Transgenic expression of *Hyp-I* gene from *Hypericum perforatum* L. alters expression of defense-related genes and modulates recalcitrance to *Agrobacterium tumefaciens*

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Declarations of interest: none

Main Conclusion

Phenolic oxidative coupling protein (*Hyp-I*) isolated from *Hypericum perforatum* L. was characterized as a defense gene involved into *H. perforatum* L. recalcitrance to *Agrobacterium tumefaciens*-mediated transformation.

Abstract

Hypericum perforatum L. is a reservoir of high-value secondary metabolites of increasing interest to researchers and to the pharmaceutical industry. However, improving their production via genetic manipulation is a challenging task as *H. perforatum* L. is recalcitrant to *Agrobacterium tumefaciens*-mediated transformation. Here, *Phenolic oxidative coupling protein* (*Hyp-1*), a pathogenesis-related (PR) class 10 family gene, was selected from a subtractive cDNA library from *A. tumefaciens*-treated *H. perforatum* L. suspension cells. The role of *Hyp-1* in defense against *A. tumefaciens* was analyzed in transgenic *Nicotiana tabacum* and *Lactuca sativa* overexpressing *Hyp-1*, and in *Catharanthus roseus* silenced for its homologous *Hyp-1* gene, *CrIPR*. Results showed that *Agrobacterium*-mediated expression efficiency greatly decreased in *Hyp-1* transgenic plants. However, silencing of *CrIPR* induced *CrPR-5* expression and decreased expression efficiency of *Agrobacterium*. The expression of core genes involved in several defense pathways was also analyzed in *Hyp-1* transgenic tobacco plants. Overexpression of *Hyp-1* led to an ample down-regulation of key genes involved in auxin signaling, microRNA-based gene silencing, detoxification of reactive oxygen species, phenylpropanoid pathway and PRs. Moreover, *Hyp-1* was detected in the nucleus, plasma membrane and the cytoplasm of epidermal cells by confocal microscopy. Overall, our findings suggest *Hyp-1* modulates recalcitrance to *A. tumefaciens*-mediated transformation in *H. perforatum* L.

Keywords: *H. perforatum* L secondary metabolites, *Agrobacterium tumefaciens*-mediated expression, *Hyp-1*, PR-10, plant defense responses

Introduction

Hypericum perforatum L., popularly known as St. John's wort, is a widely investigated medicinal plant with a history of more than 2000 years, which possesses anti-depressant, anti-inflammatory, anti-viral, anti-cancer, and anti-bacterial pharmacological properties (Patočka J 2003; Tatsis et al. 2007; Saddiqe et al. 2010). *H. perforatum* L. contains a wide range of biologically active secondary metabolites, including hypericin, hyperforin, xanthones, essential oils, phenolic acids and a broad array of flavonoids (Butterweck et al. 2003; Tatsis et al. 2007; Karppinen 2010; Saddiqe et al. 2010). Hence, metabolic engineering could offer opportunities to maximize the production of valuable substances on an industrial scale. Although genetic transformation of *H. perforatum* L. by *Agrobacterium rhizogenes* and biolistic bombardment has been reported, these protocols cannot achieve the requirements of functional genomic research (Hou et al. 2016).

A. tumefaciens is a gram-negative, soil-borne bacterium that causes crown gall disease on a broad range of host plants by integrating a transfer DNA (T-DNA) from a tumor-inducing plasmid into the host genome (Pitzschke 2013; Gohlke and Deeken 2014). This feature has been widely exploited to introduce foreign genes into the plant for functional genomics and genetic engineering purposes. However, as a bacterial pathogen, *A. tumefaciens* activates a number of defense responses in plants that include alterations in i) phytohormone metabolism (Pitzschke 2013), ii) pathogenesis-related protein syntheses (Anand et al. 2007; Pruss et al. 2008), iii) microRNA-mediated gene silencing (Dunoyer et al. 2006), iv) lignin deposition (Miedes et al. 2014; Franklin et al. 2009), v) secondary metabolite profiles (Franklin et al. 2009), and vi) production of reactive oxygen species (ROS) (Franklin et al. 2008).

A. tumefaciens-mediated transformation is the preferred method of gene transfer to plants, and it could be useful for metabolic engineering and functional genomic studies in *H. perforatum* L. However, *H. perforatum* L. recalcitrance to *A. tumefaciens*-mediated transformation constitutes a major concern in the field (Hou et al. 2016). In a previous study, *H. perforatum* L. suspension cells showed an intense oxidative burst upon interaction with *A. tumefaciens*, with 99% mortality of *A. tumefaciens* taking place in few hours while no apoptotic process in plant cells was recorded (Franklin et al. 2008).

Phenolic oxidative coupling protein (Hyp-I) was first cloned and characterized from *H. perforatum* L. cell cultures (Bais et al. 2003). The cDNA sequence of *Hyp-1* contains an open reading frame of 480 nucleotides, encoding a protein of 159 amino acids with a molecular weight of 17.8 kDa. *Hyp-1* was considered as the sole enzyme that catalyzed the conversion of emodin to the key biocompound hypericin in *H. perforatum* L. *in vitro* (Bais et al. 2003), although other reports did not confirm this catalytic activity of the protein (Kořuth et al. 2007, 2011; Michalska et al. 2010; Karppinen et al. 2016). In addition, the highest expression level of *Hyp-1* in *H. perforatum* L. was found in roots where no dark glands (sites of production of compounds of pharmacological value) are present, which left

unresolved the function of *Hyp-1* in the biosynthesis of hypericin (Kořuth et al. 2007). Furthermore, it was observed that there was no clear correlation between hypericin production and *Hyp-1* expression, since the *Hyp-1* gene was expressed in both “hypericin producing” and “hypericin lacking” species. These findings suggest that *Hyp-1* is not a limiting factor in the biosynthesis of hypericin (Kořuth et al. 2011).

In silico studies on the crystal structure of Hyp-1 evidenced that *Hyp-1* belongs to the pathogenesis-related (PR) class 10 family (Fernandes et al. 2008; Michalska et al. 2010; Sliwiak et al. 2016). PR-10 proteins are a family of small (154-163 amino acids, 15-18 KDa) and slightly acidic proteins which, unlike other PR proteins, accumulate in the cytosol. The major birch pollen allergen Bet v1 is considered the prototype for the PR-10 protein family and shares 45.1% homology to Hyp-1 (Fernandes et al. 2013; Hsu et al. 2014; Xu et al. 2014; Sliwiak et al. 2016). Some PR-10 proteins have been shown to have antifungal activities. For instance, MtPR-10 identified from *Medicago truncatula* had antifungal activity against *Aphanomyces euteiches* (Colditz et al. 2005). SsPR-10 from *Solanum surattense* inhibited hyphal growth of *Pyricularia oryzae* (Pungartnik et al. 2009). ZmPR-10.1 from *Zea mays* had antifungal activity against *Aspergillus flavus* (Xie et al. 2010). CsPR-10 from *Crocus sativus* inhibited the growth of *Fusarium oxysporum* (Gómez-Gómez et al. 2011). Besides, some PR-10s also showed antiviral and antibacterial activities. CaPR-10 from *Capsicum annuum* had antiviral activities against *Tobacco mosaic virus* (Park et al. 2004). ZmPR-10 and ZmPR-10.1 conferred antibacterial activities against *Pseudomonas syringae* pv. *tomato* DC3000 (Xie et al. 2010). Moreover, the increased expression of *Hyp-1* upon wounding and treatments with salicylic acid (SA) and abscisic acid (ABA) suggested its role in plant defense (Kořuth et al. 2013; Karppinen et al. 2016). Furthermore, upon infection by *A. tumefaciens*, *Hyp-1* expression was significantly upregulated in all analyzed species of *Hypericum* (Kořuth et al. 2013).

Gain-of-function approaches constitute useful tools for connecting genes to biological pathways. Among them, overexpression of individual genes by genetic transformation has been used to identify genes that confer stress tolerance to plants (Kondou et al. 2010). There are also abundant reports on studies in plants overexpressing PR genes, resulting in enhanced tolerance to pathogen infection. In addition, loss-of-function approaches have been exploited by plant pathologists as a parallel or complementary method to overexpression studies. Virus-induced gene silencing (VIGS) has been developed as a well-known technique based on RNA silencing to knock-down the expression of endogenous genes by utilizing recombinant viruses. Recently, *Tobacco rattle virus* (TRV)-based VIGS has been successfully used in the genetically non-tractable plant species *Catharanthus roseus* to investigate gene function (Liscombe and O'Connor 2011).

The aim of this study was to analyze the role of Hyp-1 as a candidate involved in *H. perforatum* L. recalcitrance to *Agrobacterium tumefaciens*-mediated transformation, and its impact on the expression of core genes regulating several defense pathways in *Hyp-1* transgenic tobacco plants.

Materials and methods

Plant materials and growth conditions

Seeds of *N. tabacum* were surface-sterilized by immersion in 1.5 % (v/v) bleach for 7 min, washed four times with sterile distilled water and sown onto 1% agar plates containing half strength Murashige and Skoog (MS) medium. The seeds of two varieties Batavia Laura (Casa César Santos, Portugal) and Salad bowl rossa (Vilmorin Jardin, France) of *L. sativa* were surface-sterilized by immersion in 6 % (v/v) NaClO for 15 and 10 min, respectively, then washed four times with sterile distilled water and sown onto 1% agar containing half strength MS medium. All steps were done in sterile flow chamber and seeds were grown in the tissue culture room at 25 °C with a 16-h light/8-h dark photoperiod.

Suppression subtractive hybridization (SSH) and cDNA library construction

Suspension cells of *H. perforatum* L. were treated with *A. tumefaciens* as described and a forward subtractive cDNA library was performed by using the PCR-Select cDNA Subtraction kit (Clontech, USA) according to the manufacturer's instructions. RNAs isolated from *Hypericum* suspension culture challenged with *A. tumefaciens* served as tester, while RNAs derived from control cultures served as driver, as described previously (Singh et al. 2016). Briefly, 2 µg Poly A+ RNA from tester and driver were used to prepare cDNA pools. Both cDNA s were digested with RsaI to obtain shorter, blunt-ended molecules, followed by phenol/chloroform extraction, ethanol precipitation and finally resuspension in RNase-free water. Tester cDNAs were split into two equal pools, each of which was suitable for ligation with a different adapter, supplied in the kit. After first and second hybridizations, the purified subtracted cDNAs were cloned in to pGEM-T easy cloning vector (Promega, Madison, WI, USA). Individual colonies were screened by PCR for size of inserts, and fragments above 150 bp were subjected to single-pass sequencing. Vector contamination was removed using the Vecscreen bioinformatics tool (<http://www.ncbi.nlm.nih.gov/tools/vecscreen>). The sequence similarity of obtained ESTs was analyzed by BLASTN and BLASTX and compared in different databases.

Plasmid construction and site-directed mutagenesis

For Hyp-1 overexpression, pBI121-GFP-Hyp-1 and pBI121-Hyp-1 were constructed. The full-length cDNA of the Green fluorescence protein (GFP) was amplified from the pGE vector (Received from Prof. Jim E. Brandle, Canada) (Conley et al. 2009) by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, USA) with the following forward and reverse primers: BamHI-GFP-F and GFP-SpeI-SacI-R. After sequencing, the *GFP* cDNA was digested with BamHI/SacI and inserted downstream of the *Cauliflower mosaic virus* (CaMV) 35S promoter in the binary vector pBI121 (GenBank Accession No.AF485783) to generate pBI121-GFP. To produce *Hyp-1* cDNA, a 480-nucleotide fragment was

RT-PCR-amplified from the total RNAs of a *H. perforatum* L. suspension culture treated with *A. tumefaciens*, using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, USA) and Platinum Taq DNA Polymerase High Fidelity with primers SpeI-Hyp-1-F and Hyp-1-SacI-R. The sequenced *Hyp-1* PCR fragment was finally cloned into the plasmid pBI121-GFP described above by digestion with SpeI/SacI to achieve construct pBI121-GFP-Hyp-1.

For the construction of pBI121-Hyp-1, the *GFP* gene was removed from pBI121-GFP-Hyp-1 by digestion with BamHI/SpeI. The Klenow Fragment of DNA Polymerase I (Invitrogen, USA) was used for producing blunt ends, and then the termini were ligated by T4 DNA Ligase (Thermo Scientific, USA).

To construct pBI121-GFP-Hyp-1-N, three stop codons (TAG TAA TGA) were added at 240 bp after the second ATG (nt 203) in the Hyp-1 sequence. To prepare the template pT-SpeI-Hyp-1-SacI, the plasmid pBI121-GFP-Hyp-1 and a self-ligated pGEM-T easy vector were digested by SpeI/SacI, and then ligated by T4 DNA ligase. To achieve pBI121-GFP-Hyp-1-N, the plasmid pT-SpeI-Hyp-1-SacI and the site-directed mutagenesis primers: STOP-Hyp-F and STOP-Hyp-R were used to amplify a pT-Stop codon-Hyp-1-N fragment using Platinum Taq DNA Polymerase High Fidelity. After the circularization, pT-Stop codon-Hyp-1-N was double digested with SpeI/SacI and inserted into pBI121-GFP digested with the same restriction enzymes downstream of GFP sequence.

All primer sequences are given in Table S1. All the purified recombinant plasmids were transformed into *A. tumefaciens* strain EHA105 by chemical transformation.

Agrobacterium-mediated transformation

Transgenic *N. tabacum* plants were generated by the leaf disk transformation method as described by Horsch et al. (Horsch et al. 1985). Briefly, *in vitro*-grown *N. tabacum* leaves were cut into 4-6 pieces and laid on the MSIB medium (MS medium containing IAA 0.5 mg/L; BA 2.5mg/L) for 2 days under the dark in the tissue culture room. The explants were co-incubated with *A. tumefaciens* with an optical density at 600 nm (OD_{600}) of 0.5-0.8 for 8 min under vacuum. Explants were kept in the same medium in the dark for another two days. Then explants were transferred into shooting medium (MSIB medium containing 50 mg/L Kanamycin and 500 mg/L ticarcillin), and re-cultured after every two-week period. Shoots (3-4 cm) were transferred into tubes with MS medium containing 0.5 mg/L IBA for root development.

For *L. sativa* transformation, two varieties Batavia Laura (BL) and Salad bowl rossa (SBR) were used in this study. Seedlings (4-6 days old) of *L. sativa* were punctured gently on the leaf surface near the petiolar region by scalpel blade, transferred to MS0.1BN (MS medium with 0.1 mg/L BA and 0.1 mg/L NAA) and incubated in 16-h light/8-h dark condition at 25 °C for 1 day. Explants were co-cultivated with *A. tumefaciens* suspension at OD_{600} of around 0.5 under vacuum for 10 min. Explants were then transferred to MS0.1BN plates under light for 5-7 days. Then explants were transferred to MS0.1BN medium with 50 mg/L Kanamycin + 500 mg/L ticarcillin, and re-cultured every two-weeks

until initiation of shooting. Finally, explants were transferred to MS medium with 50 mg/L Kanamycin + 500 mg/L ticarcillin for rooting and waiting for harvesting the whole transgenic plants. All the transgenic T1 plants were confirmed by PCR amplification of the *Hyp-1* and *NPTII* genes and by detection of expression of GFP fluorescence by microscopy (Leica - DM5000B + CTR5000 + ebq100, Germany). Progenies derived from self-fertilized T1 plants were germinated in MS medium containing 50 mg/L Kanamycin.

Plant total RNA isolation and quantitative real-time RT-qPCR

Total RNAs from young leaves of control and GFP-Hyp-1 transgenic *N. tabacum* plants were isolated by the CTAB method, as described (Gasic et al. 2004). The RNeasy Mini Kit (Qiagen, Germany) was used to clean up RNA including the DNase treatment according to the manufacturer's instructions. Total RNAs were quantified by NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Inc., USA) at A260/A280 and structural integrities were checked by 1.0% agarose-gel electrophoresis. 1 µg of DNase treated RNA was used to perform cDNA synthesis by Maxima First Strand cDNA Synthesis Kit according to the manufacturer's instructions.

Gene expression was monitored by RT- qPCR in 96-well plates with 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis Biodyne, Estonia). For each biological replicate, RT- qPCR reactions were performed in triplicate using 3 µl 5x EvaGreen® qPCR master mix, 200 nM of each primer, 1 µl 10x diluted cDNA template and nuclease-free water to a final volume of 15 µl. Amplification was performed on CFX96 Real-Time System C1000 Thermal Cycler (Bio-Rad Laboratories, Inc., USA) with the following conditions: 95°C for 15 min, 45 cycles of 95°C for 15 s, 60 °C for 20 s and 72°C for 20 s. Primers were designed to specifically anneal with each of the *N. tabacum* genes by using the online quantitative PCR primers design software QuantPrime (Arvidsson et al. 2008). Gene expression was normalized with the internal control *Elongation factor 1 α (EF-1 α)* and *L25 ribosomal protein* genes (Schmidt and Delaney 2010). Primer efficiencies were determined using a 4-fold serial dilution of 1 µl (10x diluted) cDNA template with 3 dilution points. The CFX Manager Software (Bio-Rad Laboratories, Inc., USA) was used to analyze data. The relative quantification of PCR products was calculated by the $2^{-\Delta\Delta CT}$ method.

For microRNA detection, the method based on adding poly(A)-tailing to validate the expression of artificial microRNAs was employed (Shi et al. 2012). For poly (A) tailing, 250 ng of total RNA, 1.25 µl 10mM ATP, 1 µl Yeast Poly(A) Polymerase (1 U/ µl) (Applied Biological Materials Inc, Canada), 5 µl 5X Poly(A) Polymerase Buffer, 2.5 µl 25 mM MnCl₂ and Nuclease-free water up to 25 µl was mixed and the reaction mixture was incubated at 37°C for 20 minutes, followed by 65°C for 20 minutes to terminate the reaction. cDNA was synthesized as follows: 25 µl Poly (A) RNA was added to 25 pmol of polyT adapter (Shi et al. 2010) and the mixture was incubated at 70°C for 2 min followed by cooling on ice for 2 min. Reactions were then mixed with 8 µl of 5X First-Strand Buffer,

1 µl of dNTP Mix (10 mM each), 1 µl of DTT (20 mM), 0.5 µl SMARTScribe Reverse Transcriptase (Clontech, USA) and sterile water up to 40 µl. The reaction mixture was incubated at 42°C for 1.5 hr. Diluted cDNA template (1µl 5x diluted) was used to conduct the qPCR reactions with gene-specific primers as described above. Gene expression was normalized to the *N. tabacum 5.8S rRNA* gene using (Shi et al. 2010). Data was analyzed as described above.

To detect the relative accumulation of *CrPR-1* and *CrPR-5* genes, one-step RT-qPCR was carried out in the Rotor-Gene 6000 real-time PCR detection system (Corbett, Australia) using *CrPR-1*- and *CrPR-5*-specific primers. To ensure the absence of DNA template in the samples, RT-qPCRs with and without reverse transcriptase were run in parallel, and *C. roseus 40S ribosomal protein S9 gene* (*CrRSP9*) (Sheshadri et al. 2018) was used to normalize gene expression. RT-qPCR was carried out at 50°C for 10 min, 95°C for 3 min, 40 cycles of 95°C for 10 s, and 60°C for 20 s. The efficiency of each pair of primers was determined by using a 5-fold serial dilution starting from 100 ng /µl RNA, with five dilution points. The Rotor-Gene 6000 software was used to confirm the synthesis of cDNA products by melting curves. All primer sequences are given in Table S1.

GUS staining assays

Fresh leaf disks were collected by sterile core borer or cut into pieces of rhombus-like shape. The disks were sterilized with 70% ethanol for 30s, 6% NaClO for 90 s, followed by washing with sterilized water. Leaf disks were kept on MSIB medium in the plant culture room for 2 days. The leaf disk transformation method was used to introduce *Agrobacterium* strain EHA105 harboring pCambia2301-GUS into plant tissues. After 5-6 days, leaf disks were incubated in the GUS staining solution (0.5 mg/µl X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylammonium salt), 20 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA (pH 8.0), 5 mM K₄Fe(CN)₆ and 5mM K₃Fe(CN)₆) at 37°C overnight. Next day, the samples were washed by 70% ethanol and the GUS activity was viewed and photographed.

Subcellular localization

For Hyp-1 subcellular localization assays, *A. tumefaciens* cultures harboring pBI121-GFP-Hyp-1 and the mCherry-labeled-plasma membrane marker PM-RK (Nelson et al. 2007) were co-infiltrated into the leaves of 5-week-old *N. tabacum* plants as described previously (Hou et al. 2011). Confocal fluorescence of GFP and mCherry were captured with Leica TCS SP2 confocal microscope. Excitation and emission settings in nanometers (nm) were 488 and 500 -to-530 respectively for GFP; 561 and 575-to-640 respectively for mCherry.

To distinguish the localization of Hyp-1 between cell wall and plasma membrane, *N. tabacum* leaves were agroinfiltrated with pBI121-GFP-Hyp-1 and free GFP as control. After 3 days, 1-cm² leaf explants were excised and incubated with 1 M mannitol for 5 min prior to observation. GFP

fluorescence patterns were examined in epidermal cells with Leica DM5000B+CTR5000+ebq100 fluorescence microscope. Images were processed with ImageJ (Schindelin et al. 2012) and Adobe Photoshop software (Adobe Systems).

Virus-induced gene silencing

For VIGS, TRV-based vectors, pTRV1 and pTRV2:00, were obtained from ABRC (<http://www.arabidopsis.org>). pTRV2: CrChlH containing a fragment of *C. roseus protoporphyrin IX magnesium chelatase subunit H (ChlH)* gene served as marker to evaluate VIGS efficiency.

A series of amino acid sequences from *Hyp-I* related genes were aligned to generate a phylogenetic tree by MEGA 6 (Tamura et al. 2013). The homologous *Hyp-I* gene from *C. roseus intracellular pathogenesis related protein (CrIPR)* (GenBank: Y10612.1) was selected to construct pTRV2: CrIPR. A 318-bp fragment from the 3' terminus of CrIPR was used to synthesize cDNA by Superscript II reverse transcriptase (Invitrogen, USA). The cDNA was amplified with Cr-IPR-specific primers by High-fidelity Taq DNA Polymerase (Roche, Germany). The PCR product was first cloned in pGEM-T easy vector. After DNA sequencing, the EcoRI-digested *CrIPR* fragment was inserted into pTRV2:00, which was previously digested with EcoRI followed by dephosphorization with alkaline phosphatase (Roche, Germany) according to the manufacturer's instructions. All the recombinant plasmids for VIGS assays were transformed into *A. tumefaciens* strain GV3101 by electroporation.

Three-to-four week-old *C. roseus* plants (Vilmorin Jardin, France) having two pairs of fully developed true leaves were used for agroinfiltration of viral vectors as described previously (Liscombe and O'Connor 2011).

Silencing of *CrIPR* mRNA was detected in upper, non-inoculated leaf tissue at 21 days post-inoculation (dpi) by semiquantitative RT-PCR, as described (Liscombe and O'Connor 2011). Cr-IPR-(C)-2-specific primers that anneal outside the region targeted for silencing were used to ensure that the endogenous gene would be tested. The *CrRSP9* described above was used as internal control. PCR cycles included denaturation at 98 °C for 30 s, annealing at 58 °C for 20 s, and elongation at 72 °C for 30 s. Aliquots were removed from the thermocycler after 36, 39, and 42 cycles. All primer sequences are given in Table S1.

Western blotting

A. tumefaciens carrying pCambia2300 containing a free *GFP* reporter gene was used to challenge silenced and control *C. roseus* plants. Samples were collected at 3 dpi and 5 dpi and western blot was carried out to measure GFP expression. Total proteins were extracted from 100 mg plant leaves in 400 µl extraction buffer containing 0.1M Tris-HCl (pH 8.0), 10mM EDTA (pH 8.0), 0.1 M LiCl, 1% SDS and 1% fresh added β-mercaptoethanol. Samples were separated on 15% SDS-PAGE gels and transferred to poly-(vinylidene difluoride) (PVDF) membranes (Amersham, GE Healthcare, Little Chalfont, Buckinghamshire, UK). The membranes were blotted with rabbit anti-GFP, N-terminal

antibody (Sigma-Aldrich, St Louis, MO, USA). Blotted proteins were detected by secondary antibody Goat anti-Rabbit IgG HRP (Sigma-Aldrich, USA) and LiteAblot® PLUS Enhanced Chemiluminescent Substrate (Euroclone, Italy).

Statistical analysis

The expression levels of target genes involved in different plant defense pathways were compared by One-way ANOVA. The relative accumulation of *PR* genes in *C. roseus* was analyzed by Student's t-test using GraphPad Prism 6 (GraphPad Software, Inc., USA).

Results

Overexpression of *Hyp-1* in *N. tabacum* and *L. sativa*

To identify genes from *H. perforatum* L. involved in defense responses against *A. tumefaciens*, a SSH-based cDNA library was performed (Singh et al. 2016). RNAs isolated from *H. perforatum* L. suspension cells challenged with *A. tumefaciens* as tester and with RNAs from control cultures as driver were used to construct the forward subtractive cDNA library from which a highly expressed *Hyp-1* gene was selected (Table S2) (Bais et al. 2003).

To characterize *Hyp-1* function in defense responses, we employed the *Agrobacterium* susceptible model plant *N. tabacum* to establish transgenic plants expressing *Hyp-1* fused at the N-terminus to GFP under the control of 35S promoter in the binary expression vector pBI121 (Fig. S1). Four transgenic tobacco plant lines, GFP-*Hyp-1*-2/-3/-4/-5, were selected by antibiotic resistance, and T-DNA insertion was confirmed by PCR. As control, besides wild type (WT), transgenic tobacco plants expressing GFP were obtained.

In order to determine whether overexpression of *Hyp-1* influences the efficiency of *Agrobacterium*-mediated expression in tobacco, we inoculated two T2 progenies derived from each of three T1 lines, GFP-*Hyp-1*-3/-4/-5, with the strain EHA105 harboring a binary plasmid to express GUS. Whereas GFP transgenic control plants showed an intense GUS staining and behaved similarly to WT plants (Fig. 1 b, c), GUS staining was absent in either of the six GFP-*Hyp-1* T2 transgenic lines (Fig. 1 d-i) or WT plants inoculated with *A. tumefaciens* containing the empty vector (Fig. 1 a).

Next, we assessed *Agrobacterium*-mediated expression in two varieties of other *Agrobacterium* susceptible plant *L. sativa*, BL and SBR, overexpressing *GFP-Hyp-1* and *Hyp-1* respectively (Fig S2 and S3), by inoculation with *Agrobacterium* harboring the GUS gene. T1 transgenic plants expressing either *GFP-Hyp-1* or *Hyp-1* showed no staining at all (Fig. 2 d, f, and Fig. 3 c) or a clear decrease in GUS staining (Fig. 2 c, e) compared to WT plants (Fig. 2 b and 3 b). Our results indicate that expression efficiencies by *A. tumefaciens* greatly decrease in tobacco and lettuce plants constitutively overexpressing *Hyp-1*, suggesting the involvement of *Hyp-1* in defense response against *A. tumefaciens*.

The conserved motif Bet.v.1 positioned at the C-terminal part of Hyp-1 (89–120 amino acid residues in Hyp-1) is common throughout the PR-10 proteins, suggesting a prominent role in protein function (Bais et al. 2003). However, there are no studies on Hyp-1 protein integrity nor function of the N-terminus of Hyp-1 up to now. So, to study the contribution of the N-terminal part of Hyp-1 and the protein integrity of Hyp-1 to the defense response against *A. tumefaciens*, we generated transgenic tobacco plants expressing the N-terminal 80 amino acids of Hyp-1 fused to GFP (Fig. S4) (GFP-Hyp-1-N plants). Three T2 progenies derived from each of two T1 lines, GFP-Hyp-1-N-2/-3, were inoculated with the *A. tumefaciens* strain harboring the GUS gene. Interestingly, T2 transgenic plants expressing GFP-Hyp-1-N showed an intense GUS staining comparable to that in WT control plants (Fig. 4b and 4Cch). This suggests that the N-terminal region of Hyp-1 is not sufficient to function as a defense protein against *A. tumefaciens*.

Subcellular localization studies

To examine the subcellular localization of Hyp-1, *Agrobacterium* strains carrying either GFP-Hyp-1 or the plasma membrane marker PM-RK were co-expressed transiently in leaf epidermal cells of WT *N. tabacum* by agroinfiltration. Two days after agroinfiltration, the fluorescence of both proteins was observed by confocal fluorescence microscopy to compare their localization. The fluorescence of GFP-Hyp-1 coincided with that of PM-RK in the plasma membrane (Fig. 5). Besides, GFP-Hyp-1 was also detected in the nucleus, but not in nucleolus, and in the cytoplasm of leaf epidermal cells of *N. tabacum*. The antibacterial defense activity of the Hyp-1 protein is retained in the GFP-Hyp-1 fusion protein (Figs. 1-3), suggesting that this subcellular localization of GFP-Hyp-1 has biological relevance.

In order to test whether Hyp-1 localized also to the cell wall, we induced cell plasmolysis by treatment of the leaf sections with 1 M mannitol to promote the detachment of the plasma membrane from the cell wall (Fig. 6 d-f, j-o), using treatment with H₂O as control (Fig. 6 a-c, g-i). In plasmolyzed cells GFP-Hyp-1 remained in the area lining the detached plasma membrane, suggesting that it was probably not present in the cell wall (Fig. 6 j-o).

We next investigated the subcellular localization of GFP-Hyp-1 in the GFP-Hyp-1-4/-5 transgenic tobacco lines. Green fluorescence was localized in the cytoplasm and nucleus of GFP-Hyp-1 expressing plants (Fig. 7 b-d), similar to the localization of transiently-expressed free GFP (Fig. 7 a). The integrity of the GFP-Hyp-1 fusion in the GFP-Hyp-1 transgenic plants was confirmed by western blot using antibodies against GFP (Fig. 7 e).

Overexpression of *Hyp-1* alters expression of multiple defense-related genes

To survey plant defense pathways involved in *Hyp-1*-mediated defense against *A. tumefaciens*, the expression levels of core genes implicated in different microbial defense pathways were determined in

GFP-Hyp-1 transgenic tobacco plants by RT-qPCR. T2 GFP-Hyp-1-3, GFP-Hyp-1-4 and GFP-Hyp-1-5 transgenic lines were selected for subsequent experiments since they exhibited higher levels of *Hyp-1* mRNA than GFP-Hyp-1-2 (Fig. 8 a).

Phytohormones such as auxin and jasmonic acid (JA) play an important role in plant defense against pathogenic microorganisms. We focused on the auxin-responsive genes *transport inhibitor response 1* (*NtaTIR1*) and *auxin responsive factor 8* (*NtaARF8*), and the JA biosynthesis gene *Lipoxygenase 1* (*NtaLOX*). The transcript levels of *NtaTIR1* and *NtaARF8* were significantly reduced in *Hyp-1* overexpression plants whereas *NtaLOX* did not show significant differences in two out of three transgenic lines tested, compared to WT control plants (Fig. 8 b-d).

PR proteins have been described as plant specific proteins that accumulate in response to pathogen interaction. To assess the involvement of PR proteins in *Hyp-1*-mediated antibacterial defense, the transcript levels of *NtaPR-10* and *NtaPR-1* were monitored by RT-qPCR (Fig. 8 e-f). Both *NtaPR-1* and *NtaPR-10* mRNAs were decreased in most of the GFP-Hyp-1 lines, especially *NtaPR-1*.

The microRNA-mediated gene silencing pathway is also involved in plant defense responses against pathogens. The key component in the miRNA pathway *ARGONAUTE 1* (*NtaAGO1*), as well as *NtamiR160*, *NtamiR164ab* and *NtamiR164c* were analyzed by RT-qPCR in *Hyp-1* overexpressing plants (Fig. 8 g-j). Results indicate that *NtaAGO1* and *Nta164ab* were significantly reduced in all transgenic lines analyzed, whereas *NtamiR160* was reduced in GFP-Hyp-1-4 and GFP-Hyp-1-5 lines which displayed the higher expression level of Hyp-1 (Fig. 8 a). There were no significant differences in *NtamiR164c* expression between transgenic and control plants.

Production of secondary metabolites via the phenylpropanoid pathway serves in plants as a defense mechanism to protect themselves from pathogens. The lignin biosynthesis genes *Phenylalanine ammonia-lyase 1, 4* (*NtaPAL1*, *NtaPAL4*), *Cinnamoyl CoA reductase* (*NtaCCR*) and *Cinnamyl alcohol dehydrogenase* (*NtaCAD*) were analyzed in *Hyp-1* overexpressing plants by RT-qPCR. Transcripts of *NtaPAL1,4* genes were downregulated in the *Hyp-1* transgenic lines, while the expression levels of *NtaCCR* and *NtaCAD* were not significantly different to those in control plants (Fig. 8 k-n). The transcripts of *Chalcone synthase* (*NtaCHS*), a key enzyme which catalyzes the first committed step in the flavonoid biosynthesis pathway, was drastically reduced in *Hyp-1* overexpressing plants (Fig. 8 o).

Lastly, transcripts of core enzymes involved in ROS homeostasis, i.e., *ascorbate peroxidase* (*NtaAPX*), *catalase* (*NtaCAT*) and *superoxide dismutase* (*NtaFe-SOD*), were also monitored in *Hyp-1* overexpressing plants by RT-qPCR. Results showed no significant differences in *NtaAPX* and *NtaCAT* expression between transgenic and control plants (Fig. 8 p, q), whereas the levels of *NtaFe-SOD* mRNA were downregulated in two out of three transgenic plants compared to control plants (Fig. 8 r).

Virus-induced gene silencing in *C. roseus*

To investigate the loss-of function phenotype of *Hyp-1*, we used the VIGS technique to knock-down

the expression of the *Hyp-1* homolog in *C. roseus*. We choose *C. roseus* because is a genetically non-tractable plant species that resembles the recalcitrance of *H. perforatum* L. to *Agrobacterium* infection (Di Fiore et al. 2004). The *Hyp-1* homolog in *C. roseus* is *intracellular pathogenesis related protein* (*CrIPR*), as revealed by phylogenetic analysis of *Hyp-1*-related genes (Fig. S5). 3-4 weeks-old *C. roseus* plants were agroinfiltrated with a TRV-based VIGS vector carrying a 318-bp fragment of *CrIPR* (pTRV2: *CrIPR*) or the empty TRV vector (pTRV2:00) as a control. Silencing of the *magnesium chelatase subunit H* (*ChlH*) gene, which provides a visual bleached leaf phenotype, was also used to monitor VIGS in *C. roseus*. At 21 dpi, photobleaching occurred in the third and fourth pairs of leaves in all pTRV2: *CrChlH* treated plants, whereas there was no visible phenotype in control and pTRV2: *CrIPR* infected plants (Fig. 9 a). Silencing efficiency of *CrIPR* was examined by semiquantitative RT-PCR in upper leaves equivalent to those showing the bleached phenotype in pTRV2: *CrChlH* infected plants. Transcripts of *CrIPR* was reduced substantially in pTRV2: *CrIPR* infected plants compared to pTRV2:00 infected, control plants (Fig. 9 b).

In order to determine whether the knock-down of *CrIPR* influences the efficiency of *Agrobacterium*-mediated expression in *C. roseus*, *CrIPR*-silenced and control plants were inoculated in the third pairs of leaves with an *A. tumefaciens* strain expressing GFP at 21 dpi. GFP expression was monitored in duplicated samples at 3 and 5 dpi by western blot (Fig. 9 c). Intriguingly, GFP accumulated at lower levels in *CrIPR*-silenced plants than in controls at both time points. Our result indicates that expression efficiency by *A. tumefaciens* is decreased in *CrIPR*-knocked-down plants.

It has been reported that silencing of PR-10-like proteins in *M. truncatula* led to induction of other PR proteins and increased tolerance to the oomycete *A. euteiches* (Colditz et al. 2007). Thus, the relative accumulation of another set of PR protein genes from *C. roseus*, *CrPR-1* and *CrPR-5*, were monitored in *CrIPR*-silenced and control plants by RT-qPCR. Interestingly, no significant difference in the expression of *CrPR-1* mRNA was found between *CrIPR*-knock-down and control plants, whereas a 40% increase of *CrPR-5* expression was measured in *CrIPR*-silenced plants compared with control plants (Fig. 9 d). These findings suggest a crosstalk between different defense responses in *CrIPR*-silenced *C. roseus* plants, which led to the up-regulation of *CrPR-5*.

Discussion

Hyp-1 was selected from a forward subtractive cDNA library of *H. perforatum* L. suspension cells treated with *A. tumefaciens*. The function of *Hyp-1* as a member of the PR-10 gene family has been barely studied (Kořuth et al. 2013). *Hyp-1* overexpression was carried out in tobacco as well as in two varieties of lettuce, two plant species amenable for *A. tumefaciens*-mediated transformation, to unveil its participation in defense against *A. tumefaciens*. Expression efficiencies of *A. tumefaciens* greatly decreased in all transgenic plants as assayed by histochemical GUS staining, indicating that *Hyp-1* participates in defense responses against *A. tumefaciens*. Previous findings indicated that GUS

staining was a good indicator of *Agrobacterium* viability, suggesting that bacteria survival was compromised in *Hyp-1* overexpressing plants (Franklin et al. 2008). In addition, transgenic tobacco plants expressing a C-terminal truncated version of the protein were used to study the contribution of the first 80 amino acids to the antibacterial activity of Hyp-1. Defense activities towards *A. tumefaciens* were highly reduced in GFP-Hyp-1-N transgenic plants, suggesting that the N terminal part of Hyp-1 is not enough to contribute to the biological function of the Hyp-1 protein. The importance of maintaining Hyp-1 protein integrity could be interpreted in the light of studies on the crystal structure of Hyp-1 (Michalska et al. 2010). The Hyp-1 molecule exhibits a typical PR-10-like protein structure, whose fold consists of seven-stranded antiparallel β -sheet wrapped around the C-terminal helix α 3. Together with another two α -helices, those main structural elements form a huge cavity within the protein interior to bound various hydrophobic ligands, like plant hormone cytokinin. Besides, a characteristic Bet.v.1 domain is positioned at 89-120 amino acid residues in Hyp-1 (Bais et al. 2003). Therefore, several of those structural motifs are lacking in the Hyp-1 mutant, which results in the loss of biological activity.

Hyp-1 protein was localized at the plasma membrane, nucleus and cytoplasm, as judged by the subcellular distribution of fluorescence derived from the transient expression of GFP-Hyp-1 in *N. tabacum* leaves. Moreover, confirmation of the integrity of the full-length fusion protein in GFP-Hyp-1 transgenic plants by Western blot confirmed that the nuclear visualization of GFP fluorescence was due to Hyp-1 accumulation in nucleus rather than the import of free GFP cleaved from the fusion protein. This study demonstrates for the first time the subcellular localization of Hyp-1 in nucleus, as had been reported for the PR-10 proteins from *Tamarix hispida* and *Vitis vinifera* (Zhang et al. 2010; He et al. 2013). The nuclear localization of Hyp-1 might play an important role in defense responses against invading pathogens, especially in pathogens like *A. tumefaciens*, whose virulence depends on integration of T-DNA into the nucleus.

To further corroborate the role of *Hyp-1* in defense responses against *A. tumefaciens*, we used a TRV-based VIGS approach to study the loss-of-function phenotype of the *Hyp-1* homologous *CrIPR* in the recalcitrant plant *C. roseus*. Contrary to what could be expected, *Agrobacterium*-mediated expression efficiency was decreased in *CrIPR*-silenced plants, which correlated with the up-regulation of *CrPR-5*. It was reported that *M. truncatula* transgenic lines silenced for *PR-10* showed a reduced colonization of the oomycete *A. euteiches*, and a suppressed infection in the roots of transgenic plants (Colditz et al. 2007). Interestingly, *MtPr-10* silencing led to the *de novo* induction of two isoforms of thaumatin-like proteins (PR-5b). Thus, antagonistic induction of PR-5 proteins, which are normally repressed due to PR-10 expression, is not an unprecedented phenomenon in plant-microbe interactions. A similar antagonistic crosstalk was observed in our *Hyp-1* overexpressing tobacco lines, where overexpression of *PR-10* from *H. perforatum* L. led to reduced expression of endogenous PR genes, *PR-1* and *PR-10*. In addition, silencing of *Polygalacturonase-inhibiting protein* in *N. benthamiana*

greatly decreased *Agrobacterium*-mediated expression efficiency, which correlated with induction of several PRs, including PR-5 (Hou et al. submitted). These findings illustrate the complex crosstalk regulating multi-factorial defense responses in plant-pathogen interactions.

A. tumefaciens as a microbial pathogen provokes a series of plant defense responses, but understanding how these responses affect pathogen infection is still unclear (Pitzschke 2013). To survey which plant defense responses could be involved in *Hyp-1*-mediated defense against *A. tumefaciens*, the expression levels of core genes from different pathways including auxin metabolism, microRNA-mediated gene silencing, lignin deposition, secondary metabolites, ROS signaling, and pathogenesis-related proteins, were determined in GFP-*Hyp-1* tobacco plants. Auxin as a phytohormone is a critical contributor in plant-pathogen interaction (Fu and Wang 2011). There are two groups of protein complexes in auxin signaling: the auxin signaling repressor auxin/indole acetic acid (Aux/IAA) and the auxin signaling receptor transport inhibitor response 1/auxin signaling F-box proteins (TIR1/AFB). Pathogen-produced IAA is perceived by TIR1 and activates TIR1/AFB to degrade Aux/IAA which negatively regulates auxin signaling by binding and inhibiting auxin responsive factors (ARFs). The released ARFs then regulate transcription of auxin-responsive genes. In addition, plant IAA biosynthesis can be up-regulated by some ARFs, and the increased IAA level causes cell wall expansion by inducing the expression of structural proteins like endo- β -1,4-glucanases (EGases) and xyloglucan endotransglycosylases (XETs). These activities participate in cell wall loosening that promotes pathogen invasion and disease development. Moreover, auxin is involved in stomata opening that allows some pathogens to gain access to the interior of plant tissues. However, plants evoke mechanisms to repress auxin signaling during pathogen infection as a defense strategy (Fu and Wang 2011). In the present study, transcripts of auxin-responsive genes *NtaTIR1* and *NtaAFR8* were remarkably reduced when *Hyp-1* was overexpressed, which suggests that *Hyp-1* overexpression could contribute to antibacterial defenses by reducing auxin signaling. Besides, overexpression of *Hyp-1* in transgenic plants led to a decrease in the transcriptional levels of *NtaAGO1* and two microRNAs, *NtamiR160* and *NtamiR164ab*. In *A. thaliana*, miR164 has been reported to target the transcripts of NAM/ATAF/CUC (NAC) domain protein *NAC1*, which acts downstream of *TIR1* to transmit auxin signals for lateral root emergence (Guo et al. 2005). A recent report suggested that miR164 and target gene *NAC4* regulate cell death during plant-pathogen interactions, possibly by repressing GDSL lipase 1 which is an important regulator of plant immune responses (Lee et al. 2017). MiR164 is also involved in plant response to abiotic stresses in *Brassica*, rice and potato (Fang et al. 2014; Bhardwaj et al. 2015; Zhang et al. 2018). MiR160 targets *ARF10*, *ARF16* and *ARF17* to down regulate auxin signaling, which in turn protect plants against bacteria and fungi (Pinweha et al. 2015; Wójcik et al. 2017). Our results offer preliminary evidence on the involvement of those two microRNAs in the regulation of plant defense against *A. tumefaciens* that needs to be further investigated.

Several key genes involved in the lignin and flavonoids biosynthesis pathways, *NtaPAL1,4*, *NtaCCR*, *NtaCAD* and *NtaCHS*, were monitored in *Hyp-1* overexpressing plants. PAL is the first enzyme of phenylpropanoid pathway, which comprises the synthesis of lignin and flavonoids (Heldt and Piechulla 2011; Bagal et al. 2012). Lignin, as a critical constituent of cell wall, plays a major role in structural defense against pathogens. Flavonoids are small molecular secondary metabolites acting as antimicrobial compounds in defense against pathogens (Mazid et al. 2011; Andersson 2012; Goyal et al. 2012). Our findings showed that *Hyp-1* overexpression in transgenic plants downregulated the expression of genes involved in the phenylpropanoid pathway (*NtaPAL1,4* and *NtaCHS*), whereas *NtaCCR* and *NtaCAD* were not changed, suggesting that overexpressing of *Hyp-1* decreased *Agrobacterium*-mediated expression efficiency independently from synthesis of lignin and flavonoids. Similarly, ROS signaling did not seem to play a significant role in *Hyp-1*-mediated defense against *A. tumefaciens* since the transcript levels of core enzymes were unaltered (*NtaAPX*, *NtaCAT*) or slightly repressed (*NtaFe-SOD*) in *Hyp-1* overexpressing plants. Nevertheless, although *Hyp-1* overexpression could induce wide changes in the gene expression pattern of defense genes, not all of these changes are necessarily involved in the molecular mechanisms underlying *Hyp-1*-mediated antibacterial defense.

Several PR-10 proteins have shown *in vitro* and *in vivo* to have DNase and RNase activities, which could help to explain the role of *Hyp-1* in antibacterial defense (Chen et al. 2006; Kim et al. 2008, 2011; Xie et al. 2010; Krishnaswamy et al. 2011; He et al. 2013). Chadha and Das (Chadha and Das 2006) reported that an RNase activity-competent version of PR-10 from peanut was internalized by hyphae of *Fusarium oxysporum* and inhibited fungal growth. Moreover, DNA fragmentation was detected in rice suspension-cultured cells treated with PBZ1, a rice PR-10 family protein (Kim et al. 2011). Therefore, the putative DNase and RNase activities of *Hyp-1* might also play an important role in the death of *A. tumefaciens* cells observed upon co-cultivation with *H. perforatum* L. (Franklin et al. 2008). In addition, our subcellular localization data suggest that *Hyp-1* localized to the plasma membrane. The plasma membrane is important during plant-microbe interactions, because it acts as a barrier between the host and the pathogen and many pathogen-associated molecular pattern recognition receptors are localized at the plasma membrane (Boller and Felix 2009). Other PR proteins with associated RNase activities have been localized in the cell plasma membrane (Jiang et al. 2015).

In summary, *Hyp-1* was selected from a forward subtractive cDNA library from *A. tumefaciens*-treated *H. perforatum* L. suspension cells. Its biological function in defense against *A. tumefaciens* was analyzed in transgenic *Nicotiana tabacum* and *Lactuca sativa* plants overexpressing *Hyp-1*, and in *C. roseus* silenced for the *Hyp-1* homologous gene. Moreover, the expression of core genes regulating several defense pathways was analyzed in *Hyp-1* overexpressing lines. *Hyp-1* was detected in the nucleus, plasma membrane and the cytoplasm of epidermal cells by confocal microscopy. Overall, our

findings suggest that *Hyp-1* from *H. perforatum* L. modulates its recalcitrance to *A. tumefaciens*-mediated transformation as part of a multi-factorial response against pathogen infection. Future research on the characterization of RNase and DNase activities of *Hyp-1* might shed light on its protective role in degrading DNA or RNA of invading pathogens in *H. perforatum* L. *Agrobacterium*-mediated genetic transformation approaches that reduce *Hyp-1* expression in *H. perforatum* L. could give a better chance at efforts aimed at the genetic manipulation of this recalcitrant and medicinal plant spp. However, antagonistic regulation of PR proteins and that of other inducible defense-related proteins that result in decreased *Agrobacterium*-mediated expression efficiency should be taken into consideration. In conclusion, the present study provides a better understanding regarding the functional characterization of *Hyp-1* gene from *H. perforatum* L., and adds significant knowledge about the multi-factorial nature of the *H. perforatum* L.-*A. tumefaciens* interaction.

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Figure legends

Fig. 1 Detection of *Agrobacterium*-mediated expression in GFP-Hyp-1 tobacco T2 lines by GUS staining. (a) Wild-type (WT) plant inoculated with *A. tumefaciens* EHA105 containing the empty vector as negative control. (b) WT plant inoculated with *A. tumefaciens* containing pCAMBIA 2301-GUS as positive control. (c) GFP transgenic line inoculated with *A. tumefaciens* containing pCAMBIA 2301-GUS as positive control. (d) GFP-Hyp-1-3-2 and (e) GFP-Hyp-1-3-3 transgenic lines inoculated with *A. tumefaciens* containing pCAMBIA 2301-GUS. (f) GFP-Hyp-1-4-1 and (g) GFP-Hyp-1-4-3 transgenic lines inoculated with *A. tumefaciens* containing pCAMBIA 2301-GUS. (h) GFP-Hyp-1-5-2 and (i) GFP-Hyp-1-5-3 transgenic lines inoculated with *A. tumefaciens* containing pCAMBIA 2301-GUS. Bars in each panel represent 10 mm

Fig. 2 Detection of *Agrobacterium*-mediated expression in Hyp-1 *L. sativa* BL T1 lines by GUS staining. (a) Wild-type (WT) plant inoculated with *A. tumefaciens* EHA105 containing the empty vector as negative control. (b) WT plant inoculated with *A. tumefaciens* containing pCAMBIA 2301-

GUS as positive control. (**c-d**) GFP-Hyp-1-1/-3 transgenic lines inoculated with *A. tumefaciens* containing GUS vector. (**e-f**) Hyp-1-6/-8 transgenic lines inoculated with *A. tumefaciens* containing GUS vector. Bars in each panel represent 10 mm

Fig. 3 Detection of *Agrobacterium*-mediated expression in *Hyp-1 L. sativa* SBR T1 lines by GUS staining. (**a**) Wild-type (WT) plant inoculated with *A. tumefaciens* EHA105 containing the empty vector as negative control (**b**) WT plant inoculated with *A. tumefaciens* containing pCambia 2301-GUS as positive control. (**c**) Hyp-1-12 transgenic line inoculated with *A. tumefaciens* containing GUS vector. Bars in each panel represent 10 mm

Fig. 4 Detection of *Agrobacterium*-mediated expression in GFP-Hyp-N tobacco T2 lines by GUS staining. (**a**) Wild-type (WT) plant inoculated with *A. tumefaciens* containing the empty vector as negative control. (**b**) WT plant inoculated with *A. tumefaciens* containing pCambia 2301-GUS as positive control. (**c-e**) GFP-Hyp-N-2 (1/-2/-3) transgenic lines inoculated with *A. tumefaciens* containing GUS vector. (**f-h**) GFP-Hyp-N-3 (1/-2/-3) transgenic lines inoculated with *A. tumefaciens* containing GUS vector. Bars in each panel represent 10 mm

Fig. 5 Subcellular localization of GFP-Hyp-1 at the plasma membrane, nucleus and cytoplasm. Leaves of *N. tabacum* were co-infiltrated with GFP-Hyp-1 and plasma membrane marker PM-RK. Panels show either monochrome green (from GFP) (**a** and **e**) or red (from mCherry) (**b** and **f**) fluorescence, overlays for colocalization studies (**c** and **g**), or bright field (**d** and **h**). Arrows indicate nucleus (N). Scale bars in all images are 25µm

Fig. 6 Fluorescence microscopy distinguish the localization of GFP-Hyp-1 between cell wall and plasma membrane. (**a-f**) GFP transient expression in leaf epidermal cells of *N. tabacum*. (**g-o**) GFP-Hyp-1 transient expression in leaf epidermal cells of *N. tabacum*. (**a-c**, **g-i**) Leaf explants incubated with H₂O prior to observation. (**d-f**, **i-o**) Leaf explants incubated with 1M mannitol prior to observation. The arrows show the plasma membrane section detached from the cell wall. Scale bars in all images are 25µm

Fig. 7 Confocal fluorescence microscopy and western blot analysis in GFP-Hyp-1 transgenic lines. (**a**) GFP transient expression in leaf epidermal cells of *N. benthamiana*. (**b**) Leaf epidermal cells of *N. tabacum* GFP-Hyp-1-4 transgenic line. (**c-d**) Leaf epidermal cells of *N. tabacum* GFP-Hyp-1-5 transgenic line. Contrary to the transient expression of free GFP, constitutive fluorescence in GFP-Hyp-1 transgenic plants were also detected in stomata guard cells (**d**). (**e**) Western blot to detect GFP-Hyp-1 expression by using antibodies against GFP. GFP+ was free GFP agroinfiltrated into *N. benthamiana* as positive control. Lane M shows the molecular weight markers in kilodalton (kDa). The panel below the western blot shows the membrane stained with Ponceau-S after blotting as control of loading. Arrows indicate nucleus (N) and stomata (S). Scale bars in all images are 25µm

Fig. 8 Quantitative real time PCR to assess core genes involved in different plant defense pathways in GFP-Hyp-1 transgenic tobacco plants. (a) Relative transcript levels of *Hyp-1*. (b-c) Relative transcript levels of *TIR1* and *ARF8*. (d) Relative transcript levels of *LOX*. (e-f) Relative transcript levels of *PR-10* and *PR-1*. (g-j) Relative transcript levels of *AGO1*, MiR160, MiR164ab and MiR164c. (k-n) Relative transcript levels of *PAL1*, *PAL4*, *CCD* and *CAD*. (k, l-o) Relative transcript levels of *PAL1*, *PAL4*, and *CHS*. (p-r) Relative transcript levels of *APX*, *CAT*, and *Fe-SOD*. 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. 9 Silencing of *CrIPR* in *C. roseus* results in decreased *Agrobacterium*-mediated expression efficiency. (a) Representative *C. roseus* plants inoculated with *Agrobacterium* strains containing pTRV2: CrChlH, pTRV2:00 and pTRV2: CrIPR at 21 days after inoculation (dpi). (b) Silencing of *CrIPR* transcripts was analyzed by semiquantitative RT-PCR at 21 dpi. The same RT reactions were used to amplify *CrRPS9* gene transcripts as a control. (c) Western blot analysis of GFP accumulation in *CrIPR*-silenced and control *C. roseus* leaves at 3 and 5 days after infiltration with an *Agrobacterium* strain expressing GFP. The panel below the western blot shows the membrane stained with Ponceau-S after blotting as control of loading. Lane M, molecular weight markers. (d) Expression analysis of *CrPR-1* and *CrPR-5* in *CrIPR*-silenced and control *C. roseus* leaves at 21 dpi. *CrRPS9* was used as reference gene. *, $P \leq 0.05$; ns, not significant

Supplementary data

Table S1 Gene-specific primers used for cloning, VIGS and gene expression analysis by real-time quantitative reverse transcriptase PCR

Table S2 Putative functional classification of transcripts identified by BLASTX analysis from two forward subtractive cDNA libraries from *Agrobacterium tumefaciens*-treated *Hypericum perforatum* L. suspension cells (12hr and 24hr)

Fig. S1 Generation of *Nicotiana tabacum* GFP-Hyp-1 overexpressing plants. (a) Wild-type *N. tabacum* control plant. (b) GFP transgenic plant as control. (c-f) GFP-Hyp-1-2/-3/-4/-5 transgenic plant lines. (g) Schematic diagram of pBI121-GFP-Hyp-1 made by software Vector NTI. (h) Confirmation of T-DNA insertion by RT-PCR

Fig. S2 Generation of *L. sativa* BL *Hyp-1* overexpression plants. (a, d) Wild-type *L. sativa* BL control plant. (b, e) BL GFP-Hyp-1 transgenic plant. (c, f) BL *Hyp-1* transgenic plant

Fig. S3 Generation of *L. sativa* SBR *Hyp-1* overexpression plants. (a, c) Wild-type *L. sativa* SBR control plant. (b, d) SBR *Hyp-1* transgenic plant

Fig. S4 Schematic diagram of pBI121-GFP-Hyp-1-N made by software Vector NTI. The cDNA

sequence encoding the N-terminal 80 amino acids of Hyp-1 (yellow arrow) followed by two stop codons was fused to GFP (orange arrow). The characteristic Bet.v.1 domain (89-120 aa residues) missing in GFP-Hyp-1 is showed by a black arrow marked with a red cross

Fig. S5 Phylogenetic relationships of *Hyp-1* homologs in plants, constructed using the program MEGA 6. The amino acid sequences were obtained from GenBank. Plant Latin names and GenBank accession numbers were used to mark the phylogenetic tree. *Hyp-1* was framed in red box and the VIGS candidate from *Catharanthus roseus* was framed in yellow box. The scale was shown in units of number of amino acid substitutions per site

GFP-Hyp-1

PM-RK

Merge

Bright field

Green fluorescent

Bright field

Merge

GFP +
H₂O GFP +
Mannitol GFP-Hyp-1
+ H₂O GFP-Hyp-1
+ Mannitol

Transgenic expression of *Hyp-1* gene from *Hypericum perforatum* L.
alters expression of defense related genes and modulates tolerance to
Agrobacterium tumefaciens

Weina Hou¹, Rupesh Kumar Singh², Pan Zhao³, Viviana Martins⁴, Emmanuel Aguilar⁵, Tomás Canto⁵, Francisco Tenllado^{5*}, Alberto Carlos Pires Dias^{1,4,6} Supplemental Data

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Fig S2. Generation of *L. sativa* BL *Hyp-1* overexpression plants. (A, D) Wild-type *L. sativa* BL control plant. (B, E) BL GFP-Hyp-1 transgenic plant. (C, F) BL Hyp-1 transgenic plant.

Fig S3. Generation of *L. sativa* SBR *Hyp-1* overexpression plants. (A, C) Wild-type *L. sativa* SBR control plant. (B, D) SBR *Hyp-1* transgenic plant.

Fig S4. Schematic diagram of pBI121-GFP-Hyp-1-N made by software Vector NTI. The cDNA sequence encoding the N-terminal 80 amino acids of Hyp-1 (yellow arrow) followed by two stop codons was fused to GFP (orange arrow). The characteristic Bet.v.1 domain (89-120 aa residues) missing in GFP-Hyp-1 is showed by a black arrow marked with a red cross.

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Transgenic expression of *Hyp-1* gene from *Hypericum perforatum* L. alters expression of defense related genes and modulates tolerance to *Agrobacterium tumefaciens*

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Supplemental Data

Table S1 Gene-specific primers used for cloning, VIGS and gene expression analysis by real-time quantitative reverse transcriptase PCR

Gene	Primers (5'-3')
Primers for plasmid construction	
BamHI-GFP-F	Forward: GTGCAC GGATCC ATGGTGAGCAAGGGCGAG ^a
GFP-SpeI-SacI-R	Reverse: CT GAGCTCACTAGT CTTGTACAGCTCGTCC ^b
SpeI-Hyp-1-F	Forward: GTGCAC ACTAGT ATGGCGGCGTACACTATT ^c
Hyp-1-SacI-R	Reverse: GTGCAC GAGCTCT TAAAGCGAAACTTCAGG ^d
STOP-Hyp-F	Forward: TAGTAATGATATTGCAAGTACAC
STOP-Hyp-R	Reverse: GAAATTTGCGGCATCAATTTTCAT
Primers for RT-qPCR	
Hyp-1	Forward: GAACGCCTGGATTTAGTGGA
	Reverse: AAGCTTGTAGGTGGCCGTAA
NtaTIR1	Forward: ACTCTTGGGCCGCTTGATACTG
	Reverse: CGCCGCAATTCTTTACAGTTCTCC
NtaARF8	Forward: GCTGCCTGCTCAAACCTCAAATGG
	Reverse: GCTGATTGCCGACTTGTTGCTG
NtaLOX1	Forward: GCAGACACTGCTTGGCATTTC
	Reverse: TGCCCGAGGTAAAGTGTATCCG
NtaPR-10	Forward: CTTGCCAATTCTACCGTCTATGCC
	Reverse: ATTAGCCGAGCTGGACGCAAAC
NtaPR-1	Forward: AGGCGTGGAACCATTAACCTTGGG
	Reverse: GCAGTCTGCAGCCAATTGAGAAAC
NtaAGO1	Forward: CATTTGGCAGCTTTCCGTGCTC
	Reverse: TGCGCTTGTGACTGATCCACTG
NtaPAL1	Forward: ATCACTGCCTCGGGTGATCTTG
	Reverse: GCCTTGGAATTAGGCCTACCAAGTG
NtaPAL4	Forward: TGCCATCTAACCTCACCGCATC
	Reverse: AGATGCCATGGCGATTTCAGCTC
NtaCCR	Forward: AGAACAAGCAGCATGGGACGAG
	Reverse: CACCAACACTGGGTTGATTGCC
NtaCAD	Forward: TGTGGTTGATGGGTGTGAAGGC
	Reverse: TCTGCCTGTGGATCTTTGACTCC
NtaCHS	Forward: CGCTCGAGTCCTTGTTGTTTGTTC
	Reverse: AAGTGGGTGTCATTGGGTCCAC
NtaCAT	Forward: ATGCAAAATCGTTCCCTGACA
	Reverse: TCGAAAAACCAGGCAAAAGTA
NtaAPX	Forward: TCAAGGCTGAGCAAGGACATGG
	Reverse: TCCTTGATGGGCTCCAAGAGTC

NtaFe-SOD	Forward: CCCAGCATTCAACAATGCAGCTC
	Reverse: TCCGTTGGGCTTCATGGACTTC
Gene	Primers (5'-3')
EF-1 α	Forward: TGAGATGCACCACGAAGCTC
	Reverse: CCAACATTGTCACCAGGAAGTG
L25	Forward: CCCCTCACCACAGAGTCTGC
	Reverse: AAGGGTGTGTTGTCCTCAATCTT
Poly(T) adapter	Forward: GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTAGCAT GC
	Reverse: GCGAGCACAGAATTAATACGAC
NtamiR160	Forward: TGCCTGGCTCCCTGTATGCCA
NtamiR164ab	Forward: AGAAGCAGGGCACGTGCA
NtamiR164c	Forward: TGGAGAAGCAGGGCACATGCT
Nta5.8S rRNA	Forward: ACGTCTGCCTGGGTGTCACA
CrPR-1	Forward: TAGTCCGAGCCAGAAAATGG
	Reverse: ACGCTTTTACCGCATCACTC
Cr-PR-5	Forward: TACTCCTCCAGCAACCCTAGC
	Reverse: CGCCGTAACTTCAACAATC
CrRSP9	Forward: GATGGTGCACGTTTCCTTTT
	Reverse: TGGGTCCTTCTCATCCAAAG
Primers for VIGS	
Cr-IPR	Forward: CGGGATTCTCCAAGGAGATGGAGGTG
	Reverse: CGGTCTAGAATAATCTGGATTGGCTTGAA
Cr-IPR-(C)-F2	Forward: GTGTTCAAGGCGTTT GTCC
	Reverse: ACCGAAATGGACCAACTTG

^a: The nucleotides in *italic* and **bold** indicate the introduced restrictive cleavage site BamHI

^b: The nucleotides in *italic* and **bold** indicate the introduced restrictive cleavage sites SpeI-SacI

^c: The nucleotides in *italic* and **bold** indicated the introduced restrictive cleavage site SpeI

^d: The nucleotides in *italic* and **bold** indicated the introduced restrictive cleavage site SacI

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Supplemental Data

Table S2 Putative functional classification of transcripts identified by BLASTX analysis from two forward subtractive cDNA libraries from *Agrobacterium tumefaciens*-treated *Hypericum perforatum* L. suspension cells (12hr and 24hr)

Clone No	Time	Hits	Accession	Description	E value	Origin
13, 14	12h	PLN02378	PLN02378	Glutathione S-transferase DHAR1 (responsive to exogenous toxins)	6.43e-90	<i>Corchorus capsularis</i>
91	12h	PLN02906	PLN02906	Xanthine dehydrogenase (disease resistance)	1.08e-62	<i>Vicia caroliniana</i>
94	12h	Bet_v1-like	cd07816	Hyp-1 (disease resistance)	1.22e-09	<i>Hypericum maculatum</i>
168	12h	PLN02243	PLN02243	S-adenosylmethionine synthase/polygalacturonase-inhibiting protein (Pathogen responsive)	2.65e-07	

Clone No	Time	Hits	Accession	Description	E value	Origin
13	24h	xylanase_inhibitor_I_lik TAXi_N	pfam14543	TAXI-I inhibits degradation of xylan in the cell wall (pathogen responsive)	2.63e-30	<i>Gossypium raimondii</i>
8, 11, 92, 143, 158	24h	Bet_v1-like	cd07816	Hyp-1 (PR-10)	1.36e-123	<i>Hypericum maculatum</i>
13	24h	GST_C_Tau	cd03185	GST_N family, Glutathione S-transferase (Pathogen responsive)	1.59e-11	<i>Manihot esculenta</i>
16	24h	PLN02582	pfam13292	1-deoxy-D-xylulose-5-phosphate synthase (Pathogen responsive)	2.61e-75	<i>Jatropha curcus</i>
17	24h	PLN00113	PLN00113	Leucine-rich repeat (LRR) protein (Pathogen Responsive)	1.60e-04	<i>Populus euphratica</i>
19	24h	PLN02243	PLN02243	S-adenosylmethionine synthase (Pathogen Responsive)	1.36e-123	<i>Noccaea caerulea</i>]
72	24h	SPFH_like_u4	cd03407	SPFH (Stomatin, prohibitin, flotillin, and HflK/C) superfamily (Pathogen responsive)	2.34e-16	<i>Citrus sinensis</i>