Running head: Control of *N. attenuata* seedling growth by *HSPRO*

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Title: HSPRO controls early Nicotiana attenuata seedling growth during interaction with the fungus Piriformospora indica.

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Abstract

In a previous study aimed at identifying regulators of *Nicotiana attenuata* responses against chewing insects, a 26-nucleotide tag matching the *HSPRO* (ORTHOLOG OF SUGAR BEET *HsI*<sup>pro-1</sup>) gene was found to be strongly induced after simulated herbivory (Gilardoni *et al.*, 2010). Here we characterized the function of *HSPRO* during biotic interactions in transgenic *N. attenuata* plants silenced in its expression (ir-*hspro*). In wild-type plants (WT), *HSPRO* expression was not only induced during simulated herbivory but also when leaves were inoculated with *Pseudomonas syringae* pv. *tomato* (Pst DC3000) and roots with the growth-promoting fungus *Piriformospora indica*. Reduced *HSPRO* expression did not affect the regulation of direct defenses against *Manduca sexta* herbivory or Pst DC3000 infection rates. However, reduced *HSPRO* expression positively influenced early seedling growth during interaction with *P. indica*; fungus-colonized ir-*hspro* seedlings increased their fresh biomass by 30% compared to WT. Grafting experiments demonstrated that reduced *HSPRO* expression in roots was sufficient to induce differential growth promotion in both roots and shoots. This effect was accompanied by changes in the expression of 417 genes in colonized roots, most of which were metabolic genes. The lack of major differences in the metabolic profiles of ir-*hspro* and WT colonized roots (as analyzed by liquid chromatography-time-of-flight-mass spectrometry) suggested that accelerated metabolic rates were involved. We conclude that *HSPRO* participates in a whole-plant change in growth physiology when seedlings interact with *P. indica*.

**Keywords:** HSPRO, *Nicotiana attenuata*, *Piriformospora indica*, growth, FACs, defense
Introduction

*Nicotiana attenuata* is a wild annual tobacco plant native to the deserts of Southwestern US and it germinates after fires from long-lived seed banks to form monocultures in post-fire nitrogen-rich soils (Baldwin and Morse, 1994). As a result of its life history, *N. attenuata* grows rapidly after seed germination and the control of seedling growth is critical for plant fitness since young seedlings are more vulnerable to environmental stresses. In addition to water availability and high temperatures and light intensities, *N. attenuata* plants interact with unpredictable communities of beneficial and non-beneficial organisms in their natural environment (Baldwin and Preston, 1999; Barazani et al., 2005; Long et al., 2010). With regard to biotic interactions, *N. attenuata* (and plants in general) readjust their metabolic and growth programs to meet the new requirements of *de novo* biosynthesis of direct (e.g., accumulation of toxic metabolites) and indirect (e.g., production of volatiles) defense responses as well as to induce tolerance mechanisms (e.g., C and N bunkering in roots) or to facilitate symbiotic interactions (Rosenthal and Kotanen, 1994; Bardgett et al., 1998; Schwachtje and Baldwin, 2008). Activation of these responses requires metabolic energy and the redirection of carbon (C), nitrogen (N) and additional resources throughout the whole body of the plant (Schwachtje and Baldwin, 2008; Bolton, 2009). With the aim of identifying regulatory components of the pathways mediating defense and tolerance responses against lepidopteran larvae in *N. attenuata*, a SuperSAGE (serial analysis of gene expression) approach was recently performed by our group to quantify the early transcriptional changes elicited by the insect elicitor N-linolenoyl-glutamic acid (18:3-Glu) (Gilardoni et al., 2010). The analysis targeted mRNAs encoding rare transcripts constitutively expressed and showing rapid and transient induction after 18:3-Glu elicitation. Among the approximately 500 differentially expressed transcripts, more than 25% corresponded to putative regulatory components (Gilardoni et al., 2010). One of these components was a homolog of a group of proteins denominated Putative Nematode Resistance Protein (PNRP) or HSPRO based on their homology to Hs1pro-1 from *Beta procumbens* (sugar beet)(Cai et al., 1997).

The study describing the role of Hs1pro-1 was the first to provide functional information for this group of proteins, and Hs1pro-1 was originally identified as a gene conferring resistance to the beet cyst nematode *Heterodera schachtii* (Schmidt)(Cai et al., 1997). Subsequent studies performed in different plant species suggested however that this group of proteins has a more general role in the regulation of plant responses to biotic and abiotic
stresses. For example, the Arabidopsis thaliana genome encodes for two homologs of B. procumbens Hs1pro-1, HSPRO1 and HSPRO2 and these two genes have been categorized as “general stress signaling” genes (Baena-Gonzalez and Sheen, 2008). The expression of Arabidopsis HSPRO genes is not induced by nematode feeding (Puthoff et al., 2003) but it can be differentially induced by salicylic acid (SA), P. syringae pv. tomato (Pst DC3000) and Xanthomonas campestris pv. campestris infection, the bacterial elicitor flg22, phosphate starvation, salt stress, drought, wounding and UV-B (Hammond et al., 2003; Zipfel et al., 2004; Cominelli et al., 2005; Gissot et al., 2006; Fujita et al., 2007; Kudla et al., 2007; Murray et al., 2007; Walley et al., 2007). Genetic disruption of HSPRO2 in Arabidopsis increased susceptibility to Pst DC3000 (Murray et al., 2007) and its ectopic expression confers increased resistance against oxidative stress (Luhua et al., 2008). The role of HSPRO genes in these processes is at present unknown. Interestingly, in Arabidopsis, it has been shown that HSPRO1 and HSPRO2 interact with the AKINβγ (adaptor–regulator related to the SNF1/AMPK family) subunit of the SnRK1 (SNF1-related protein kinase) complex (Gissot et al., 2006). This complex is a heterotrimeric protein kinase complex related to the Saccharomyces cerevisiae SNF1 (sucrose non-fermenting 1) kinase (Baena-Gonzalez and Sheen, 2008). Plant SnRK1s are central regulators of metabolism via the control of gene expression and enzyme activity (Halford et al., 2003; Lovas et al., 2003; Schwachtje et al., 2006; Baena-Gonzalez and Sheen, 2008).

In this study, we analyzed the role of the HSPRO gene in N. attenuata during diverse biotic interactions, including M. sexta herbivory, Pst DC3000 infection and association with the growth-promoting fungus Piriformospora indica. P. indica is a root-colonizing basidiomycete of the order Sebacinales (Varma et al., 1999; Weiss et al., 2004) and is closely related to fungal clones isolated from soil samples collected from the rhizosphere of N. attenuata in its natural habitat (Barazani et al., 2005). P. indica has the ability to colonize roots of different plant species including N. attenuata, thereby initiating a mutualistic interaction resulting in plant growth promotion (Sahay and Varma, 1999; Barazani et al., 2005; Achatz et al., 2010; Fakhro et al., 2010). The results demonstrated that HSPRO is not involved in the regulation of traits associated with direct defense responses against M. sexta herbivory or performance of Pst DC3000 during infection, but is a negative regulator of N. attenuata early seedling growth stimulated by P. indica.

Results
Sequence and localization analyses of *N. attenuata* HSPRO

From the SuperSAGE analysis published recently by Gilardoni *et al.* (2010), a 26-nt tag (UniTag-6205) was identified as a tag whose abundance was 18-fold enriched in leaves of *N. attenuata* plants within 30 min of elicitation with the fatty acid-amino acid conjugate (FAC) 18:3-Glu. The full-length cDNA corresponding to UniTag-6205 was obtained by 5’ and 3’ rapid amplification of cDNA ends (RACE) and it was found to encode for an open reading frame of 1,437 bases and for a predicted polypeptide of 478 amino acids (molecular weight: 54 kDa). The protein presented 45% to 70% amino acid sequence identity to the sugar beet Hs1pro-1 protein and to several homologs in other plant species including HSPRO1 and HSPRO2 from Arabidopsis (Fig. 1a and Suppl. Figs. S1 and S2). For consistency with the Arabidopsis nomenclature, we named the *N. attenuata* homolog HSPRO. The phylogenetic analysis of the 21 closest homologs of HSPRO from different plant species found in GenBank showed that amino acid similarity between paralogs was higher than between orthologs, with sequences from legumes, monocotyledonous and Arabidopsis species clustering together (Fig. 1b). Interestingly, HSPRO sequences from monocots clustered closer to sequences from the moss *Physcomitrella patens* than did to sequences from dicots (Fig. 1b), thus not following the phylogeny of the taxa shown. In silico analysis predicted cytosolic localization for HSPRO and this prediction was confirmed by expressing an HSPRO-EGFP (Enhanced Green Fluorescent Protein) C-terminal fusion protein in leaf protoplasts and analysis by fluorescence microscopy (Fig. 1c).

**HSPRO expression is induced by different biotic stress-associated treatments**

Analysis of HSPRO mRNA levels in *N. attenuata* plants showed that this gene was not only differentially induced by 18:3-Glu but also by *M. sexta* and *S. exigua* OS in leaves (with *M. sexta* OS being the strongest inducer: ~12-fold) when compared to wounding (control treatment; Fig. 2a). FAC elicitation induces a strong JA burst in *N. attenuata* plants (Kallenbach *et al.*, 2010) and analysis of HSPRO expression in plants deficient in either JA (ir-lox3) and JA-Ile (ir-jar4/6) production or JA signaling (ir-coi1) showed that the induction of HSPRO was negatively affected by JA production (*i.e.* increased HSPRO mRNA accumulation in ir-lox3 plants compared to WT (control)) but not affected in plants deficient in JA-Ile accumulation or *COII* expression (Fig. 2b). Moreover, the induction of HSPRO expression depended on SIPK (Salicylic acid Induced Protein Kinase), a known regulator of
JA-mediated responses in *N. attenuata* (Wu *et al.*, 2007). HSPRO mRNA levels were induced 2.5-fold after 10 h of Pst DC3000 infection and 30-fold after 1 h of exogenous SA treatment (Fig. 2c and Suppl. Fig. S3) whereas they were not induced by *Agrobacterium tumefaciens* infection compared to control treatment (Fig. 2c). Thus, similar to other plant species, HSPRO responded to multiple biotic stress-associated stimuli.

Analysis of tissue-specific expression showed that HSPRO was ubiquitously expressed with the highest levels of expression in flower parts, in particular the corolla (Fig. 2d).

**Generation of *N. attenuata* plants with stably reduced levels of HSPRO expression**

To examine in further detail the function of HSPRO, stably transformed *N. attenuata* plants with reduced expression of this gene were generated by inverted repeat-mediated RNA interference (ir-RNAi; see Materials and Methods for a detailed description of the generation of these plants). Two homozygous independently transformed lines, named ir-hspro1 and ir-hspro2, were selected and used for all the experiments described below. These lines harbored a single T-DNA insertion in their genomes (Fig. 3a) and the levels of HSPRO mRNA were reduced on average by 93 % (ir-hspro1) and 95 % (ir-hspro2) compared to WT plants after 18:3-Glu elicitation (a condition that maximizes HSPRO expression; Fig. 3b). A third line, ir-hspro3 harbored two T-DNA insertions in its genome (Figs. 3a) and it was used only for a selected number of experiments. The levels of HSPRO mRNA in this line were reduced by 91 % compared to WT (Fig. 3b).

The growth and morphology of ir-hspro plants grown under standard chamber and glasshouse conditions were indistinguishable from those of WT at all stages of development (Figs. 3c and 3d; see also below).

**HSPRO does not affect defense responses against *M. sexta* herbivory and Pst DC3000 infection**

Based on the strong expression response of HSPRO to *M. sexta* OS and FACs (Fig. 2a), we first assessed whether ir-hspro plants were more susceptible to the attack of *M. sexta* larvae. The results showed that the performance of these larvae (evaluated as the gain of body mass as a function of time) was similar between ir-hspro and WT plants (Suppl. Fig. S4a). Consistently, the quantification of the JA-inducible defense metabolites nicotine, chlorogenic acid (3-O-caffeoylquinic acid), and rutin (quercetin-3-O-rutinoside) after elicitation with *M.
*sexta* OS showed that their amounts were similar in leaves of ir-*hspro* and WT plants (Suppl. Fig. S4b-d).

Second, based on the high levels of *HSPRO* expression in flower parts and in particular the corolla (Fig. 2d), we assessed whether this gene participates in the regulation of traits (defensive and non-defensive) associated with the interaction of insects with flowers. The analyzed traits were: 1) the production of benzyl acetone (BA) in corollas, 2) the volume of nectar in flowers, 3) the amount of nicotine in nectar, 4) the amount of sugar in nectar, and 5) the level of TPI (trypsin protease inhibitor) activity in ovaries and anthers. These traits were quantified in WT and ir-*hspro* plants grown either under control conditions or under the attack of *M. sexta* caterpillars for 15 consecutive days. All samples were collected at the end of the treatment (15 days). The results showed that the analyzed traits did not differ between ir-*hspro* and WT plants (Suppl. Fig. S5).

Finally, based on the induction of *HSPRO* mRNA levels by SA and Pst DC3000 infection (Fig. 2c), ir-*hspro* and WT plants were infected with this pathogen by leaf infiltration. In two independent experiments, the number of colony forming units (CFU) retrieved per area of infected leaves were similar between WT and ir-*hspro* plants at 24, 48 and 72 h post-infection (data not shown).

**HSPRO participates in the mechanisms that regulate growth promotion by the fungus Piriformospora indica**

The induction of *HSPRO* mRNA levels by diverse biotic related-stresses prompted us to investigate the interaction of ir-*hspro* plants with the growth promoting fungus *Piriformospora indica*. This fungus has the capacity to establish symbiotic associations with roots of a broad range of plant species including *N. attenuata* (Varma et al., 1999; Barazani et al., 2005; Waller et al., 2005; Qiang et al., 2011). To study the interaction between ir-*hspro* seedlings and *P. indica*, we used a previously described plate system (Camehl et al., 2011) (Fig. 4a). In this system, the hyphae reached the seedling’s roots between day 4 and 5 after the start of the experiment (i.e. transfer of seedlings to plates containing *P. indica*; see Materials and Methods for a detailed description of the system used). Unless noted, all the experiments were conducted with seedling tissue harvested at day 14 after the start of the experiment. In WT seedlings, *HSPRO* transcripts were undetected in roots of control treatment but strongly induced upon *P. indica* colonization (Fig. 4b). In colonized roots of ir-*hspro* seedlings, the level of this mRNA was also increased but it remained at less than 8% of WT levels (Fig. 4b).
The quantification of root, shoot and seedling fresh biomasses showed that *P. indica*-colonized ir-hspro seedlings gained on average 30% more biomass than *P. indica*-colonized WT seedlings (Fig. 4c-e). This experiment was repeated seven times with consistent results (Suppl. Table SI). The differential growth promotion of ir-hspro seedlings varied between 15 to 73% depending on the experiment and the length of the incubation period (10 or 14 days) with an average growth promotion of 32% (Suppl. Table SI). Thus, in addition to the growth promotion effect of *P. indica* observed on WT seedlings (Fig. 4c-e and Suppl. Table SI), there was an enhanced differential growth promotion on ir-hspro seedlings. The difference in growth was maintained as the seedlings were transferred to soil and grown in the glasshouse for maturation. In this case, the rosette diameter was determined as a parameter of growth (Suppl. Fig. S6a). At the end of the rosette expansion period (i.e. start of reproductive phase [bolting]), the rosette diameter was similar between the two genotypes (Suppl. Fig. S6a). A higher percentage of ir-hspro plants bolted one day earlier than WT (25% and 42% of ir-hspro1 and ir-hspro2, respectively) and the rate of stalk elongation and flowering time were similar between the genotypes (Suppl. Fig. S6b-d). These results showed that the growth of *P. indica*-colonized ir-hspro seedlings was primarily accelerated during the early stages of seedling growth without consequences for the final plant size at the mature stage.

To analyze if the differential growth promotion of ir-hspro seedlings during interaction with *P. indica* was the result of a differential assimilation of CO₂ produced by the fungus, ir-hspro and WT seedlings and *P. indica* were grown together but physically separated from one another in a three-sector split-plate system (Suppl. Fig. S7a). This setting allowed for the exchange of CO₂ between organisms in the absence of physical contact. No differential growth promotion was observed between WT and ir-hspro seedlings in this experiment (Suppl. Fig. S7b-d), indicating that physical interaction between roots and *P. indica* was required to differentially stimulate the growth of ir-hspro seedlings.

**Analysis of *P. indica*-root interactions and *P. indica*-induced changes in phytohormone levels**

Microscopy analysis of *P. indica*-colonized roots of WT and ir-hspro seedlings at two different times (days 7 and 14 of the plate system) showed a close association between roots and the fungal hyphae (Suppl. Fig. S8). Similar to other plant species (Varma et al., 1999; Stein et al., 2008; Schafer et al., 2009; Lee et al., 2011; Zuccaro et al., 2011), the fungus colonized the maturation zone of the root without a strong association with the elongation
zone and root tip (Suppl. Fig. S8). Also similar to previous observations performed with *N. attenuata* seedlings and *Sebacina vermifera* (a closely related Sebacinales species) (Barazani *et al.*, 2005), we could not detect fungal structures in the roots characteristic of endomycorrhiza (*e.g.*, arbuscules and intracellular vesicles). Root growth and hair density after *P. indica* colonization were not different between WT and ir-hspro seedlings (Suppl. Fig. S9) and the number of secondary roots per seedling was also similar between genotypes (WT: 5.25 ±0.33; ir-hspro1: 4.50 ± 0.36; ir-hspro2: 4.92 ± 0.23; n=12). Quantification of *P. indica*-root colonization by quantitative amplification of the *P. indica* EF1A gene (Deshmukh *et al.*, 2006) showed a lower tendency of root colonization of ir-hspro seedlings compared to WT seedlings however the differences were not statistically significant (Fig. 4f). Hence, the differential growth promotion of ir-hspro seedlings was not associated with increased *P. indica* root colonization or root growth.

During colonization of Arabidopsis roots by *P. indica*, the regulation of root cell death by the fungus plays an important role (Jacobs *et al.*, 2011; Qiang *et al.*, 2011). When roots of *N. attenuata* WT and ir-hspro seedlings were analyzed for cell death by trypan-blue staining in both, the absence and presence of *P. indica* (at day 14 on the plate system), no differences in the staining pattern were observed between plant genotypes (Suppl. Fig. S10). It has been reported that the interaction of *P. indica* and closely related Sebacinales species with roots involves changes in phytohormone accumulation and signaling (Barazani *et al.*, 2007; Stein *et al.*, 2008; Vadassery *et al.*, 2008; Schafer *et al.*, 2009; Camehl *et al.*, 2010). Quantification of JA, SA, ABA and ethylene (ET) levels in *P. indica*-colonized WT and ir-hspro seedlings (at day 14 of the plate system) showed that the levels of SA were reduced ~2-fold by root colonization but they did not differ between genotypes (Suppl. Fig. S11a). JA and ABA levels were not affected by root colonization while ET levels were induced. However, the levels of these phytohormones were similar between genotypes (Suppl. Fig. S11b and data not shown). Interestingly, the levels of colneleic acid (CA), a divinyl-ether (DVE) derived from the action of 9-lipoxygenase (9-LOX), were strongly induced (from less than 1 nmol gFW⁻¹ to 40 nmol gFW⁻¹) by *P. indica* in roots of both ir-hspro and WT seedlings (Suppl. Fig. S11c). These results suggested that similar to other plant species (Camehl *et al.*, 2010; Jacobs *et al.*, 2011; Leon-Morcillo *et al.*, 2012), some defense-associated responses were triggered by *P. indica* in *N. attenuata* roots.
Gene expression profiling of ir-hspro roots reveals significant changes in metabolic processes during P. indica colonization

To gain further insight into the mechanisms affected in ir-hspro plants, changes in gene expression in roots of ir-hspro and WT seedlings were analyzed. RNA was isolated from roots of WT and ir-hspro seedlings grown for 14 days on the plate system either in the absence or presence of P. indica, and changes in gene expression were evaluated with an Agilent custom-array containing 43,533 N. attenuata probes (Gilardoni et al., 2011). This array represented approximately 70 to 80% of the N. attenuata transcriptome (Gase and Baldwin, 2012). Genes were considered to be differentially regulated when \( \log_2(\text{fold-changes; FCs}) \) were larger or equal to 1 or smaller or equal to -1 (ir-hspro vs. WT) and \( q \)-values were lower than 0.05 (corresponding to a false discovery rate (FDR) less than 5%). Using these conditions, transcripts corresponding to 11 genes were differentially expressed in control roots of ir-hspro seedlings (Fig. 5a and Suppl. Table SII) while 417 genes were differentially expressed in P. indica-colonized roots of ir-hspro seedlings (Fig. 5b and Suppl. Table SII). In control roots, nine transcripts were up- and two down-regulated while in colonized roots, 293 transcripts were up- 124 were down-regulated (Fig. 5a and b). Eight genes (all of unknown function) were differentially up-regulated in both control and P. indica-colonized roots (Fig. 5c and Suppl. Table SII).

In P. indica-colonized roots and based on the biological process (BP), 60.6% of the annotated genes were involved in metabolic processes while 18.1% in responses to stimuli (Fig. 5d). Analysis of enzyme codes (EC; Suppl. Tables SII and SIII), revealed that the most prevalent changes in gene expression occurred in enzymes involved in metabolic processes associated with the metabolism of starch and sugars, purines, nicotinate and nicotinamide, and membrane glycerophospholipids (Table I). Moreover, several genes involved in the transport of metabolites or ions were also affected in their expression (Table I). GO categorization by molecular function (MF) showed that genes encoding for enzymes with acyltransferase (14.2%), hydrolase (12.7%), and nucleotide binding (14.2%) activities were the most prevalent genes changing expression levels in ir-hspro roots (Fig. 5e and Table II). The changes in the expression of genes involved in metabolic processes were consistent with the differential growth rate of ir-hspro seedlings, and showed that the growth response was accompanied by significant changes in metabolic gene expression. The changes in the expression of genes involved in responses to stimuli (as the second most prevalent group of
genes; Fig. 5d) most likely reflected the processes affected in ir-hspro seedlings that were more directly connected with the interaction of roots with P. indica. The expression of several genes associated with phytohormone signaling was affected in ir-hspro roots and these included genes associated with JA (jasmonate zim-domain protein [JAZ], coronatine-insensitive 1 [COI1]), auxin (auxin response factor [ARF]) and ABA (abscisic acid insensitivity 1b [ABI1b]) signaling (Table II).

Interaction of P. indica with ir-hspro roots does not affect the accumulation of polar metabolites

The changes in the expression of multiple genes involved in metabolic processes prompted us to investigate if the accumulation of primary and secondary metabolites was affected during the association of roots from ir-hspro seedlings with P. indica. For this purpose, we profiled small polar metabolites extracted from P. indica-colonized roots of WT and ir-hspro seedlings by LC-ToF-MS (liquid chromatography-time-of-flight-mass spectrometry). Root samples were harvested from seedlings grown for 14 days on the plate system and polar metabolites were extracted (see Materials and Methods for details). Ions were selected using the ESI (electrospray ionization) interface in both positive and negative ion modes and those metabolites eluting from the LC column between 125 and 550 sec and having m/z values ranging from 90 to 1400 were selected for analysis. After data analysis (see Material and Methods for a detailed description), no significant differences in the accumulation of ions in roots of ir-hspro and WT seedlings were detected in the negative ion mode (data not shown) and in the positive ion mode, the abundance of only three ions (out of more than 2,500 identified) changed significantly between these genotypes (Suppl. Table SIV). The intensities of these ions were however low and the fold changes small (between 2 and 2.8-fold down-regulated in ir-hspro roots).

Reduced HSPRO expression in roots is sufficient to control differential growth promotion in the whole seedling

We reasoned that if HSPRO had a general role associated with the control of growth instead of a more direct role in the control of the association of P. indica with roots, grafting experiments in which root stocks and shoot scions were reduced or not in HSPRO expression, could provide important information about the function of this gene. Hence, shoot scions and root stocks from either WT or ir-hspro seedlings were reciprocally grafted (Fig. 6a) and the
root, shoot and seedling biomasses were quantified after 19 days of seedling growth in either the presence or absence of *P. indica*. A differential growth promotion was observed (compared to the WT/WT seedlings) in all cases in which either the root stock or the shoot scion belonged to ir-hspro seedlings (Fig. 6b to d). This differential growth promotion was similar to the grafted parental seedlings (ir-hspro-1/ir-hspro-1 and ir-hspro-2/ir-hspro-2; Fig. 6b to d).

**Discussion**

As mentioned in the introduction, the function of *Hs1pro-1* in sugar beet was originally associated with resistance to cyst nematodes, however, several subsequent studies performed in different plant species have suggested that homologs of this gene have a more general role in the plant’s response to environmental stresses (see Introduction for references). Consistent with the observation that HSPRO homologs are induced by multiple stresses in Arabidopsis, we found that *N. attenuata* HSPRO mRNA levels were induced by multiple biotic stress-associated stimuli including simulated lepidopteran herbivory, SA application, *Pst* DC3000 infection, and *P. indica* root colonization (Figs. 2 and 4b and Suppl. Fig. S3b and S9).

*N. attenuata* HSPRO is a negative regulator of seedling growth induced by *P. indica*

Microarray analysis of *P. indica*-colonized roots showed that silencing HSPRO expression brought about significant changes in gene expression and that the largest fraction (~60%) of these genes were involved in metabolic processes (Fig. 5 and Table I). These changes in gene expression were consistent with the accelerated growth of ir-hspro seedlings; increased growth rates are accompanied by increased metabolic rates to meet growth demands (e.g., cell walls and cellular membranes). Additionally, 18% of the genes affected in their expression in roots of ir-hspro seedlings were categorized as “responses to stimuli and stresses” (Fig. 5). The genes in this category probably reflected those genes having a more direct association with the interaction of roots with *P. indica* (Table II). In the absence of *P. indica* colonization, changes in gene expression in roots of ir-hspro plants were very small, with only 11 genes changing expression compared to WT seedlings (Fig. 5a). These results were consistent with a function of HSPRO in the control of metabolism during stress responses.

Similar to the interaction of roots from different plant species with arbuscular mycorrhizal (AM) fungi (Strack et al., 2003; Hause and Fester, 2005; Herrera-Medina et al.,
2007), the interaction between P. indica and plant roots is controlled by multiple phytohormones including auxin and cytokinins (Vadassery et al., 2008), gibberellins (Schafer et al., 2009), ET (Barazani et al., 2007; Camehl et al., 2010), SA and JA (Jacobs et al., 2011). Changes in the mRNA levels of auxin and ET signaling components and a cytokinin biosynthesis gene were affected in colonized roots of ir-hspro seedlings (Table II). Moreover, changes were also detected in the expression of COII and a JAZ homolog (Table II), two components of the JA-signaling pathway (Xie et al., 1998; Turner, 2007; Paschold et al., 2008). Together the results suggested that the phytohormone-signaling network was affected in ir-hspro plants and that these changes are probably part of the mechanisms effecting differential growth promotion of these plants during interaction with P. indica.

Differential expression of genes involved in ET and JA signaling in roots of WT and ir-hspro seedlings were not accompanied however by the differential accumulation of these phytohormones. The levels of JA and ABA in roots did not change during P. indica root colonization compared to uncolonized roots (Suppl. Fig. S11b) and although ET and SA levels were affected by P. indica root colonization the levels were similar between WT and ir-hspro seedlings (Suppl. Fig. S11a). The reduction in SA levels in P. indica-colonized roots may reflect the suppression of defense and cell death responses in N. attenuata seedlings. Lower SA levels in tobacco and Arabidopsis have been correlated with higher Glomus mosseae (Herrera Medina et al., 2003) and P. indica (Jacobs et al., 2011) root colonization, respectively. However, the accumulation of CA, a divinyl-ether strongly induced in leaves of Solanaceae species upon infection by Phytophthora species (Weber et al., 1999; Bonaventure et al., 2011), was also strongly induced in roots by P. indica in both ir-hspro and WT seedlings, indicating that oxylipin-related defense pathways were activated by this fungi (Suppl. Fig. S11c). CA is a DVE which is strongly induced in leaves of tobacco (N. tabacum) and potato (Solanum tuberosum) plants in response to pathogens such as Phytophthora parasitica and P. infestans (Weber et al., 1999; Gobel et al., 2002; Fammartino et al., 2007). It has been shown that DVEs have antimicrobial properties by, for example, inhibiting mycelial growth and spore germination of some Phytophthora species (Prost et al., 2005). Interestingly, during the interaction of G. intraradices with tomato (Solanum lycopersicum) roots, there is a strong induction of genes involved in the formation of oxylipins derived from 9-LOX activity, and it has been suggested that 9-LOX products may control AM fungal spread in roots (Leon-Morcillo et al., 2012).
Gene expression analysis by microarray hybridization showed only three genes directly associated with defense responses and differentially expressed in *P. indica*-colonized roots of ir-*hspro* seedlings (Suppl. Table SII). These observations were consistent with the current understanding of the mechanisms involved in the association of plant growth-promoting rhizobacteria and fungi with roots; the activation of immune responses by plants and their suppression by microorganisms underlay the establishment of long-term mutualistic interactions (Preston, 2004; Van Wees et al., 2008; Zamioudis and Pieterse, 2012).

Grafting experiments demonstrated that the silencing of *HSPRO* expression in roots was sufficient to induce differential growth promotion in both roots and shoots of ir-*hspro* seedlings. Thus, the effect on shoot growth was dependent on the function of HSPRO in the roots. Because small silencing RNAs generated in shoots of *N. attenuata* seedlings can be transported to roots (but not vice versa) and reduce gene expression of the targeted gene in the latter tissue (Fragoso et al., 2011), the similar effect on growth promotion observed in WT(shoot)/ir-*hspro*(root) and ir-*hspro*(shoot)/WT(root) grafted seedlings was most likely explained by shoot-induced silencing of *HSPRO* expression in roots of ir-*hspro*(shoot)/WT(root) grafted seedlings (Fig. 6a). Microscopy analysis of *P. indica*-colonized and uncolonized roots did not reveal morphological changes in roots of ir-*hspro* seedlings compared to WT, changes in the association pattern of *P. indica* with the maturation zone of the root, or differential root cell death (Suppl. Figs. S8, S9 and S10). The microbe-mediated stimulation of plant growth has been associated with improved plant nutrition via increase uptake of growth-limiting soil nutrients. Since *P. indica* root colonization and root growth was not different between ir-*hspro* and WT seedlings and seedlings were grown under conditions of high nutrient availability (media or soil), it is unlikely that increased uptake of growth-limiting nutrients was a main factor influencing the *P. indica*-induced differential growth promotion of ir-*hspro* seedlings. Barazani et al. (2005) showed that the nutritional status of *N. attenuata* plants colonized by *S. vermifera* and *P. indica* does not depend on nitrogen and phosphorous supply, and similar results were reported by Achatz et al. (2010) in *P. indica*-barley symbiosis. Since growth and metabolic rates are mutually dependent, further experiments will be required to disentangle the role of *HSPRO* in the control of growth and/or metabolism. As mentioned in the introduction, the Arabidopsis HSPRO1 and HSPRO2 proteins interact with the AKINβγ subunit of the SnRK1 complex (Gissot et al., 2006). Thus, although speculative at this point, one possible scenario is that HSPRO affects *N. attenuata* seedling growth and/or metabolism via its association with SnRK1.
What’s the role of HSPRO in responses against insects and pathogenic bacteria?

Herbivore attack elicits metabolically costly defenses that can decrease plant fitness by limiting metabolic resources otherwise invested in growth and reproduction (Schwachtje and Baldwin, 2008; Bolton, 2009). The performance of *M. sexta* larvae and the *M. sexta*-OS elicited induction of the defense-associated metabolites nicotine, chlorogenic acid and rutin were similar between ir-hspro and WT plants (Suppl. Fig. S4). Moreover, flower traits associated with the interaction of plants with insects were not affected in *M. sexta*-attacked or unattacked ir-hspro plants (Suppl. Fig. S5). Thus, these results suggested that HSPRO was not directly involved in the regulation of induced defenses or plant-insect association traits.

Similarly, the performance of Pst DC3000 on leaves of ir-hspro plants was unaffected compared to WT plants. These results contrasted those made with Arabidopsis, where plants disrupted in *HSRO2* expression supported increased Pst DC3000 growth in leaves (Murray et al., 2007). However, similar to *HSRO2* expression in Arabidopsis (Murray et al., 2007), *N. attenuata* HSPRO was also induced by SA and Pst DC3000 infection (Fig. 2 and Suppl. S3), suggesting these two genes have both overlapping and divergent functions during bacterial infection in these two plant species.

Although speculative at this point, one possible scenario is that HSPRO regulates plant tolerance mechanisms against insect herbivores. In *N. attenuata*, the mRNA levels of GAL83 (a β-subunit of SnRK1) are rapidly repressed upon insect herbivory and it has been shown that GAL83 is involved in the regulation of tolerance to insect herbivory via the control of resource allocation in roots (Schwachtje et al., 2006). Thus, one possibility is that the HSPRO gene product in *N. attenuata* participates in the regulation of tolerance mechanisms associated with the regulation of SnRK1 activity.

Plants responses to insect herbivores can also involve changes in the association of roots with belowground beneficial microorganisms such as growth-promoting fungi and rhizobacteria (Bardgett et al., 1998; Pineda et al., 2010). For example, insect herbivory can affect mycorrhizal colonization (Gehring and Whitham, 1991; Gehring and Bennett, 2009) or the composition of the root-associated microbial community (Stultz et al., 2009). Thus, although speculative at this point, but given the results obtained with *P. indica*, an alternative (but not excluding) scenario is that HSPRO participates in mechanisms that control the interaction of herbivore-attacked plants with belowground microorganisms (e.g., via changes in nutrient allocation and root exudates). Plants interacting with beneficial microbes can
benefit from an increase in tolerance to herbivory, for example, by affecting C and N reallocation used for tissue regrowth after herbivory (Bardgett et al., 1998). Additionally, plants can also benefit via increased resistance to plant pathogens (Pineda et al., 2010). However, association with beneficial microorganisms can also reduce plant fitness by compromising induced defense responses against insect herbivores (Barazani et al., 2005). Thus, a delicate balance of interactions between roots and microorganisms is required to optimize plant fitness in nature and \textit{HSPRO} may play a role in this process. These hypotheses are the focus of future research.

Conclusions

The results presented in this study have unraveled the important role that \textit{HSPRO} has in the control of early \textit{N. attenuata} seedling growth stimulated by the growth-promoting fungus \textit{P. indica}. Since the effect on growth was only observed when ir-hspro seedlings were colonized by this fungus, and \textit{HSPRO} expression was induced by multiple stress-associated stimuli, the results suggested that \textit{HSPRO} plays an important role in growth and/or metabolism readjustment during stress responses. Although speculative, the control over metabolism during insect herbivory could involve the regulation of resource partitioning between shoots and roots and its resulting consequences in the interaction of roots with soilborne microbes. The results opened new hypotheses on how this control may be achieved, and the interaction of HSPRO with components of the SnRK1 complex appears as one potential scenario. Future work will focus on the disentangling of the \textit{HSPRO}-dependent mechanisms underlying the regulation of growth/metabolism during stress responses.

Materials and Methods

Please refer to online Suppl. Experimental Procedures S1 for additional experimental details.

\textit{Plant growth and treatments}

Seeds of the 31\textsuperscript{st} generation of an inbred genotype of \textit{Nicotiana attenuata}, originally collected from southwestern Utah in 1988 were used for all experiments. For glasshouse experiments, seeds from WT and genetically transformed plants were germinated as previously described (Krügel et al., 2002). Plants were grown in the glasshouse under high-pressure sodium lamps (200-300 \textmu mol s\textsuperscript{-1} m\textsuperscript{-2} light) with a day/night cycle of 16 h (26-
28°C/8 h (22-24°C) and 45 to 55% humidity. For plate experiments with *P. indica* see section “*P. indica* maintenance and inoculation of *N. attenuata* seedlings” below. *N. attenuata* ir-lox3 (Allmann et al., 2010), ir-jar4/6 (Kang et al., 2006), ir-coi1 (Paschold et al., 2008) and ir-sipk (Wu et al., 2007) plants have been previously described.

For wounding and elicitation treatments, leaves were wounded by rolling a fabric-pattern wheel three times on each side of the midvein and the wounds were supplemented either with 10 µL of 0.01% (v/v) Tween-20 in water (wounding treatment), 10 µL of 18:3-Glu (0.03 nmol/µL; FAC elicitation) or 10 µL of *M. sexta* or *Spodoptera exigua* OS (OS elicitation). For analysis of gene expression, *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000) and *Agrobacterium tumefaciens* (GV3101) were grown on Luria-Bertani (LB) liquid medium containing 25 µg mL⁻¹ Rifampicin until OD₆₀₀=0.5 to 0.6. Bacteria were pelleted and re-suspended in 10 mM MgCl₂ to a final OD₆₀₀=0.02 (10⁷ colony forming units (CFU) mL⁻¹). This suspension was syringe-infiltrated into leaves (1 mL leaf⁻¹). As control treatment, leaves were infiltrated with an aqueous solution of 10 mM MgCl₂. For infection assays with Pst DC3000, the bacteria pellet was re-suspended in 10 mM MgCl₂ to a final OD₆₀₀=0.001 (10⁵ CFU mL⁻¹) and the suspension was syringe-infiltrated into leaves (1 mL leaf⁻¹). Leaf discs of 8 mm in diameter were harvested at different times (0, 1, 2 and 3 days), ground in 0.3 mL sterile water and after centrifugation, 1/10 serial dilutions of the supernatant were plated out on LB agar plates containing 25 µg mL⁻¹ Rifampicin. Plates were incubated for two days at 28°C and the CFU were counted. For SA treatment, a solution of 300 µM SA dissolved in 0.2% (v/v) Tween-20/water was used. The solvent alone was used as control treatment. Tissue expression profile of *HSPRO* was evaluated by collecting different plant tissues from WT *N. attenuata* plants; rosette leaves and roots were collected from 30 day-old (rosette stage) plants whereas stalks, stalk leaves, sepals, pistils, corolla, and stamens were collected from 50 day-old plants.

**Generation of stably silenced lines**

A PCR fragment generated with primers ir-hspro-fwd and ir-hspro-rev (Suppl. Table SV) and *HSPRO* cDNA as template was subcloned using *SacI* and *XhoI* (New England Biolabs, Frankfurt, Germany) restriction sites into the pSOL8 transformation vector (Bubner et al., 2006) as an inverted-repeat construct. This construct was used to transform *N. attenuata* WT plants using *Agrobacterium*-mediated transformation and plant regeneration as previously described (Krügel et al., 2002). T₁ transformed plants were analyzed for T-DNA
insertion number by DNA gel blot hybridization (see below). Segregation analysis for hygromycin resistance in T2 seedlings was performed on agar plates supplemented with hygromycin (0.035 mg mL⁻¹). Two lines, ir-hspro1 and ir-hspro2 had a single T-DNA insertion in their genomes, and were used for most of the experiments. A third line, ir-hspro3 had two T-DNA insertions and was used for some experiments. Efficiency of gene silencing (HSPRO mRNA levels) in ir-hspro plants was evaluated by qPCR (see below) after 1 h of 18:3-Glu elicitation using the primers listed in Suppl. Table SV. For Southern blot analysis, genomic DNA from WT and ir-hspro plants was isolated by the cetyltrimethylammonium bromide (CTAB) method. DNA samples (5 µg) were digested with EcoRV (New England Biolabs) overnight at 37°C according to commercial instructions and separated on a 0.8% (w/v) agarose gel using standard conditions. DNA was blotted onto Gene Screen Plus Hybridization Transfer membranes (Perkin Elmer Life and Analytical Sciences, Boston, MA) using the capillary transfer method. A gene-specific probe for the hygromycin resistance gene hptII was generated by PCR using the primers HYG1-18 and HYG3-20 (Suppl. Table SV). The probe was labeled with [α-³²P]dCTP (Perkin Elmer) using the Rediprime II kit (Amersham Pharmacia, Freiburg, Germany) according to commercial instructions.

**P. indica maintenance and colonization of N. attenuata seedlings**

*P. indica* was maintained on Kaefer medium (KM; a modified *Aspergillus* minimal medium) (Pham GH, 2004) containing 1% (w/v) agar. For seedling colonization, 9 cm discs of polyamide mesh (pore 70 µm/thickness 80 µm; SEFAR Gmbh, Wasserburg, Germany) were placed on top of 9 cm agar plates containing modified plant nutrient culture medium (5 mM KNO₃, 2 mM MgSO₄, 2 mM Ca(NO₃)₂, 0.01 µM FeSO₄, 70 µM H₂BO₃, 14 µM MnCl₂, 0.5 µM CuSO₄, 1 µM ZnSO₄, 0.2 µM Na₂MoO₄, 0.01 µM CoCl₂, 10.5 g L⁻¹ agar, pH 5.6, 0.6% (w/v) agar). Two 7-day-old *N. attenuata* seedlings germinated on Gamborg B5 medium (pH: 6.8; 0.6 % (w/v) agar) were laid on the polyamide discs at a distance of 1 cm from an agar plug placed in the center of the plate and containing a 2-week-old *P. indica* culture (Fig. 4a). Agar plugs without fungus were used as control. The plates were incubated horizontally for 10 or 14 days at 21°C and light was supplied from the side for 16 h day⁻¹ with a white fluorescent light source (80 µmol m⁻² s⁻¹). The fresh biomass of total seedlings, roots and shoots was determined with a microbalance. Seedling grafting was performed as previously described (Fragoso *et al.*, 2011). After grafting, seedlings were first kept for 5 days on Gamborg’s B5 medium containing 0.8% (w/v) agar for recovery and were then transferred to
agar plates covered with polyamide mesh discs and pre-incubated (7 days before) with *P. indica* agar plugs. The fresh biomass of total seedlings, roots and shoots was determined in this case 19 days after the transferring of the seedlings to the *P. indica*-containing plates (due to the slower growth of grafted seedlings compared to intact seedlings).

**Quantitative real-time PCR**

Total RNA was extracted using the TRIzol® reagent (Invitrogen, Karlsruhe, Germany) and 5 µg of total RNA were reverse transcribed using oligo(dT)$_{18}$ and SuperScript reverse transcriptase II (Invitrogen). Quantitative real-time PCR (qPCR) was performed with a Mx3005P Multiplex qPCR system (Stratagene, La Jolla, CA) and the qPCR Core kit for SYBR® Green I (Eurogentec, Liege, Belgium). Relative quantification of *HSPRO* mRNA levels was performed by the comparative $\Delta$Ct method using the elongation factor 1A (*Na-EF1A*) mRNA as an internal standard (Gilardoni *et al.*, 2010). The sequences of the primers used for qPCR are listed in Suppl. Table SV. All the reactions were performed using the following qPCR conditions: initial denaturation step of 95°C for 10 min, followed by 40 cycles each of 95°C for 15 s and 60°C for 1 min, with a final extension step of 95°C for 15 s and 60°C for 30 sec. All samples were obtained from at least three independent biological replicates ($n=3$) for each time point, plant genotype, and treatment.

For quantification of *P. indica* colonization rates, DNA was extracted from *P. indica*-colonized roots and control roots by the CTAB method. qPCR was performed using SYBR® Green and 20 ng of isolated DNA as template. Copy number of the *P. indica* translation elongation factor 1a (*Pi-EF1A*) gene (Deshmukh *et al.*, 2006) relative to the *Na-EF1A* gene was used to quantify colonization rates of *P. indica* by the comparative $\Delta$Ct method. The primers used are listed in Suppl. Table SV.

**Statistical analysis**

Statistics were calculated using the SPSS software version 17.0. The data was subjected either to one-way analysis of variance (ANOVA; and means were compared by the Tukey post-hoc test). For analysis of differences in bolting and flowering time the Kolmogorov-Smirnov (KS) test was used. The number of replicates ($n$) used in each experiment are detailed in the Figure’s captions.
Accession numbers

Data from this article can be found under the following accession numbers: Na-HSPRO (JQ354963; GenBank database), Agilent Chip platform (GPL13527; NCBI GEO database), microarray data (GSE35086; NCBI GEO database).

Suppl. Material

Suppl. Experimental Procedures S1.

Table S1. Analysis of WT and ir-hspro seedling’s biomasses during P. indica-root colonization and control treatments.

Table SII. List of genes changing expression in control and P. indica-colonized roots of ir-hspro seedlings compared to WT.

Table SIII. List of annotated genes involved in metabolism and with enzyme codes differentially expressed in P.indica-colonized roots of ir-hspro seedlings.

Table SIV. List of ions with differential accumulation in P. indica-colonized roots of ir-hspro seedlings compared to WT seedlings (positive mode of ionization).

Table SV. List of primers.

Figure S1. Alignment of N. attenuata HSPRO protein sequence with close homologs in different plant and moss species.

Figure S2. Alignment of N. attenuata HSPRO protein sequence with homologs in A. thaliana and B. procumbens.

Figure S3. Analysis of HSPRO expression in WT plants and in transgenic plants reduced in MAP kinase expression.

Figure S4. Analysis of defense responses against M. sexta in WT and ir-hspro plants.

Figure S5. Analysis of flower traits associated with the interactions of N. attenuata plants with insects.

Figure S6. Analysis of growth and developmental parameters of P. indica-colonized plants grown in the glasshouse.

Figure S7. Analysis of growth promotion of WT and ir-hspro seedlings induced by P. indica in a split-plate system.

Figure S8. Laser confocal microscopy analysis of roots from P. indica-colonized WT and ir-hspro seedlings.

Figure S9. Root morphology of P. indica-colonized WT and ir-hspro seedlings.

Figure S10. Analysis of root cell death in WT and ir-hspro seedlings.
Figure S11. Analysis of SA, JA and CA levels in roots of WT and ir-hspro seedlings.

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Figure Legends

Fig. 1. Analysis of HSPRO amino acid sequence and cellular localization.

(a) Schematic protein sequence alignment of N. attenuata HSPRO (JQ354963), Arabidopsis HSPRO1 (At2g4000) and HSPRO2 (At3g55840), and B. procumbes Hs1pro-1 (U79733 plus DQ148271). The cartoon above the sequences shows the % of similarity (green bars within the overlapping regions represent identical amino acids in the four sequences). See Suppl. Fig. S2 for a detailed amino acid alignment. (b) Phylogenetic analysis of HSPRO proteins from
different organisms. The tree was constructed using the Geneious Pro software (5.3.4) with the Jukes-Cantor genetic distance model and the Neighbor Joining tree building method with bootstrapping (602 random seed, 100 replicates and 50% support threshold). See Suppl. Fig. S1b for a reference to accession numbers. (c) Arabidopsis mesophyll protoplasts were isolated and transiently transfected with vectors carrying either EGFP alone (cytosolic localization), EGFP C-terminal fusions with RGA (RGA-EGFP; nuclear localization), and EGFP C-terminal fusions with HSPRO (HSPRO-EGFP) under regulation of the CaMV35S promoter. After transfection, protoplasts were incubated for 15 h in the dark at room temperature and images were taken with a Zeiss Axioplan fluorescence microscope with standard settings for EGFP.

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The levels of HSPRO mRNA were analyzed by qPCR in leaves of WT and transgenic N. attenuata plants after different treatments and in different plant organs and tissues. mRNA levels are expressed relative to the levels of the reference gene Na-EF1A. Quantification was performed by the ΔCt method (n=3 to 6; bars= ±S.E.). (a) Elicitation of leaves from WT plants with OS from M. sexta and S. exigua larvae, synthetic 18:3-Glu, or wounding. One way-ANOVA with Tukey post-hoc test (M. sexta OS vs. wounding); ***: P<0.001. (b) Elicitation of leaves from WT and transgenic lines with synthetic 18:3-Glu. One way-ANOVA with Tukey post-hoc test (ir-lox3 vs. WT); **: P<0.01. (c) Infection of leaves from WT plants with Pst DC3000 and A. tumefaciens (GV3101). One way-ANOVA with Tukey post-hoc test (Pst DC3000 vs. control); **: P<0.01; ***: P<0.001. (d) HSPRO mRNA levels in different organs and tissues of WT N. attenuata plants. Relative levels of HSPRO mRNA in roots were set arbitrarily to one. One way-ANOVA with Tukey post-hoc test (roots vs. other tissues); *: P<0.05; **: P<0.01; ***: P<0.001.

**Fig. 3. Characterization of ir-hspro plants.**

(a) Southern blot analysis of the ir-hspro transgenic lines. Genomic DNA from four independent ir-hspro N. attenuata lines (1 to 4) and WT plants was digested with EcoRV and resolved by agarose gel electrophoresis. A 32P-labeled fragment corresponding to the hygromycin resistance gene nptII was used as a probe. The white arrows point to individual T-DNA insertions (lane 2): ir-hspro1; lane (3): ir-hspro2; lane (4): ir-hspro3). (b) Analysis of HSPRO mRNA levels in leaves of ir-hspro lines at 1 h after 18:3-Glu elicitation (n=6; bars=
Relative mRNA levels were quantified as detailed in caption of Fig. 1. One way-ANOVA with Tukey post-hoc test (WT vs. ir-hspro); ***: P<0.001. (c) Morphology of WT and ir-hspro plants at the late elongation stage. (d) Rosette growth curve (measured as rosette diameter) of WT and ir-hspro plants (n=8 to 20; bars= ±S.E.).

**Fig. 4. Induction of differential growth promotion of ir-hspro seedlings by *P. indica***.
(a) Plate system used for the experiments. Five days after germination in standard agar media, two seedlings were transferred onto a nylon mesh covering an agar plate at 1 cm distance from a plug transferred from a 2-week old *P. indica* culture. (b) Analysis of *HSPRO* mRNA levels in roots of WT and ir-hspro seedlings during interaction with *P. indica* and in control treatment (absence of *P. indica*). Root samples were harvested at day 14 (n=3; bars= ±S.E.) and mRNA levels were quantified as detailed in caption of Fig. 1. b.d.: below detection limit.
(c to e) Determination of the fresh biomass of total seedlings, shoots, and roots was performed with a microbalance after 14 days of seedling growth on the plate system (n=18 to 20; bars= ±S.E.); One way-ANOVA with Tukey post-hoc test (WT vs. ir-hspro during *P. indica* colonization); **: P<0.01; ***: P<0.001. (f) Quantification of *P. indica* root colonization. DNA was extracted from roots of WT and ir-hspro seedlings colonized by *P. indica* at day 14 and fungal colonization was determined by qPCR based on the relative abundance of the Pi-EF1a gene compared to the Na-EF1a gene (n=16 to 18; bars= ±S.E.). b.d.: below detection limit.

**Fig. 5. Microarray and Gene Ontology (GO) analysis of differentially expressed genes in *P. indica*-colonized roots of ir-hspro and WT seedlings**
(a) Distribution of FC (fold changes) of genes expressed differentially in roots of ir-hspro seedlings (ir-hspro vs. WT) (b) Distribution of FC of genes expressed differentially in roots of ir-hspro seedlings colonized by *P. indica* (ir-hspro vs. WT). (c) Venn diagram of the number of genes differentially expressed in control and colonized roots of ir-hspro seedlings compared to WT. The number in the intersection represents the genes differentially expressed in the two groups. (d,e) Annotated genes differentially expressed in *P. indica*-colonized roots of ir-hspro seedlings were categorized based on (d) biological processes and (e) molecular function, using the Blast2Go software. The numbers between brackets represent the
percentage (%) of genes in the metabolism category (M) or in the stress response category (RS).

**Fig. 6. Reciprocal grafting of ir-hspro and WT seedlings and determination of seedling biomass during root colonization by *P. indica.***

(a) Scheme of the grafting combinations used. (b to d) Determination of the fresh biomass of total seedlings, shoots, and roots was performed with a microbalance after 19 days of seedling growth on the plate system. One way-ANOVA with Tukey post-hoc test (WT vs. ir-hspro during *P. indica* colonization); *: \( P<0.05 \); **: \( P<0.01 \); ***: \( P<0.001 \) (\( n=18 \) to 20; bars=±S.E.).
# Tables

Table I. List of selected genes differentially expressed in *P. indica*-colonized roots of ir-
*hspro* seedlings and involved in metabolic processes.

| Gene ID | FC\(^a\) | SD | q value | Gene description                  | Enzyme Codes                  |
|---------|---------|----|---------|-----------------------------------|-----------------------------|
| Na_08858 | 3.4     | 1.1 | 2.8E-02 | alpha amylase precursor           | EC:3.2.1.60; EC:3.2.1.1     |
| Na_36852 | 3.0     | 1.0 | 8.7E-03 | UDP-glucuronate 4-epimerase 3-like| EC:5.1.3.6                   |
| Na_32458 | 2.8     | 1.2 | 1.7E-02 | granule-bound starch synthase      | EC:2.4.1.21                  |
| Na_31027 | 2.7     | 0.8 | 6.7E-03 | beta-glucosidase 41               | EC:3.2.1.21                  |
| Na_36520 | 2.6     | 0.5 | 4.9E-03 | beta-galactosidase precursor      | EC:3.2.1.23                  |
| Na_02547 | 2.8     | 0.8 | 1.0E-02 | UDP-glucuronate 4-epimerase 3-like| EC:5.1.3.6                   |
| Na_02156 | 3.0     | 1.1 | 2.9E-03 | beta-glucosidase 41               | EC:3.2.1.21                  |
| Na_32458 | 2.8     | 1.2 | 1.7E-02 | granule-bound starch synthase      | EC:2.4.1.21                  |
| Na_31027 | 2.7     | 0.8 | 6.7E-03 | beta-glucosidase 41               | EC:3.2.1.21                  |
| Na_36520 | 2.6     | 0.5 | 4.9E-03 | beta-galactosidase precursor      | EC:3.2.1.23                  |

\(\text{Starch and sugar metabolism}\)

| Na_12907 | 3.3     | 1.0 | 1.4E-02 | nucleoside-triphosphatase         | EC:3.6.1.15                  |
| Na_09592 | 2.8     | 0.2 | 4.0E-02 | adenosinetriphosphatase           | EC:3.6.1.3                   |
| Na_26486 | 2.6     | 0.3 | 3.5E-02 | guanylate kinase                  | EC:2.7.4.8                   |
| Na_12713 | 2.1     | 0.1 | 1.1E-02 | 5'-nucleotidase                   | EC:3.1.3.5                   |

\(\text{Purine metabolism}\)

| Na_38363 | 2.9     | 0.6 | 2.3E-02 | nicotinamidase                    | EC:3.5.1.19                  |
| Na_12713 | 2.1     | 0.2 | 1.1E-02 | 5'-nucleotidase                   | EC:3.1.3.5                   |
| Na_34109 | 2.0     | 0.5 | 2.9E-02 | aldehyde oxidase                  | EC:1.2.3.1                   |

\(\text{Nicotinate and nicotinamide metabolism}\)

| Na_39990 | 2.1     | 0.3 | 5.7E-03 | glycerophosphodiester phosphodiesterase | EC:3.1.4.46 |
| Na_12017 | 2.0     | 0.2 | 3.7E-02 | glycerol-3-phosphate dehydrogenase  | EC:1.1.1.94; EC:1.1.1.8 |

\(\text{Glycerophospholipid metabolism}\)

| Na_05066 | 3.6     | 0.9 | 4.0E-03 | peptide transporter protein       |                             |
| Na_13676 | 3.2     | 0.7 | 2.0E-03 | monosaccharide-sensing protein     |                             |
| Na_27616 | 3.2     | 1.0 | 2.5E-02 | amino acid permease 6              |                             |
| Na_09592 | 2.8     | 0.2 | 4.0E-02 | abc transporter family protein     |                             |
| Na_34386 | 2.3     | 0.3 | 4.9E-02 | aminophospholipid atpase           |                             |
| Na_25349 | 2.2     | 0.1 | 1.1E-03 | abc transporter c family member 4-like |                           |
| Na_21786 | 2.2     | 0.2 | 4.8E-02 | two-pore calcium channel           |                             |
| Na_10110 | 2.1     | 1.1 | 4.9E-02 | potassium channel                  |                             |
| Na_27994 | 0.5     | 0.1 | 2.1E-03 | lipid-transfer protein             |                             |
| Na_24731 | 0.5     | 0.1 | 7.1E-03 | bidirectional sugar transporter sweet2-like |                     |

\(\text{a: (fold change; ir-}\)\*hspro\* vs. WT)
Table II. List of selected genes differentially expressed in *P. indica*-colonized roots of ir-*hspro* seedlings and involved in regulatory processes

| Gene ID    | FC*  | SD  | q value    | Gene description                                      |
|------------|------|-----|------------|-------------------------------------------------------|
| Na_10354   | 11.5 | 2.1 | 7.2E-05    | transcription factor LIM1                              |
| Na_40479   | 2.2  | 0.5 | 2.8E-02    | NAC domain transcription factor                       |
| Na_31687   | 0.5  | 0.1 | 7.8E-03    | NPL6-like                                             |
| Na_26270   | 0.5  | 0.1 | 2.3E-02    | retinoblastoma-related protein 1-like (RB1-like)      |
| Na_06561   | 0.5  | 0.1 | 2.1E-02    | MYB transcription factor                              |
| Na_29071   | 0.4  | 0.1 | 1.5E-03    | MYB transcription factor                              |
| Na_36458   | 3.0  | 1.1 | 4.8E-03    | serine threonine protein kinase                       |
| Na_25553   | 3.0  | 0.6 | 2.3E-02    | protein kinase domain-containing protein              |
| Na_18646   | 2.8  | 1.0 | 1.7E-02    | CBL-interacting serine threonine-protein kinase       |
| Na_41571   | 2.6  | 0.5 | 2.2E-03    | protein kinase 1b                                     |
| Na_18066   | 2.6  | 0.4 | 6.9E-03    | receptor like protein kinase -like                    |
| Na_38660   | 2.5  | 0.5 | 4.8E-02    | protein kinase family protein                         |
| Na_15274   | 2.4  | 0.5 | 1.3E-02    | TCTR2 protein                                         |
| Na_26181   | 0.5  | 0.1 | 2.5E-02    | leucine-rich repeat receptor-like protein kinase       |
| Na_21188   | 0.5  | 0.0 | 4.9E-03    | leucine-rich repeat protein kinase-like protein        |
| Na_36236   | 3.1  | 1.0 | 3.2E-03    | zeatin o-glucosyltransferase                          |
| Na_13465   | 2.6  | 1.0 | 4.1E-02    | ABA responsive transcription factor                   |
| Na_43242   | 2.5  | 0.6 | 4.1E-02    | indole-3-acetic acid-amido synthetase                 |
| Na_20394   | 2.3  | 0.7 | 2.2E-02    | jasmonate zim-domain protein (JAZ)                    |
| Na_04958   | 2.3  | 0.1 | 2.6E-03    | coronatine-insensitive 1 (COI1)                       |
| Na_15777   | 2.0  | 0.5 | 5.0E-02    | auxin response factor (ARF)                           |
| Na_28242   | 2.0  | 0.5 | 3.0E-02    | abscisic acid insensitivity 1b (ABI1b)                |
| Na_24453   | 0.5  | 0.0 | 3.6E-04    | auxin-induced saur-like protein                       |
| Na_10858   | 0.5  | 0.1 | 2.8E-02    | ethylene-responsive transcription factor WIN1         |
| Na_00974   | 2.6  | 1.0 | 3.1E-02    | vicilin-like antimicrobial peptides 2-1-like          |
| Na_40698   | 2.0  | 0.4 | 7.2E-03    | disease resistance protein                            |
| Na_42497   | 0.5  | 0.1 | 6.8E-03    | callose synthase 10-like                              |

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- **F**: Quantification of *P. indica* root colonization. DNA was extracted from roots of WT and ir-hspro seedlings colonized by *P. indica* at day 14 and fungal colonization was determined by qPCR based on the relative abundance of the Pi-EFla gene compared to the Na-EFla gene (*n*=16 to 18; bars= S.E.). b.d.: below detection limit.
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