealed an important role in alleviating biotic as well as abiotic stresses [112]. In *P. purpurea*, the higher transcript levels of BRs at the first hours of challenging with *P. cinnamomi* suggest their role in modulation of *P. purpurea* immunity.

3.13 SUBTILISIN

SUBTILISIN-LIKE PROTEASES, also named subtilases, are a very diverse family of serine proteases present mostly in plants. In *Lycopersicon esculentum*, Vera and Cornejero [113] have reported the accumulation of SUBTILASE P69 in tomato leaves infected with *Citrus exocortis.* Since then, a huge amount of research has been developed aiming at understanding the role of subtilases in regulation of plant pathogen interactions. Plant subtilases present a large spectrum of biological function, including response to biotic and abiotic stresses (for a review see Schaller *et al*. [114]). In *P. purpurea* challenged with *P. cinnamomi*, the *SUBTILISIN* transcript levels decrease from 12 to 24 hpc, increasing again to 48 hpc, though they maintain high vlaues (Fig. 2). Ramírez *et al*. [115] have identified an extracellular SUBTILASE in *Arabidopsis* and reported that this SUBTILASE (SBT3.3) enhances innate immune response, while loss of function enhances susceptibility. According to the same authors, SBT3.3 is required for expression of SA responsive genes. Figueiredo *et al*. [116] have stated that several SUBTILISIN-LIKE proteases are associated to plant-pathogen resistance, and that they may play important roles both in pathogen recognition and in initiation of signaling cascades, leading to the activation of defence-related genes. Ramírez *et al*. [115] reported that SUBTILASES may be linked to immune priming events. They also pointed out that expression of SBT3.3 is rapidly demanded during the activation of innate immunity, preceding the activation of SA responsive genes and responding very rapidly to H_2O_2, a common ROS species generated very early during PAMP recognition by PRRs, leading to activation of innate immunity. So, SBT3.3 represents a new regulator of primed immunity.

The role of SUBTILISIN in defence of *Solanum* sp. against *P. infestans* has been recently investigated by Wang *et al*. [117]. According to these authors, in plant-microbe interactions, the cysteine-rich protein PC2 secreted from the potato late blight pathogen *P. infestans* induces immunity in *Solanum* plant species, which occurs only after cleavage by plant apoplastic SUBTILISIN-LIKE proteases, such as tomato P69B. The plant protease cleaves a pathogen apoplastic protein to activate defence responses achieving immunity, while the pathogen deploys protease inhibitors as a counter-defence measure to evade recognition by the host. In *P. purpurea*, the high transcript level of SUBTILISIN from 12 to 48 hpc may account for the role of this protein in the cleavage of *P. cinnamomi* proteases released by the pathogen.

3.14 CYTOCHROMES (CYPs)

In challenged *P. purpurea*, *CYTOCHROMES* transcripts exhibit log2FoldChange values higher than 4 along the time schedule under study (Fig. 2). According to Xu *et al*. [118], CYPs participate in the regulation of JA biosynthesis for plant defence. According to Park *et al*. [119], allene oxide synthase (AOS), which catalyzes the dehydration of

hydroperoxide to an instable allene oxide in the JA biosynthetic pathway, plays an important role in wound-induced defence against biotic attacks. In a recent review, Pandian *et al*. [120] have postulated that as a response to environmental stresses, several *CYP* genes are expressed. The same authors provided an overview on the different roles of CYP 450 in plants and emphasized the important role of CYPs in the crosstalk between abiotic and biotic stress responses. Previously, Yan *et al*. [121] reported the induction of *GmCYP82A3*, a gene from soybean CYP82 family, after *P. sojae* infection, salinity, drought stresses, and treatment with methyl jasmonate (MeJA) or ethephon (ETH). The same authors have observed consistent CYP high expression levels in resistant cultivars. According to the same authors, over-expression of *GmCYP82A3* in *N. benthamiana* plants induced strong resistance to *Botrytis cinerea* and *P. parasitica.* The enhanced resistance of transgenic plants was accompanied with increased expression of the JA/ET signaling pathway-related genes [121]. The high overexpression of CYTOCHROMES in *P. purpurea* may well correlate with the innate immunity of this plant to *P. cinnamomi.*

3.15 PENTATRICOPEPTIDE

PENTATRICOPEPTIDE REPEAT (PPR) proteins constitute one of the largest protein families in land plants, most species possessing more than 400 members. In challenged *P. purpurea,* the transcript abundance of this gene is very high at 12 hpc, and decreasesuntil 48 hpc, the log2FoldChange remaining higher than 2.5 at this last time point (Fig. 1). According to Barkan and Small [122], a PPR protein targeted to mitochondria or chloroplasts binds one or several organellar transcripts and influences their expression by altering RNA sequence, turnover, processing, or translation. Their combined action has tremendous effects on organelle biogenesis and function and, consequently, on photosynthesis, respiration, plant development and environmental responses. In poplar (*P. trichocarpa*) genome, Xing *et al*. [123] have reported the molecular mechanisms of these *PtrPPR* genes in response to environmental stresses. According to these authors, poplar genome-wide transcriptomic analysis revealed that 154 of the *PtrPPR* genes are induced by biotic and abiotic treatments, including *Marssonina brunnea* infection, SA, or methyl jasmonate (MeJA). PPR proteins are among the nucleus-encoded chloroplast RNA-binding proteins (RBPs), and most of the chloroplast PPR proteins characterized, so far, participate mainly in the stabilization of mRNAs [124]. The same authors pointed out that PPR287 affects the level of chloroplast rRNAs, which is essential for chloroplast biogenesis and function. Since an nortriterpenoid (phlomispurpentaolone) was shown to be constitutively produced by roots, and to exhibit anti-*Phytophthora* activity [19], the up-regulation of the gene coding for these PPR proteins may account for the maintenance of chloroplasts integrity following pathogen infection, which is essential for the accomplishment of the biosynthetic pathway of the triterpenoid compound, that after excretion from *P. purpurea* roots to the rhizosphere, acts as a cytotoxic compound against *P. cinnamomi* present in the soil.

3.16 HEAT

Plants possess several tandem repeats containing proteins belonging to different families. HEAT repeat protein domain has been suggested to bind multiple ligands to establish a rapid response to external stimuli, namely biotic and abiotic stress. In *Arabidopsis*, Monaghan and Li [125] identified a HEAT repeat protein domain (ILA) responsible for innate immunity against *P. syringae*. The high transcript levels of HEAT in challenged *P. purpurea* along the three time points after challenge, its log2FoldChange value attaining 6 at 48 hpc (Fig. 2), account for its role in enhancing the innate immune

response to *P. cinnamomi* challenge by interaction with other repeat containing proteins. According to Sharma and Pandey [126], in plants, most repeat family proteins are associated with different domain arrangements which could be correlated with the response to stress adaptation and tolerance.

3.17 Retrotransposons

Retrotransposons comprise the most common class of transposable elements (TEs) in eukaryotes and represent up to 90% of plant genomes [127]. The transcriptome analysis of *P. purpurea* challenged with *P. cinnamomi* revealed very high values for the general category of retrotransposons (increasing from more than 4 to more than 5 along the three time points studied). To McClintock [128], transcriptional elements can be considered as motors of adaptative genetic changes following stress and play an important role in the genome response to environmental signals. According to Grandbastien [129], the transcriptional activation of several well-characterized plant retrotransposons is tightly linked to molecular pathways activated by stress and this activation is under the control of *cis*-regulatory sequences strikingly similar to those of plant defence genes. Transposable DNAs are important coordinators in many biological processes such as maintenance of chromosome integrity and creation of novel regulatory networks [130]. More recently, Lai and Eulgem [131] emphasized the contribution of transposable elements to the diversification of disease resistance genes, which are among the fastest evolving genes. In *Arabidopsis*, Zervudacki *et al*. [132] demonstrated a link between response of a retroelement to biotic stress and its co-option for immunity regulation. Pouteau *et al*. [133] have reported that the TE (Tnt1) transcript accumulates in response to microbial elicitors from *Trichoderma viride* and *Phytophthora* spp. The high trancript levels of retrotransposons, increasing along the 3 time points, suggest that challenging *P. purpurea* with *P. cinnamomi* may induce transcription of retrotransposons as reported by Pouteau *et al*. On this subject, it is reasonable to continue following McClintock [128] according to whom a goal for the future would be to determine the extent of knowledge the cell has of itself, and how it utilizes this knowledge in a "thoughtful" manner when challenged. Much work has to be done in the future in order to understand the precise role of retrotransposons' transcription in plant immunity. Many other transcripts related to secondary metabolism and vesicle trafficking have been detected but will not be considered in this paper.

4. Conclusions

The top-down approach of *Phlomis purpurea* transcriptome after challenging with *Phytophthora cinnamomi*, allowed the identification of a gene network accounting for the innate immunity of this plant against *P. cinnamomi.* The analysis of *P. purpurea* transcripts selected illustrates the complexity of molecular interactions and the crosstalk among the different proteins and corresponding genes expressed since pathogen recognition. The data obtained support the assumption that *P. purpurea* presents innate immunity against *P. cinnamomi*. The analyses of these transcripts bring new insights into the coordinated co-expression of genes related to plant immunity, providing a comprehensive understanding of the complex network underlying plant defence responses.

In summary, the up-regulation of WAKs accounts for their role in pathogen detection through the external binding domain and in signaling through its internal kinase domain. Hormones (SA, JA and ET) combined with G-proteins, calcium, CaM and CRT, all of them presenting a characteristic profile along the time scale, may act together with several transcription factors, and may function in signal transduction that will be at the base of the defence response involving the correct functioning of plastids and endoplasmic reticulum/golgi complex, followed by vesicle

trafficking for exocytosis of phlomispurpentaolone through the cell wall. Taken together, these results show the complexity of the interplay of a set of genes and the corresponding encoded proteins responsible for the innate immunity of *P. purpurea*, initiated as soon as the plant recognizes the cinnamomins released by the pathogenic oomycete *P. cinnamomi*. Multiple mechanisms may be at the base of *P. purpurea* innate immunity. The set of genes discussed allowed us to recognize the following categories: genes regulating signal perception; defence-related signal components; transcription factors regulating gene expression; genes regulating secondary metabolite production. Transcription factors (TFs) in particular, remain potential genomic candidates for wide application in crop breeding, to enable plants to withstand unfavorable conditions.

The knowledge gathered from this transcriptomic analysis may contribute to deciphering the interplay among the different components of the immune system and their use in breeding programs for crop improvement. Improving our understanding on the mechanism(s) of pathogen infection and on host/pathogen interactions may open new opportunities for defining strategies to maximize the control of pathogen infection and minimize consequent crop yield losses. New knowledge is becoming available for use from both host and pathogen viewpoints; the challenge now, is to transfer this new knowledge from the laboratory into the field in order to breed resistant cultivars through introgressions supported by genomic approaches, prospects that can be implemented in the future to enable better disease management. Although knowledge on the complex phenomenon of plant immunity has drastically increased, studies have to be continued in order to enable a precise understanding of the crosstalk among plant receptors, signal transducers, transcription factors, gene expression and metabolites production.

Research on plant immunity will continue to be driven by the need to overcome disease pressures associated with forestry and agricultural practices and climate change. Knowledge on receptors, as initiators of stress responses, and on transcription factors, as well as on retrotransposons, may be on the forefront of efforts to develop crop resistance. Such discoveries may constitute important tools for engineering resistance to pathogens as well as promising targets for marker-assisted selection and breeding crops by design, using new technologies namely the CRISPR/Cas9 system. The data reported will help to promote sustainability of modern agriculture which undoubtedly, will benefit from increased productivity, while preserving the environment. Further research on the coordinated network encompassing innate immunity in *P. purpurea* will be of great interest towards incorporating knowledge on plant immunity into disease management programmes.

Author Contribution

M.S.S.P. and A.C. conceived and designed the research framework; A.B.performed the experiments; M.S.S.P. and A.C. analyzed the data and wrote the manuscript; A.C. and M.S.S.P. supervised the work and finalized this manuscript.All authors read and approved the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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