Transcriptional responses of *Hypericum perforatum* cells to *Agrobacterium tumefaciens* and differential gene expression in dark glands

Weina Hou ^{A, B}, Rupesh Kumar Singh ^C, Viviana Martins ^B, Francisco Tenllado ^{D,*}, Gregory Franklin ^A, Alberto Carlos Pires Dias ^{A, B, E, *}

^A Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), Department of Biology, University of Minho, 4710-057, Braga, Portugal

^B Centre of Molecular and Environmental Biology (CBMA), Department of Biology, University of Minho, 4710-057, Braga, Portugal

^C Centro de Química de Vila Real (CQ-VR), UTAD, 5000-801, Vila Real, Portugal

^D Departamento de Biotecnología Microbiana y de Plantas, Centro de Investigaciones Biológicas Margarita Salas, CSIC, Madrid, 28040, Spain

^E Center of Biological Engineering (CEB), University of Minho, 4710-057, Braga, Portugal

*Correspondence: Email: tenllado@cib.csic.es; phone number: 34-91 837 31 12; acpdias@bio.uminho.pt.

Abridged title: Gene expression elicited by *Agrobacterium* in *H. perforatum*

Summary Text for the Table of Contents:

Hypericum perforatum L., known as St. John's wort, is a medicinal plant with anti-depressant, anti-inflammatory, and anti-cancer properties. *H. perforatum* is recalcitrant to transformation by *Agrobacterium tumefaciens*, which limits metabolic engineering studies. We explored the expression profiles of key genes involved in several plant defense responses to get insights into the molecular basis of this recalcitrance. Our results suggest that secondary metabolites could impair pathogen's growth, making *H. perforatum* recalcitrant to *A. tumefaciens*-mediated transformation.

Abstract

Hypericum perforatum L. is a well-known medicinal plant which possesses secondary metabolites with beneficial pharmacological properties. However, improvement in the production of secondary metabolites via genetic manipulation is a challenging task as *H. perforatum* remains recalcitrant to *Agrobacterium tumefaciens*-mediated transformation. Here, the transcripts of key genes involved in several plant defense responses (secondary metabolites, RNA silencing, reactive oxygen species (ROS) and specific defense genes) were investigated in *H. perforatum* suspension cells inoculated with *A. tumefaciens* by quantitative real-time RT-PCR. Results indicated that key genes from the xanthone, hypericin and melatonin biosynthesis pathways, the ROS-detoxification enzyme *HpAOX*, as well as the defense genes *Hyp-1* and *HpPGIP*, were all up-regulated to rapidly respond to *A. tumefaciens* elicitation in *H. perforatum*. By contrast, expression levels of genes involved in hyperforin and flavonoid biosynthesis pathways were markedly down-regulated upon *A. tumefaciens* elicitation. In addition, we compared the expression patterns of key genes in *H. perforatum* leaf tissues with and without dark glands, a major site of secondary metabolite production. Overall, we provide evidence for the up-regulation of several phenylpropanoid pathway genes in response to elicitation by *Agrobacterium*, suggesting that production of secondary metabolites could modulate *H. perforatum* recalcitrance to *A. tumefaciens*-mediated transformation.

Additional keywords: Polygalacturonase-inhibiting protein, Phenolic oxidative coupling protein, alternative oxidase, plant defence

Introduction

H. perforatum L.(St. John's wort), is a well-known medicinal plant with a history of more than 2000 years distributed world-widely. *H. perforatum* has attracted attention due to its pharmacological properties like anti-depressant, anti-inflammatory, anti-bacterial, anti-viral, and anti-cancer(Saddiqe *et al.* 2010). A wide range of biologically active secondary metabolites has been detected in this species, including hypericin, hyperforin, xanthones, essential oils, phenolic acids, and a broad array of flavonoids (Karppinen 2010; Saddiqe *et al.* 2010). Several of these biologically active components are highly accumulated in secretory structures known as dark glands, which are present in leaf margins, stems, flower petals and stamens of *H. perforatum* (Zobayed *et al.* 2006; Karppinen and Hohtola 2008; Beerhues 2011). *H. perforatum* remains recalcitrant to *Agrobacterium tumefaciens*-mediated transformation (Hou *et al.* 2016). Moreover, increased accumulation of important bio-compounds was observed upon co-cocultivation of *H. perforatum* cell suspension cultures with *A. tumefaciens* (Franklin *et al.* 2009). These findings suggested that the production of antimicrobial secondary metabolites may underlie the *H. perforatum* recalcitrance to *A. tumefaciens*-mediated transformation (Franklin *et al.* 2008; 2009; Hou *et al.* 2016).

Plant secondary metabolites have an essential role in defense against pathogens (Goyal *et al.* 2012). The major active secondary metabolites in *H. perforatum*, i.e., flavonoid, xanthone, hyperforin and hypericin, are polyketide derivatives, and the key reactions of their biosynthesis pathways are catalyzed by type III polyketide synthases (PKSs) (Beerhues 2011; He *et al.* 2012). To date, five type III PKS proteins have been reported from genus *Hypericum*, including chalcone synthase (CHS), benzophenone synthase (BPS), octaketide synthase (OKS), PKS1 and PKS2 (Karppinen and Hohtola 2008; He *et al.* 2012). Phenylalanine ammonia-lyase (PAL) as the first committed enzyme catalyzes the vital step of the general phenylpropanoid pathway at the link between primary and secondary metabolism (Gaid *et al.* 2012; Klejdus *et al.* 2013). Besides, CHS, a superfamily of PKSs, catalyzes the first committed step in the flavonoid biosynthesis pathway (Zhou *et al.* 2013; Meng *et al.* 2015). The biosynthesis of xanthone also occurs through the phenylpropanoid pathway and BPS, another superfamily of PKSs, is employed as the critical enzyme to catalyze the synthesis of the carbon skeleton of xanthone (Liu *et al.* 2003; Belkheir *et al.* 2016). In previous studies, it was reported the increased production of xanthone as well as

the transcriptional upregulation of *PAL*, *4CL* (4-coumarate:CoA ligase) and *BPS* in *H. perforatum* cell suspension cultures in response to elicitation by *A. tumefaciens* (Franklin *et al.* 2009; Hou *et al.* 2016). OKS is considered to form the precursor of hypericin in *H. perforatum*. *In situ* hybridization assays confirmed the role of PSK2 in the synthesis of hypericin in dark glands of *H. perforatum* (Karppinen and Hohtola 2008; Karppinen *et al.* 2008). Expression of PKS1 was highly correlated with hyperforin synthesis in *H. perforatum* (Karppinen and Hohtola 2008). Melatonin belongs to another major group of secondary metabolites, and is considered to act as an antioxidant or growth promoter in *H. perforatum*. A putative melatonin biosynthesis pathway including phosphoribosylanthranilate transferase (PAT) and indole-3-glycerol phosphate synthase (IGPS) has been described (He *et al.* 2012).

~~Plant defense against *A. tumefaciens* is a multifaceted response, which includes not only secondary metabolites, but also the small RNA-mediated gene silencing pathway (Dunoyer *et al.* 2006; Peláez and Sanchez 2013). RNA silencing is induced by double-stranded RNA (dsRNA) which is subsequently processed by dsRNA-specific RNase III enzyme (Dicer-like protein) into 21-25 nucleotide long small interfering RNAs (siRNAs). Then, the siRNAs are recruited into the RNA-induced silencing complex, including ARGONAUTE (AGO) proteins, to guide the sequence-specific degradation of complementary RNAs. In plants, RNA-dependent RNA polymerases (RDRs) have been reported to generate secondary siRNAs in order to amplify defense signals (Li *et al.* 2017). In the RDRs-mediated RNA silencing pathway, Suppressor of Gene Silencing 3 (SGS3) is required to convert single-stranded RNA to dsRNA to produce siRNAs (Li *et al.* 2017).~~

In our previous studies, two defense genes, *Phenolic oxidative coupling protein* (*Hyp-1*) and *Polygalacturonase-inhibiting protein* (*PGIP*) were selected from a forward subtractive cDNA library from *H. perforatum* cell suspension cultures elicited with *A. tumefaciens*. Overexpression of *Hyp-1*, a pathogenesis-related (PR) class 10 family gene, in tobacco and lettuce greatly decreased *Agrobacterium*-mediated expression efficiency in transgenic plants (Hou *et al.* 2020a). HpPGIP belongs to the leucine-rich repeat (LRR) superfamily that specifically recognizes and inhibits polygalacturonase, a cell wall degrading enzyme secreted during infection by pathogenic microorganisms (Meshram and Srivastava 2013). The role of *HpPGIP* in defense against *A. tumefaciens* was analyzed in transgenic tobacco. Results showed that overexpression of *HpPGIP*

alone and fused with *Hyp-1* reduced *Agrobacterium*-mediated expression efficiency in transgenic plants (Hou *et al.* 2020b). These findings suggest that both *Hyp-1* and *HpPGIP* modulate recalcitrance to *A. tumefaciens*-mediated transformation in *H. perforatum*.

Defense responses in *H. perforatum* against *A. tumefaciens* probably involve multiple pathways and activation of several genes that remain yet to be fully understood. In this study, the transcripts of core genes involved in the different defense responses described above, as well as genes from the small RNA-mediated gene silencing pathway, *ARGONAUTE* (*AGO*), *RNA-dependent RNA polymerases* (*RDR6*) and *Suppressor of Gene Silencing 3* (*SGS3*) (Dunoyer *et al.* 2006; Li *et al.* 2017; Peláez and Sanchez 2013), and the reactive oxygen species (ROS)-detoxification enzymes alternative oxidase (*AOX*) and catalase (*CAT*) (Velada *et al.* 2016), were analyzed by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) in *H. perforatum* cell suspensions challenged with *A. tumefaciens*. In addition, gene expression was analyzed in dark glands obtained from *H. perforatum* seedlings to further understand the biogenesis of secondary metabolites and their involvement in the plant defense responses.

Materials and methods

Plant material

Seeds of *H. perforatum* variety “Helos” (Richters seeds, ON, Canada) were put in Eppendorf tubes of 2.0 mL containing 1.0 mL sterile water with 10 μ L of Tween[®] 20 (Sigma-Aldrich, USA) and kept at 4°C in the dark. After 4 h, the solution was removed and the seeds were washed four times with sterilized distilled water followed by 6% active chlorine for 3 min followed by washing in sterilized distilled water. Seeds were germinated on 1/2 MS medium containing 1% agar. Seedlings were grown for two weeks and samples of leaf margins with dark glands were collected and denoted as with dark glands (*HpWith*), while middle part of leaf served as without glands (*HpWithout*). All steps were conducted in a laminar flow cabinet, and seeds were germinated and grown in a plant tissue culture room at 25°C under a 16-h light/8-h dark photoperiod. *A. tumefaciens* preparation

A. tumefaciens EHA105 containing recombinant plasmid pCambia1301 was incubated in 200 mL Luria-Bertani (LB) medium containing antibiotic at 28°C overnight until optical density (OD)₆₀₀=0.6, then 200 μ M acetosyringone (AS) was added. After the gently centrifuge, the pellet

of the *A. tumefaciens* was suspended in 2 mL Murashige and Skoog (MS) medium with OD₆₀₀=1.0 as the bacterium stock for next step.

Elicitation of H. perforatum cells with A. tumefaciens

H. perforatum cell suspensions were elicited with *A. tumefaciens* and the details of the experimental procedure were described previously (Franklin *et al.* 2008). Briefly, *H. perforatum* cell suspension cultures were established from stem segments of the variety “Helos” (Richters seeds, ON, Canada) as described (Dias *et al.* 2001). The suspended *H. perforatum* cell suspensions were harvested in a sterile beaker at a final concentration of 5,000 cells mL⁻¹ in 80 mL MS medium containing 100 µM AS, after five days of subculture. Then the flakes of *H. perforatum* cell suspensions were inoculated with the agrobacterium stock. Cell samples were collected after co-cultivation periods of 12 h (Hp-AT 12h) and 24 h (Hp-AT 24h). A control condition corresponding to *H. perforatum* suspensions only, 0 h (Hp-AT 0h), was included. The treatment and control cultures were incubated at 16-h light/8-h dark photoperiod at 25°C in a growth chamber during co-cultivation.

Plant total RNA isolation and first-strand cDNA synthesis

Samples (0.12-0.15g) were grounded in liquid nitrogen and mixed with 0.9 mL of preheated (65°C) extraction buffer containing 2% CTAB (w/v), 2% polyvinylpyrrolidone (PVP) K-30 (w/v), 100 mM Tris HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl and 2% β-mercaptoethanol (v/v). The homogenate was incubated at 65°C for 10 min and 0.9 mL of chloroform/isoamyl alcohol (24:1, v/v) was added into the mixture, vortexed, and centrifuged at 12,000 x g for 15 min at 4°C. The resulting upper aqueous phase was transferred into another tube containing 100 µL 3 M sodium acetate (pH 5.5) and 600 µL isopropanol. The mixture was incubated at -80°C for 30 min, followed by centrifugation at 12,000 x g for 30 min at 4°C. The supernatant was decanted and the pellet was dried and dissolved in 250-375 µL RNase free TE (pH 8.0). Equal volume of 8 M LiCl was added, mixed well and incubated at -80°C for 4-5 h, followed by centrifugation at 13,000 rpm for 30 min at 4°C. The supernatant was decanted and the pellet was rinsed with 75% ethanol and collected by centrifugation at 12,000 x g for 5 min at 4°C. The dried pellet was finally dissolved in 45 µL of RNase-free water and stored at -80°C. To clean up the RNA samples, the RNeasy Mini Kit (Qiagen, Germany) was used according to the manufacturer’s instructions. NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Inc., USA) was used

to quantify total RNA at A260/A280, and 1.0% agarose-gel electrophoresis was employed to check structural integrities of total RNA. Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, USA) was utilized to perform cDNA synthesis by using 1µg DNase-treated RNA, according to the manufacturer's instructions.

Quantitative real-time RT-PCR

qRT-PCR was used to analyze gene expression in 96-well plates with 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis Biodyne, Estonia). For each biological replicate, qRT-PCR reactions were performed in triplicates containing 5x EvaGreen® qPCR master mix (3 µL), 200 nM of each primer, 10x diluted cDNA template (1µL) and nuclease-free water up to a final volume of 15 µL. The CFX96 Real-Time System C1000 Thermal Cycler (Bio-Rad Laboratories, Inc., USA) was used to perform amplification under the following conditions: initial denaturation at 95°C for 15 min, 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 20 s and elongation at 72°C for 20 s. Gene expression was normalized with internal control *glyceraldehyde-3-phosphate dehydrogenase A subunit (GAPDH)* and *18S ribosomal RNA (18S rRNA)* (Jepson *et al.* 2014). Target genes were examined by using gene specific primers (Table 1). A four-fold serial dilution of 1 µL 10x diluted cDNA template was used to determine the efficiency of primers with three dilution points. The data were analyzed by using CFX Manager Software (Bio-Rad Laboratories, Inc., USA). The relative quantification of PCR products was calculated by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

The transcript levels of target genes from *H. perforatum* were compared with controls by One-way ANOVA or Student's t-test using GraphPad Prism 6 (GraphPad Software, Inc., USA). In the figures, the values were marked with asterisks to denote the significance levels compared with control: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; and ****, $P \leq 0.0001$; ns, stands for not significant.

Results

Expression of H. perforatum genes in response to A. tumefaciens elicitation

194 To evaluate the possible involvement of secondary metabolites derived from *H. perforatum* in
 195 the defense response against *A. tumefaciens*, the expression of a series of genes involved in the
 196 synthesis of secondary metabolites was investigated in suspension cells challenged with *A.*
 197 *tumefaciens* by qRT-PCR. The expression of *HpPAL*, the first enzyme of the phenylpropanoid
 198 pathway which participates in the flavonoid and xanthone biosynthetic routes, was increased up
 199 16-fold after 12 h of co-cultivation with *A. tumefaciens*, and returned to basal levels at 24 h (Fig.
 200 1a). The expression of *HpCHS*, a gene involved in the flavonoid biosynthesis pathway, was
 201 down-regulated by 25% after 12 h and further decreased by 77.5% after 24 h (Fig. 1b). The
 202 expression of the xanthone biosynthesis gene *HpBPS* was up-regulated by 29-fold in *A.*
 203 *tumefaciens*-challenged cell cultures after 12 h, but this effect was not observed after 24 h (Fig.
 204 1c). The transcriptional level of *HpPKS1*, which encodes an enzyme in the hyperforin
 205 biosynthesis pathway, was down-regulated significantly by 75% after 12 h and reached its lowest
 206 level, 99% reduction, after 24 h (Fig. 1d). The expression of the hypericin biosynthesis genes
 207 *HpOKS* and *HpPKS2* were both increased up 6.5-fold and 39-fold (Fig. 1e, f), respectively, after
 208 12 h, and later on returned to the same level as control in the case of *HpOKS* and to 4-fold higher
 209 in the case of *HpPKS2* after 24 h. The analysis of two key genes involved in the melatonin
 210 biosynthesis pathway, *HpPAT* and *HpIGPS*, showed that there were no significant differences in
 211 *HpPAT* expression during elicitation by *A. tumefaciens* (Fig. 1g), whereas the transcriptional
 212 level of *HpIGPS* was increased significantly by 85.7% after 12 h co-cultivation with *A.*
 213 *tumefaciens* (Fig. 1h). Graphic representations of the putative biosynthesis pathways of
 214 flavonoids, xanthone, hyperforin, hypericin and melatonin in *H. perforatum* are shown in Fig. 2a.
 215 The relative expression levels of core genes in those secondary metabolic biosynthesis pathways
 216 at 12 and 24 h are indicated using the fold change color scale (Fig. 2b).

217 The small RNA-mediated gene silencing pathway plays a significant role in plant defense against
 218 pathogens. Thus, the expression of several key genes involved in this pathway, namely *HpRDR6*
 219 and *HpSGS3* and *HpAGO5* was evaluated after co-cultivation with *A. tumefaciens*. The results
 220 showed that *HpRDR6* transcripts increased by 25% after 12 h elicitation (Fig. 3a). In contrast,
 221 the expression of *HpSGS3* was significantly down-regulated by 85% and 77.5% after 12 and 24 h,
 222 respectively (Fig. 3b), whereas there was no significant difference in the expression of *HpAGO5*
 223 upon elicitation (Fig. 3c). The relative expression levels of those genes are indicated using the
 224 fold change color scale in Fig. 3d.

ROS signaling is a major process underlying plant response to biotic stress. We monitored the expression of two genes encoding core enzymes involved in ROS homeostasis, *HpCAT* and *HpAOX*, after elicitation by *A. tumefaciens*. The expression of *HpAOX* was up-regulated 2-fold after 12 h of co-cultivation with *A. tumefaciens* (Fig. 4a), while *HpCAT* expression was not significantly affected (Fig. 4b).

Two genes associated with the defense response to *A. tumefaciens* in *H. perforatum*, namely *Hyp-1* and *HpPGIP*, were also monitored by qRT-PCR. Both genes were strongly up-regulated by 24-fold and 9-fold (*Hyp-1*) and 24-fold and 3.8-fold (*HpPGIP*) after 12 h and 24 h elicitation with *Agrobacterium*, respectively (Fig. 4c,d). The fold variation in the expression of these genes is also presented in Fig. 4e.

Comparison of gene expression in H. perforatum tissues with or without dark glands

To gain knowledge on the participation of dark glands in defense responses against pathogens, leaf tissues of *H. perforatum* with (HpWith) or without (HpWithout) glands were collected, and the expression of the target genes studied previously was investigated by qRT-PCR. The expression of *HpPAL* was not significantly different in tissues with and without glands (Fig. 5a). *HpCHS* showed a lower level of expression, 25% reduction, in HpWith compared to HpWithout (Fig. 5b), while *HpBPS* was up-regulated by 5-fold in HpWith compared to HpWithout (Fig. 5c). The hyperforin biosynthesis gene *HpPKS1* accumulated 25% less in gland-containing samples (Fig. 5d); conversely, the hypericin biosynthesis genes *HpOKS* and *HpPKS2* were highly accumulated in HpWith, 99-fold increase, compared to HpWithout (Fig. 5e,f). Moreover, there were not statistical differences in the expression of the melatonin biosynthesis genes *HpPAT* and *HpIGPS* between HpWith and HpWithout (Fig. 5g,h). The fold variation in the expression of all these secondary metabolite biosynthesis genes is also presented in Fig. 5i.

HpRDR6, *HpSGS3* and *HpAGO5*, which are involved in gene silencing pathways, were similarly expressed in tissues with or without dark glands (Fig. S1). Moreover, the ROS homeostasis gene *HpAOX2* was equally expressed in HpWith and HpWithout tissues (Fig. 6a), while *HpCAT* was more expressed, 20% increase, in tissues containing glands (Fig. 6b). Finally, transcripts of the defense genes *Hyp-1* and *HpPGIP* were accumulated in HpWith at higher levels, 20% and 70% respectively, than in HpWithout tissues (Fig. 6c,d). The fold variation in the expression of these genes is shown in Fig. 6e.

Discussion

A. tumefaciens is widely used to transfer genes into plant systems for functional genomics and genetic engineering purposes. However, as a bacterial pathogen, *A. tumefaciens* evokes a series of host defense responses that preclude the genetic manipulation of several important plant species. In particular, *H. perforatum* is recalcitrant to *A. tumefaciens*-mediated transformation. This drawback constitutes a major limitation to improve the production of valuable substances of interest to researchers and the pharmaceutical industry in *H. perforatum* (Hou *et al.* 2016; Saddiqe *et al.* 2010). In this study, we monitored the expression of defense-related genes in *H. perforatum* cells upon co-cultivation with *A. tumefaciens* to get insights into the molecular basis of this recalcitrance. To date, several type III PKS proteins responsible for secondary metabolites production have been reported from the genus *Hypericum*, including CHS, BPS, OKS, PKS1 and PKS2 (Liu *et al.* 2003; Karppinen and Hohtola 2008; Karppinen *et al.* 2008). The transcripts of *HpPAL* and *HpBPS* were found to be strongly up-regulated in *H. perforatum* elicited by *A. tumefaciens*, as revealed by qRT-PCR analysis. The rapidly induced expression of *HpPAL* and *HpBPS* suggested that the xanthone biosynthesis pathway was activated after infection with the pathogen, in agreement with previous data reporting that several xanthones synthesized by *H. perforatum* cells showed antimicrobial activities (Franklin *et al.* 2009). The biosynthetic route leading to hypericin is still poorly understood, however it is presumed to be biosynthesized via a polyketide pathway in which OKS and PKS2 act as key enzymes (Karppinen *et al.* 2010). Hypericin biosynthesis is believed to be regulated by transcriptional control of key enzymes participating in the pathway (Pavlik *et al.* 2007). *In situ* hybridization analysis showed that *HpPKS2* transcripts were localized in leaf margins, flower petals, stamens and dark glands where hypericin accumulated specifically, indicating that *HpPKS2* may play a role in the biosynthesis of hypericin (Karppinen *et al.* 2008). In the present study, the transcript levels of *HpPKS2* and *HpOKS* were significantly up-regulated in *H. perforatum* elicited by *A. tumefaciens*. The accumulation of both transcripts reached a maximum at 12 h after *Agrobacterium* elicitation, and then declined at 24 h. The rapidly induced expression of *HpOKS* and *HpPKS2* indicates that the hypericin biosynthesis pathway is activated immediately after infection with *A. tumefaciens*. Indeed, it was reported the induction of hypericin accumulation in *H. perforatum* in response to the fungi *Colletotrichum gloeosporioides*, *Phytophthora capsici* and *Diploceras hypericinum* (Sirventia and Gibson 2002; Çirak *et al.* 2005).

Melatonin is another major group of secondary metabolites in *H. perforatum*, although its biosynthesis pathway is poorly understood. Nonetheless, a putative melatonin biosynthesis pathway with the participation of nine enzymes including *HpIGPS* has been described (He *et al.* 2012). In the current study, *HpIGPS* was observed to be up-regulated in *H. perforatum* treated with *A. tumefaciens*. The maximum accumulation was observed at 12 h after *Agrobacterium* elicitation, suggesting that the melatonin biosynthesis pathway was activated immediately after the pathogen was sensed by *H. perforatum* cells. A role of melatonin in plant-pathogen interactions has been discussed. Exogenous melatonin treatments improved resistance of *Malus prunifolia* against the fungus *Diplocarpon mali* (Yin *et al.* 2013). In addition, the application of melatonin induced several pathogenesis-related protein genes, as well as a series of defense-related genes, in *Arabidopsis* plants infected with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Lee *et al.* 2014). On the other hand, *HpPKS1* and *HpCHS*, key enzymes involved in the hyperforin and flavonoids biosynthesis pathways respectively, were markedly down-regulated upon *A. tumefaciens* elicitation, suggesting that these secondary metabolites do not contribute to the defense response against *Agrobacterium* in *H. perforatum*.

Intriguingly, the transcripts of two genes, *Hyp-1* and *HpPGIP*, which have been demonstrated to play a role in defense against *Agrobacterium* (Hou *et al.* 2020a,b), were highly expressed upon *A. tumefaciens* elicitation. *In situ* RNA hybridization studies localized the *Hyp-1* transcripts in mesophyll cells and vasculature of *H. perforatum* (Karppinen *et al.* 2016). A structural study revealed that *Hyp-1* was crystallized in complex with melatonin, and concluded that *Hyp-1* may be able to storage and transport melatonin under stress conditions in *H. perforatum* (Sliwiak *et al.* 2016).

ROS signaling is a key player underlying plant response to pathogen attack (Dangl and Jones 2001). The transcript of *HpAOX2*, an enzyme that acts to decrease formation of harmful ROS, was rapidly up-regulated in *H. perforatum* elicited by *A. tumefaciens* at 12 h, indicating that ROS scavenging was activated shortly after infection with the pathogen. Indeed, *H. perforatum* suspension cells showed an intense oxidative burst upon interaction with *A. tumefaciens* (Franklin *et al.* 2008). Besides the ROS-scavenging enzyme systems, xanthonenes and melatonin have been reported to act as powerful antioxidants, thus promoting stress resistance in plants (Franklin *et al.* 2009). In our study, *A. tumefaciens* induced the expression of genes involved in the biosynthesis of xanthone and melatonin (*HpPAL*, *HpBPS* and *HpIGPS*), thus increasing the

accumulation of secondary metabolites that could impair *A. tumefaciens* growth and, on the other side, providing protection to *H. perforatum* against oxidative damage.

RNA silencing is part of the innate immunity system utilized by plants to defense against diverse microbes, including bacteria such as *A. tumefaciens* and *P. syringae* (Dunoyer *et al.* 2006; Peláez and Sanchez 2013). It was reported that an endogenous small interfering RNA was specifically induced by *P. syringae* with the participation of RDR6 and SGS3 in *Arabidopsis* (Katiyar-Agarwal *et al.* 2006). In our study, expression of *HpRDR6* was up-regulated in *H. perforatum* cells challenged with *A. tumefaciens*, within 12 h, while *HpSGS3* was down-regulated at both 12 h and 24 h after elicitation. Besides, AGO5 expression did not vary significantly at any time-point. Our data indicate that diverse RNA silencing pathways might contribute in different ways to the defense responses against *A. tumefaciens* in *H. perforatum*.

To further understand the involvement of dark glands in the biosynthesis of secondary metabolites and defense responses, we compared the expression patterns of the target genes in *H. perforatum* leaf tissues with and without dark glands. The *HpBPS*, *HpOKS* and *HpPKS2* genes responsible for xanthonenes and hypericin production, respectively, were up-regulated in tissues containing dark glands compared to tissues lacking these glands. Similarly, the defense genes *Hyp-1* and *HpPGIP* were also up-regulated in tissues containing glands. By contrast, *HpPKS1*, which catalyzes the first step of the hyperforin biosynthesis pathway, and *HpCHS* involved in flavonoids synthesis, were both down-regulated in tissues containing dark glands. It has been reported that dark glands are the site of preferential accumulation of hypericin in *H. perforatum*, whereas hyperforin accumulates in minute amounts in dark glands compared to other tissues (Soelberg *et al.* 2007; Zobayed *et al.* 2006). Thus, there was a good correlation between the expression profiles of secondary metabolite biosynthesis genes upon *A. tumefaciens* elicitation, up- or down-regulated, and the expression patterns shown by these genes in dark glands. Altogether, these findings suggest that dark glands of *H. perforatum* participate in defense responses against *A. tumefaciens* by the synthesis of secondary metabolites and the defense proteins Hyp-1 and HpPGIP.

In summary, we provide evidence for the up-regulation of several phenylpropanoid pathway genes in *H. perforatum* in response to *Agrobacterium* infection, suggesting that secondary metabolites could impair the growth of the pathogen, making *H. perforatum* recalcitrant to *A.*

tumefaciens-mediated transformation. Our results provide a basis for further elucidating the molecular mechanism of *H. perforatum* recalcitrance, as well as the biogenesis of active compounds in dark glands of this medicinal plant spp.

Acknowledgments

The author acknowledges the financial support provided by the Fundação para a Ciência e a Tecnologia (FCT) project (PTDC/AGR-GPL/119211/2010), to G.F., grant UID/BIA/04050/2013 (POCI-01-0145FEDER-007569), PhD grant (SFRH/BD/52561/2014), under the Doctoral Programme “Agricultural Production Chains – from fork to farm” (PD/00122/2012) to A.C.P., and by Ministry of Economy and Competitiveness of Spain (grant BIO2016-75619-R [AEI/FEDER, UE]) to F.T.

The authors declare no conflicts of interest

References

- Beerhues L (2011) Biosynthesis of the active *Hypericum perforatum* constituents. *Med Aromat Plant Sci Biotechnol* **5**:70–77.
- Belkheir AK, Gaid M, Liu B, Hänsch R, Beerhues L (2016) Benzophenone synthase and Chalcone synthase accumulate in the mesophyll of *Hypericum perforatum* leaves at different developmental stages. *Front Plant Sci* **7**:1–9.
- Çirak C, Aksoy HM, Ayan AK, Sağlam B, Kevseroğlu K (2005) Enhanced hypericin production in *Hypericum perforatum* and *Hypericum pruinatum* in response to inoculation with two fungal pathogens. *Plant Protect. Sci.* **41**:109–114.
- Dangl JL, Jones, JD (2001) Plant pathogens and integrated defence responses to infection. *Nature* **411**:826–833.
- Dunoyer P, Himber C, Voinnet O (2006) Induction, suppression and requirement of RNA silencing pathways in virulent *Agrobacterium tumefaciens* infections. *Nat Genet.* **38**:258–263.
- Franklin G, Conceição LFR, Kombrink E, Dias ACP (2008) *Hypericum perforatum* plant cells reduce *Agrobacterium* viability during co-cultivation. *Planta* **227**:1401–1408.

Franklin G, Conceição LFR, Kombrink E, Dias ACP (2009) Xanthone biosynthesis in *Hypericum perforatum* cells provides antioxidant and antimicrobial protection upon biotic stress. *Phytochemistry* **70**:60–68.

Gaid MM, Sircar D, Müller A, Beuerle T, Liu B, Ernst L, Hänsch R, Beerhues L (2012) Cinnamate: CoA ligase initiates the biosynthesis of a benzoate-derived xanthone phytoalexin in *Hypericum calycinum* cell cultures. *Plant Physiol* **160**:1267–1280.

Galla G, Vogel H, Sharbel TF, Barcaccia G (2015) De novo sequencing of the *Hypericum perforatum* L. flower transcriptome to identify potential genes that are related to plant reproduction sensu lato. *BMC Genomics* **16**:254–275.

Goyal S, Merillon J, Ramawat KG (2012) Secondary metabolites and plant defence. In: Mérillon J., Ramawat K. (eds) Plant Defence: Bi-ological Control. Progress in Biological Control, vol 12. Springer, Dordrecht

He M, Wang Y, Hua W, Zhang Y, Wang Z (2012) De novo sequencing of *Hypericum perforatum* transcriptome to identify potential genes involved in the biosynthesis of active metabolites. *PLoS One* **7**: 1–10.

Hou WN, Shakya P, Franklin G (2016) A perspective on *Hypericum perforatum* genetic transformation. *Front Plant Sci* **7**:1–12.

Hou WN, Singh RK, Zhao P, Martins V, Aguilar E, Canto T, Tenllado F, Dias ACP (2020a) Transgenic expression of *Hyp-1* gene from *Hypericum perforatum* L. alters expression of defense-related genes and modulates recalcitrance to *Agrobacterium tumefaciens*. *Planta*. **251**:13.

Hou WN, Singh RK, Zhao P, Martins V, Aguilar E, Canto T, Tenllado F, Franklin G, Dias ACP (2020b) Overexpression of *polygalacturonase-inhibiting protein (PGIP)* gene from *Hypericum perforatum* alters expression of multiple defense-related genes and modulates recalcitrance to *Agrobacterium tumefaciens* in tobacco. *J Plant Physiol* **253**, 153268.

Jepson C, Karppinen K, Daku RM, Sterenberg BT, Suh DY (2014) *Hypericum perforatum* hydroxyalkylpyrone synthase involved in sporopollenin biosynthesis-Phylogeny, site-

402 directed mutagenesis, and expression in nonanther tissues. *FEBS J* **281**:3855–3868.

403 Karppinen K (2010) Biosynthesis of hypericins and hyperforins in *Hypericum perforatum* L.(St.
404 John's wort)—precursors and genes involved. PhD thesis.

405 Karppinen K, Hohtola A (2008) Molecular cloning and tissue-specific expression of two cDNAs
406 encoding polyketide synthases from *Hypericum perforatum*. *J Plant Physiol* **165**:1079–1086.

407 Karppinen K, Hokkanen J, Mattila S, Neubauer P, Hohtola A (2008) Octaketide-producing type
408 III polyketide synthase from *Hypericum perforatum* is expressed in dark glands
409 accumulating hypericins. *FEBS J* **275**:4329–4342.

410 Katiyar-Agarwal S, Morgan R, Dahlbeck D, Borsani O, Villegas A Jr, Zhu JK, Staskawicz BJ,
411 Jin H (2006). A pathogen-inducible endogenous siRNA in plant immunity. *Proc Natl Acad*
412 *Sci U S A.* **103**:18002-18007.

413 Klejdus B, Kováčik J, Babula P (2013) PAL inhibitor evokes different responses in two
414 *Hypericum* species. *Plant Physiol Biochem* **63**:82–88.

415 Li F, Wang Y, Zhou X (2017) SGS3 cooperates with RDR6 in triggering geminivirus-induced
416 gene silencing and in suppressing geminivirus infection in *Nicotiana Benthiana*. *Viruses*
417 **9**:247–258.

418 Liu B, Falkenstein-Paul H, Schmidt W, Beerhues L (2003) Benzophenone synthase and chalcone
419 synthase from *Hypericum androsaemum* cell cultures: cDNA cloning, functional expression,
420 and site-directed mutagenesis of two polyketide synthases. *Plant J* **34**:847–855.

421 Meng C, Zhang S, Deng YS, Wang GD, Kong FY(2015) Overexpression of a tomato flavanone
422 3-hydroxylase-like protein gene improves chilling tolerance in tobacco. *Plant Physiol*
423 *Biochem* **96**:388–400.

424 Pavlík M, Vacek J, Klejdus B, Kuban V (2007) Hypericin and hyperforin production in St.
425 John's wort in vitro culture: influence of saccharose, polyethylene glycol, methyl jasmonate,
426 and *Agrobacterium tumefaciens*. *J. Agric. Food Chem.* **55**:6147–6153

427 Peláez P, Sanchez F (2013) Small RNAs in plant defense responses during viral and bacterial
428 interactions : similarities and differences. *Front Plant Sci.* **4**:1–16.

429 Saddiqe Z, Naeem I, Maimoona A (2010) A review of the antibacterial activity of *Hypericum*
430 *perforatum* L. *J Ethnopharmacol* **131**:511–521.

431 Soelberg J, Jørgensen LB, Jäger AK (2007). Hyperforin accumulates in the translucent glands of
432 *Hypericum perforatum*. *Annals of botany* **99**:1097–1100.

433 Sirventa T, Gibsonb D (2002) Induction of hypericins and hyperforin in *Hypericum perforatum*
434 L. in response to biotic and chemical elicitors. *Physiological and Molecular Plant*
435 *Pathology*. **60**:311–320.

436 Sliwiak J, DauterZ, Jaskolski M (2016) Crystal structure of Hyp-1, a *Hypericum perforatum* PR-
437 10 protein, in complex with melatonin. *Front. Plant Sci.* **7**:1–10.

438 Velada I, Cardoso HG, Ragonezi C, Nogales A, Ferreira A, Valadas V, Arnholdt-Schmitt B
439 (2016) Alternative oxidase gene family in *Hypericum perforatum* L.: Characterization and
440 expression at the post-germinative phase. *Front Plant Sci* **7**:1–16.

441 Velada I, Ragonezi C, Arnholdt-Schmitt B, Cardoso H (2014) Reference genes selection and
442 normalization of oxidative stress responsive genes upon different temperature stress
443 conditions in *Hypericum perforatum* L. *PLoS One* **9**:1–24.

444 Zhou B, Wang Y, Zhan Y, Li Y, Kawabata S (2013) Chalcone synthase family genes have
445 redundant roles in anthocyanin biosynthesis and in response to blue/UV-A light in turnip
446 (*Brassica rapa*; Brassicaceae). *Am J Bot* **100**:2458–2467.

447 Zobayed SMA, Afreen F, Goto E, Kozai T (2006) Plant-environment interactions: Accumulation
448 of hypericin in dark glands of *Hypericum perforatum*. *Ann Bot* **98**:793–804.

449

450 Table 1 Gene-specific primers used for gene expression analysis by quantitative real-time PCR.

Gene	Primers (5'-3')	References
HpGAPDH	Forward: 5'-ATGGACCATCAAGCAAGGACTG-3' Reverse: 5'-GAAGGCCATTCCAGTCAACTTC-3'	Jepson et al., 2014
Hp18SrRNA	Forward: 5'-AAACGGCTACCACATCCAAG-3' Reverse: 5'-CAACCCAAAGTCCAACACTACG-3'	
HpPAL	Forward: 5'-TGTGAGGCAACGTGTGAATAG A-3' Reverse: 5'-TCCCATAGAAGGACCAAGCAA-3'	Velada et al. 2014
HpCHS	Forward: 5'-GCGCTGCATCGATCATCA-3' Reverse: 5'-CAGCTCGAACAAGGGCTTTT-3'	Velada et al., 2016
HpBPS	Forward: 5'-TGCTCTACAATCAGGGGTGC-3' Reverse: 5'-CGCATGGAAAGTCATGGCAG-3'	This study
HpPKS1	Forward: 5'-TGTACGTCTCATCCAGTGAGC-3' Reverse: 5'-ACACCACCGTAACAGCCTAAG-3'	Jepson et al., 2014
HpPKS2	Forward: 5'-TACACAAGGAGACACCCAAGC-3' Reverse: 5'-GGAAGTGGCAACATAGCATCC-3'	K Karppinen, 2010
HpOKS	Forward: 5'-TGCGAGTTGATGGTGTGCGAG-3' Reverse: 5'-ATATTGGGCGCTCTGTAGGC-3'	This study
HpPAT	Forward: 5'-ACCAGGGTTGGCTTTGAC C-3' Reverse: 5'-TTCTTCCTCCGTCCTCTCTCC-3'	Velada et al., 2014
HpIGPS	Forward: 5'-AACGGGGCCGGATAATTC-3' Reverse: 5'-CACCAAAACCGCTCTCACAC-3'	
Hyp-1	Forward: 5'-GAACGCCTGGATTTAGTGGA-3' Reverse: 5'-AAGCTTGTAGGTGGCCGTAA-3'	This study
HpPGIP	Forward: 5'-CTCAGATTCTTGAGGCTTGACT-3' Reverse: 5'-ATCGACCCAGATAGCTTGTGT-3'	This study
HpAGO5	Forward: 5'-ACCAGCAGACCCACCCACTA-3' Reverse: 5'-GCATAGCTTGTGTTGTGAGACTTTG-3'	This study
HpRDR6	Forward: 5'-GGCTTTCTCTTCTCCATTCCA-3' Reverse: 5'-AGCAATACAATCACCCGTCA-3'	Galla et al., 2015
HpSGS3	Forward: 5'-CCAGGGCTCAAGAAATTACCA-3' Reverse: 5'-CACGAAGTCCCTCCATCTCCTT-3'	
HpAOX2	Forward: 5'-TCAACGCCTACTTTGTGATCTATCTC-3' Reverse: 5'-AATGGCCTCTTCTTCCAAATAGC-3'	Velada et al., 2016
HpCAT	Forward: 5'-CTTCGTTTCGTTTCGTTCTCCG-3' Reverse: 5'-GATCCAACGGTGAGGGAGTG-3'	This study

451

452

Figures Legends

Fig. 1. Expression variation of genes involved in secondary metabolites biosynthesis pathways in response to *Agrobacterium tumefaciens*. Relative transcript levels of (a, b) *PAL* and *CHS* from flavonoid biosynthesis pathway, (c) *BPS* from xanthone biosynthesis pathway, (d) *PKS1* from hyperforin biosynthesis pathway, (e, f) *OKS* and *PKS2* from hypericin biosynthesis pathway, and (g, h) *PAT* and *IGPS* from melatonin biosynthesis pathway, as detected by qRT-PCR. Asterisks denote the significance levels compared with control: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$ and ns denotes not significant.

Fig. 2. Putative secondary metabolite synthesis pathways in *H. perforatum* L. and fold variation in key genes in response to *Agrobacterium tumefaciens* (a) Biosynthesis pathways of flavonoids, xanthone, hyperforin, hypericin, and melatonin. Red arrow denotes increased expression of transcripts; Green arrow denotes reduced expression of transcripts. (b) Fold variation in relative transcript levels of genes from those pathways according to the indicated fold change color scale. *PAL*, phenylalanine ammonia-lyase; *C4H*, cinnamate 4-hydroxylase; *4CL*, 4-coumarate-CoA ligase; *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *BPS*, benzophenone synthase; *PKS1*, polyketide synthase 1; *MAT*, dimethylallyltranstransferase; *OKS*, octaketide synthase; *PSK2*, polyketide synthase 2; *AS*, anthranilate synthase; *PAT*, phosphoribosylanthranilate transferase; *PAI*, phosphoribosylanthranilate isomerase; *IGPS*, indole-3-glycerol phosphate synthase.

Fig. 3. Expression variation of genes involved in gene silencing pathway in response to *Agrobacterium tumefaciens*. Relative transcript level of (a) *RDR6*, (b) *SGS3* and (c) *AGO5* as detected by qRT-PCR. (d) Fold variation in relative transcript levels of genes according to the indicated fold change color scale. Asterisks denote the significance levels compared with control: **, $P \leq 0.01$; ***, $P \leq 0.001$ and ns denotes not significant.

Fig. 4. Expression variation of genes involved in ROS homeostasis and defense in response to *Agrobacterium tumefaciens*. Relative transcript level of (a) *AOX2*, (b) *CAT*, (c) *Hyp-1* and (d) *PGIP*, as detected by qRT-PCR. (e) Fold variation in relative transcript levels of genes according to the indicated fold change color scale. Asterisks denote the significance levels compared with control: *, $P \leq 0.05$; **, $P \leq 0.01$; ****, $P \leq 0.0001$ and ns denotes not significant.

Fig. 5. Expression variation of genes involved in secondary metabolites biosynthesis pathways in *H. perforatum* L. leaves with (HpWith) or without (HpWithout) glands. (a, b) Relative transcript levels of *PAL* and *CHS* from flavonoid biosynthesis pathway, (c) *BPS* from xanthone biosynthesis pathway, (d) *PKS1* from hyperforin biosynthesis pathway, (e, f) *OKS* and *PKS2* from hypericin biosynthesis pathway, (g, h) *PAT* and *IGPS* from melatonin biosynthesis pathway, as detected by qRT-PCR. (i) Fold variation in relative transcript levels of genes according to the indicated fold change color scale Asterisks denote the significance levels compared with control: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$ and ns denotes not significant.

Fig. 6. Expression variation of genes involved in ROS homeostasis and defense in *H. perforatum* L. leaves with (HpWith) or without (HpWithout) glands. (a, b) Relative transcript levels of *AOX2* and *CAT* from ROS pathway, and (c, d) *Hyp-1* and *HpPGIP*, as detected by qRT-PCR. (e) Fold variation in relative transcript levels of genes according to the indicated fold change color scale. Asterisks denote the significance levels compared with control: *, $P \leq 0.05$; **, $P \leq 0.01$ and ns denotes not significant.

Fig. S1. Expression variation of genes involved in RNA silencing pathway in *H. perforatum* L. leaves with (HpWith) or without (HpWithout) glands. (a) Relative transcript level of *RDR6*, (b) *SGS3*, and (c) *AGO5*, as detected by qRT-PCR. Asterisks denote the significance levels compared with control: ns denotes not significant.

(a)

(b) Flavonoids biosynthesis pathway

Xanthone biosynthesis pathway

Hyperforin biosynthesis pathway

Hypericin biosynthesis pathway

Melatonin biosynthesis pathway

Fold increase

Fold decrease

12h 24h

Fold increase

(i) Flavonoids biosynthesis pathway

HpPAL

HpCHS

Xanthone biosynthesis pathway

HpPAL

HpBPS

Hyperforin biosynthesis pathway

HpPKS1

Hypericin biosynthesis pathway

HpOKS

HpPKS2

Melatonin biosynthesis pathway

HpPAT

HpIGPS

Fold increase

Fold decrease

With glands

(a)

HpAOX2

(b)

HpCAT

(c)

Hyp-1

(d)

HpPGIP

(e) ROS pathway

HpAOX2 *HpCAT* *Agrobacterium* defense genes*Hyp-1* *HpPGIP*

With glands

Fold increase

No changes

0-50%

51-100%