Barley Leaf Transcriptome and Metabolite Analysis Reveals New Aspects of Compatibility and *Piriformospora indica*–Mediated Systemic Induced Resistance to Powdery Mildew

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Colonization of barley roots with the basidiomycete fungus *Piriformospora indica* (Sebacinales) induces systemic resistance against the biotrophic leaf pathogen *Blumeria graminis* f. sp. *hordei* (*B. graminis*). To identify genes involved in this mycorrhiza-induced systemic resistance, we compared the leaf transcriptome of *P. indica*-colonized and noncolonized barley plants 12, 24, and 96 h after challenge with a virulent race of *B. graminis*. The leaf pathogen induced specific gene sets (e.g., LRR receptor kinases and WRKY transcription factors) at 12 h postinoculation (hpi) (prepenetration phase) and vesicle-localized gene products 24 hpi (haustorium establishment). Metabolic analysis revealed a progressing shift of steady state contents of the intermediates glucose-1-phosphate, uridinediphosphate-glucose, and phosphoenolpyruvate 24 and 96 hpi, indicating that *B. graminis* shifts central carbohydrate metabolism in favor of sucrose biosynthesis. Both *B. graminis* and *P. indica* increased glutamine and alanine contents, whereas substrates for starch and nitrogen assimilation (adenosinediphosphate-glucose and oxoglutarate) decreased. In plants that were more *B. graminis* resistant due to *P. indica* root colonization, 22 transcripts, including those of pathogenesis-related genes and genes encoding heat-shock proteins, were differentially expressed $\geq$ twofold in leaves after *B. graminis* inoculation compared with non-mycorrhized plants. Detailed expression analysis revealed a faster induction after *B. graminis* inoculation between 8 and 16 hpi, suggesting that priming of these genes is an important mechanism of *P. indica*-induced systemic disease resistance.

Powdery mildew is an important disease of crop plants, including wheat and barley. In barley, the disease is caused by the obligate biotrophic fungus *Blumeria graminis* f. sp. *hordei* (*B. graminis*). Interaction of compatible and incompatible races of this fungus with barley is well characterized on the microscopic level (Aist and Bushnell 1991; Ellingboe 1972; Huckelhoven et al. 1999) and on the level of transcriptional changes of the host (Bischof et al. 2011; Wise et al. 2007). The recent sequencing of the *B. graminis* genome (Spanu et al. 2010) and feasibility of functional tests (Douchkov et al. 2005; Nowara et al. 2010) make it an even more valuable model biotrophic pathogen.

To fight agronomic losses due to powdery mildew, a better understanding of molecular mechanisms of disease resistance is required. Resistance to pathogens is categorized into basal resistance triggered by the perception of pathogen-associated molecular patterns (PAMPs) present in a wide range of pathogens, and resistance (*R*)-gene-mediated resistance of specific plant cultivars which developed the ability to recognize specific pathogen-derived proteins (effectors) or pathogen-induced changes of host molecules triggered by specialized pathogens which overcome PAMP-triggered resistance (Jones and Dangl 2006). *R* genes have been widely used in crop breeding programs but were not durable in most cases (Johnson 1984). A potentially powerful approach to enhance resistance against a broad range of pathogens is the activation of acquired resistance. It can be induced locally or systemically by encounters with incompatible or necrotizing pathogens (Durrant and Dong 2004; Langen and Kogel 2005; Ryals et al. 1996; Sticher et al. 1997). This type of resistance is termed systemic acquired resistance (SAR), characterized by systemic upregulation of salicylic acid (SA)-regulated transcripts (e.g., of pathogenesis-related [PR] genes). Also, nonpathogenic root-colonizing bacteria can provide resistance in distal tissues, a mechanism termed induced systemic resistance (ISR), which requires jasmonic acid signaling and also ethylene signaling (Van Loon et al. 1998). In addition, several natural and chemical compounds (Kuc 2001) can enhance the plants' capacity to mount a defense response, a process also known as priming (Conrath et al. 2002).

Root colonization by the basidiomycete fungus *Piriformospora indica* protected various host plants, including cereals, against abiotic and biotic stresses (Serfling et al. 2007; Stein et al. 2008; Waller et al. 2005). Colonization of roots by *P. indica*

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is restricted to the root cortex, and *Arabidopsis* plants defective in the jasmonate defense pathway are compromised in *P. indica*-induced resistance; therefore, the underlying mechanism was classified as ISR (Stein et al. 2008; Waller et al. 2005). Furthermore, experiments under field conditions showed that *P. indica* is also able to increase grain yield in barley (Achatz et al. 2010; Waller et al. 2005). This is consistent with reports that showed growth promotion, yield increase, and abiotic stress tolerance in a range of mono- and dicotyledonous plants colonized with the endophytic fungus (Fakhro et al. 2010; Peskan-Berghofer et al. 2004; Pham et al. 2004; Sherameti et

al. 2008; Verma et al. 1998). Therefore, ISR strategies based on *P. indica* and related *Sebacinales* spp. could provide an approach to enhance resistance against a broad range of pathogens without high metabolic cost.

Microscopic investigation of *B. graminis*-challenged barley leaves from plants colonized with *P. indica* revealed an enhanced active defense, visible as an increase in the number of effective papillae formed in response to attempted *B. graminis* penetration of epidermal cells (Waller et al. 2005). However, root colonization by *P. indica* did not elevate SA-regulated defense genes in barley leaves (Waller et al. 2005), pointing to a mechanism different from the well-characterized SAR.

To further characterize the molecular basis of the observed systemic resistance induced by *P. indica*, we analyzed the leaf transcriptome and quantified 70 leaf metabolites. We analyzed expression data of *P. indica*-colonized and noncolonized barley leaves at three defined stages of the interaction with a virulent race of *B. graminis*. Specific leaf metabolite patterns could be obtained for both *B. graminis* challenge and systemic effects of the root endophyte. Among several hundred *B. graminis*-induced barley probe sets, 22 transcripts were identified as differentially induced in leaves of *P. indica*-colonized plants during the early interaction with *B. graminis*, providing molecular evidence for the priming processes induced by root mycorrhization.

RESULTS

Diverse sets of barley genes are induced at distinct stages of the interaction with *B. graminis*.

To analyze the barley leaf transcriptome, the Affymetrix Barley1 22K Gene Chip (Close et al. 2004) was used. *P. indica* colonized and noncolonized 3-week-old barley plants were inoculated with *B. graminis* or mock inoculated and third leaves were harvested at 12 h postinoculation (hpi), corresponding to the recognition and pre-penetration phase; 24 hpi, corresponding to haustorium establishment; and 96 hpi, corresponding to biotrophic fungal development. A leaf segment test (Waller et al. 2005) was used to measure the outcome of the *B. graminis* attack on *P. indica* colonized and noncolonized plants. In the three independent experiments used for transcriptome analysis,

Fig. 1. Powdery mildew-regulated transcripts in barley leaves. Venn diagram showing numbers of *Blumeria graminis* f. sp. *hordei*-responsive transcripts ($\geq$ twofold upregulated [red] or downregulated [blue]; false discovery rate < 0.05) in barley leaves 12, 24, and 96 h postinoculation (hpi) with the pathogen. Mean fold-inductions of transcript levels were calculated from levels detected in three independent experiments by hybridization to the Affymetrix Barley1 GeneChip.

GO Term (Biological Process)	SIZE	FDR
12 hpi <i>Bgh</i> upregulated		
apoptosis	63	0.0001
intracellular protein transport	32	0.0031
response to wounding	12	0.0113
defense response	42	0.0147
protein folding	43	0.0271
protein ubiquitination	18	0.0295
24 hpi <i>Bgh</i> upregulated		
defense response	42	0.0
protein folding	43	0.0008
96 hpi <i>Bgh</i> upregulated		
protein folding	43	0.0048
defense response	42	0.0077
cell wall macromolecule catabolic process	16	0.0291
12 hpi <i>Bgh</i> downregulated		
photosynthesis	28	0.0005
carbon fixation	14	0.0075
24 hpi <i>Bgh</i> downregulated		
response to auxin stimulus	20	0.0010
carbon fixation	14	0.0010
96 hpi <i>Bgh</i> downregulated		
response to auxin stimulus	20	0.0103
transcription	135	0.0337
photosynthesis	28	0.0441

Fig. 2. Functional gene sets overrepresented among *Blumeria graminis* f. sp. *hordei*-regulated barley transcripts 12, 24, and 96 h postinoculation (hpi). Functional gene sets significantly (false discovery rate < 0.05) overrepresented among *B. graminis*-responsive transcripts 12, 24, and 96 hpi. Enrichment analysis was done using the gene set enrichment analysis algorithm (Subramanian et al. 2005). Functional categories (gene ontology [GO] terms) were obtained from the AgriGO database (Du et al. 2010). Number of transcripts contained in respective categories is given as size. Only categories with sizes larger than 10 are displayed.

B. graminis colony numbers were reduced by 33 to 59% in *P. indica*-colonized plants.

Comparing *B. graminis*-inoculated with mock-inoculated leaves, 1,339 up- and 639 downregulated transcripts were detected at 12 hpi (mean of three experiments $\geq$ twofold or $\leq$ twofold, false discovery rate [FDR] < 0.05), representing approximately 9.5% of all probe sets on the Gene Chip. At 24 hpi, 350 up- and 286 downregulated and, at 96 hpi, 81 up- and 84 downregulated transcripts were detected (Fig. 1). In all, 187 transcripts affected by *B. graminis* at 12 hpi were also present among those detected at 24 hpi, while 92 overlapped between 24 and 96 hpi. In total, 34 transcripts were *B. graminis* regulated at all three time points (Fig. 1). On the other hand, among 1,339 upregulated transcripts at 12 hpi, 1,185 were specific for this time point and not significantly regulated more than twofold at either of the two other time points (Supplementary Table 1).

Using gene-set enrichment analysis, we identified functional gene categories (gene ontology [GO] terms) within *B. graminis* up- and downregulated barley transcripts. Functional categories significantly overrepresented among upregulated

transcripts were “defense response” and “protein folding” at all three time points (Fig. 2). Interestingly, the categories “photosynthesis” and “carbon fixation” were overrepresented among downregulated transcripts at all three time points. Transcripts upregulated at 12 hpi were enriched in the categories “apoptosis”, “intracellular protein transport”, and “protein ubiquitination” (Fig. 2).

Analysis of powdery mildew-regulated barley transcript clusters reveals significant overrepresentation of specific functions.

To identify co-regulated transcripts and to characterize relevant functions during *B. graminis* pathogenesis, we performed k-means clustering of differentially expressed transcripts and analyzed GO enrichment of the resulting 12 clusters (Fig. 3). Among three clusters representing transcripts expressed at higher levels after *B. graminis* inoculation 12 hpi, clusters 2 and 7 showed a significant overrepresentation of the GO terms “protein kinase activity” and “transcriptional regulation”, concomitant with expected signaling in response to the pathogen at this time point. Among two clusters representing transcripts

Fig. 3. Overview of 12 different clusters of *Blumeria graminis* f. sp. *hordei*-regulated transcripts. Using the k-means clustering algorithm, 12 clusters were calculated based on the list of transcripts differentially expressed in at least one comparison. Prior to cluster analysis, the data was standardized. Each diagram shows the relative transcript levels 12, 24, and 96 h after mock (control) and *B. graminis* treatment, respectively. Gray curves indicate relative transcript levels of the genes contained in the cluster and red curves indicate mean transcript levels. Number of genes contained in each cluster is given within the respective diagram. In each cluster image, significantly overrepresented prominent functional (F) and cellular (C) transcript categories are indicated. Number of genes within the cluster belonging to the respective indicated category is given in parentheses. A detailed list of overrepresented functional categories is provided in Supplementary Table 2.

upregulated by *B. graminis* at all time points, cluster 10 was significantly enriched in genes of the functional categories “kinase activity”, “transcription factor activity”, apoptosis, and “protein transport/localization”.

Transcripts induced predominantly at 24 hpi, represented by cluster 11, showed no overrepresentation of functional GO terms but of vesicular localization. Vesicle transport is regarded as important for pathogen responses and also for establishment of the haustorium, which takes place from 24 hpi onward. “Vesicular localization” was also overrepresented in cluster 3 (*B. graminis* upregulated 12 and 24 hpi), and 24- and 96-hpi *B. graminis*-upregulated transcripts (cluster 9) were enriched in the categories “structural molecules” and “structural constituents of ribosome”.

Beside those gene classes induced after *B. graminis* inoculation, several clusters represented patterns corresponding to *B. graminis*-repressed transcripts. No significant overrepresentation of functional categories was detected for clusters 5 and 6 (downregulated 12 hpi), while cluster 12 (including transcripts downregulated by *B. graminis* at all three time points) was characterized by overrepresentation of the category “DNA binding”. The prevalence of GO terms related to signal transduction processes among the 12-hpi *B. graminis*-regulated transcripts became also apparent when visualizing the functional category “Regulation” using the MapMan tool (Usadel et al. 2005): Large-scale changes were visible within the subcategories “transcription factors”, “receptor kinases”, “calcium signaling”, and “hormone-related transcripts” (Supplementary Fig. 1). Among the MapMan category transcription factors, several transcripts $\geq$ twofold upregulated at 12 hpi were identified (Supplementary Fig. 3 and 6). Most of these transcription factors exhibited a transient increase at 12 hpi with no or only weak *B. graminis*-dependent expression at 24 and 96 hpi. Likewise, receptor-like kinases were also transiently upregulated at 12 hpi (Supplementary Fig. 2), as were transcripts belonging to the category “phenylpropanoid and lignin metabolism” (Supplementary Fig. 4).

Powdery mildew induces a shift of steady-state contents of the intermediates G1P, UDP-Glc, and PEP at 24 and 96 hpi.

Using the same leaf material as for transcriptome analysis, more than 70 key metabolites of primary metabolism, including free sugars, free amino acids, carboxylates, phosphorylated intermediates, and antioxidants, were assessed at 12, 24, and 96 hpi (Horst et al. 2010) (Supplementary Table 3). In those samples taken at the end of the light period, intermediates of both glycolysis (in particular, phosphoenolpyruvate [PEP] and 3-phosphoglyceric acid [3-PGA]) and the tricarboxylic acid (TCA) cycle (in particular, citrate and oxoglutarate) were progressively diminished with the course of *B. graminis* infection between 24 and 96 hpi compared with the respective mock-treated controls (Fig. 4A to D and M), indicating altered flux into the anaplerotic TCA cycle that, among other functions, provides carbon skeletons for nitrogen assimilation into glutamine. Consequently, the glutamine/glutamate ratio (Fig. 4L) and the contents of almost all other amino acids, including the nonproteinogenic amino acids citrullin, ornithine, and GABA (Fig. 4M), increased strongly in leaves infected with *B. graminis*. Furthermore, *B. graminis*-infected leaves exhibited a shift in carbon partitioning between sucrose and starch in favor of sucrose (Fig. 4K). This is also reflected by an increased abundance of the intermediates of sucrose biosynthesis such as glucose-1-phosphate and UDP-glucose, and diminished steady state content of the starch precursor ADP-glucose (Fig. 4G to I and M). Concomitant with the alterations in major carbohydrate metabolism, we observed that the electron transport

rate of *B. graminis*-infected leaves was progressively diminished by 18% to $47.1 \mu\text{mol m}^{-2} \text{s}^{-1}$ compared with $57 \mu\text{mol m}^{-2} \text{s}^{-1}$ of uninfected control leaves at 96 hpi, while the CO_2 assimilation rate slightly decreased to $7.3 \mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ in *B. graminis*-infected leaves compared with $7.8 \mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ in control leaves.

***P. indica* root colonization modulates expression of a specific set of *B. graminis*-regulated transcripts in the leaf.**

To detect gene candidates associated with *P. indica*-induced *B. graminis* resistance, gene expression patterns of both *P. indica*-colonized and noncolonized barley leaves at 12, 24, and 96 hpi were compared. In the absence of *B. graminis*, only weak fold-change differences in transcript levels were detectable in *P. indica*-colonized plants: seven genes showed a ≥ 1.5 -fold differential expression (mean of three independent experiments, FDR < 0.05) and only one was downregulated ≥ 2 -fold (Supplementary Tables 4 and 5). However, gene set enrichment analysis identified functional gene categories affected by *P. indica* (e.g., at 12 and 96 hpi) “endoplasmic reticulum (ER)-related” among upregulated transcripts and “photosynthesis” among downregulated transcripts (Supplementary Table 6). In the presence of *B. graminis*, transcripts that accumulated to higher levels in *P. indica*-colonized plants compared with noncolonized plants were significantly enriched in the functional category “defense response” at 12, 24, and 96 hpi and protein folding at 12 and 24 hpi, while “photosynthesis/chloroplast” was enriched among downregulated transcripts 24 and 96 hpi.

We identified transcripts in *B. graminis*-inoculated barley leaves that were differentially expressed between *P. indica*-colonized and noncolonized plants: 41 transcripts were consistently up- or downregulated in *B. graminis*-infected leaves at least twofold in all three independent experiments due to the presence of *P. indica*. Selected transcripts were further subjected to independent confirmation by quantitative real-time polymerase chain reaction (QPCR). Those genes with an expression pattern independently confirmed in two QPCR experiments were chosen for further analysis: At 12 and 24 hpi, 12 (11 up- and 1 downregulated) and 10 (6 up- and 4 downregulated) transcripts, respectively, fulfilled these criteria (Supplementary Table 7). Among these, *Barley chemically induced 7* (BCI-7) and three PR genes (PR2, PR1 [PRB1-2], and PR5 [TLP5]) exhibited the most pronounced *P. indica*-dependent upregulation, while downregulated transcripts included a putative zinc finger protein, an HGA1 glycosyltransferase, Contig23097_at (unknown function), and sucrose:fructan 6-fructosyltransferase, which catalyzes an essential step in fructan biosynthesis.

***P. indica* induces earlier and faster expression of defense-related genes in the leaf during the *B. graminis* prepenetration phase.**

To gain more information about *P. indica*-primed genes during specific phases of the *B. graminis*-barley interaction, we analyzed expression of candidate genes in independent time-course experiments by QPCR. Expression patterns for transcripts coding for PR1, PR2, PR5, and heat-shock protein 70 (Hsp70) are shown in Figure 5. Enhanced expression of these four genes in *P. indica*-colonized plants after *B. graminis* infection was confirmed for the 12- and 14-hpi time points. In addition, earlier *B. graminis*-dependent induction in *P. indica*-colonized plants was detected for PR1 and PR2 already at 10 hpi. PR2 showed higher transcript levels in *P. indica* colonized plants at 14 to 20 hpi, while PR1 transcripts were induced to similar levels in *P. indica*-colonized and control plants at these later time points. Transcripts of Hsp70 were also induced by *B. graminis* transiently between 12 and 16 hpi.

Fig. 4. Changes in barley leaf metabolites in response to *Blumeria graminis* f. sp. *hordei* (*B. graminis*) leaf infection and root colonization with *Piriformospora indica*. Metabolite contents in barley leaves sampled at the end of the light phase were determined from four independent subpools of the leaf material that was used for transcript profiling at 24 and 96 h postinoculation (hpi) (left and right bracket, respectively). **A to L**, Leaf contents of the indicated metabolites. Control plants, black bars; *P. indica* colonized plants, gray bars; *B. graminis*-infected plants, white hatched bars; *P. indica*-colonized and *B. graminis*-infected plants, gray hatched bars. Values are means of four biological replicates $\pm$ standard error taken from one representative experiment. Data shown were reproduced with similar results in a total of three independent experiments. Significant differences to mock-treated plants with $P < 0.05$ are indicated with an asterisk, while significant differences between *B. graminis*- and *B. graminis* + *P. indica*-treated leaves with $P < 0.05$ are indicated by a cross above the respective bars. Significance was calculated using the Welch-Satterthwaite test. **M**, Schematic diagram summarizing the *B. graminis*-induced changes in barley leaf primary metabolism from all three independent experiments. Color shading indicates the experimental robustness of the given differences. Gray, no difference between *B. graminis*-infected and control leaves; red, increased in *B. graminis*-infected leaves; dark red, significantly increased steady state content in *B. graminis*-infected leaves at at least one time point in all three experiments, with $P < 0.05$ or increased content in *B. graminis*-infected leaves at both time points in all three experiments with $P < 0.1$; blue, decreased in *B. graminis*-infected leaves; dark blue, significantly decreased steady state content in *B. graminis*-infected leaves at at least one time point in all three experiments with $P < 0.05$, or decreased content in *B. graminis*-infected leaves at both time points in all three experiments with $P < 0.1$; pale blue, decreased content in *B. graminis*-infected leaves at one time point in more than one experiment with $P < 0.1$. 3-PGA, 3-phosphoglyceric acid; 6-PG, gluconate-6-phosphate; ADP-Glc, adenosinediphosphate-glucose; AsA, ascorbic acid; Ery4P, erythrose-4-phosphate; Fru, fructose; Fru6P, fructose-6-phosphate; Fru1,6bP, fructose-1,6-bisphosphate; GABA, γ -aminobutyric acid; Glc, glucose; Glc1P, glucose-1-phosphate; Glc6P, glucose-6-phosphate; GSH, glutathione; PEP, phosphoenolpyruvate; α -KG, α -ketoglutarate; RubP, ribulose-1,5-bisphosphate; Suc, sucrose; Suc6P, sucrose-6-phosphate; TrioseP, triosephosphates; UDP-Glc, uridinediphosphate-glucose; amino acids are abbreviated by standard three-letter code.

BCI-7, a transcript elevated during SA- or benzothiadiazole (BTH)-induced resistance (Besser et al. 2000), showed the highest *P. indica*-dependent differential response at 12 hpi. QPCR confirmed that expression was elevated 2.1-, 4.6-, and 3.9-fold in *P. indica*-colonized plants at 12 hpi with *B. graminis* in the three microarray experiments. In an independent experiment, *BCI-7* transcript levels were induced earlier and stronger (approximately 20-fold at 10 hpi) in *P. indica*-primed leaves compared with *B. graminis*-inoculated plants that were not colonized with *P. indica* (Fig. 6). In all experiments, induction of *BCI-7* transcript levels during the compatible interaction with *B. graminis* was highly transient for a time period of approximately 4 h approximately 12 hpi.

Some *P. indica*-induced changes of leaf metabolites are similar to *B. graminis*-induced changes.

Similarities between *P. indica* and *B. graminis* effects on the barley leaf were apparent as reduced levels of transcripts re-

lated to the functional gene categories photosynthesis and carbon fixation. Some changes of leaf metabolite abundance caused by *B. graminis* infection were also observed in leaves of *P. indica*-colonized plants not challenged with *B. graminis* (Fig. 4) and might relate to metabolic shifts due to the primed state of the leaf. ADP-glucose, starch, and oxoglutarate contents were constantly diminished upon root colonization by *P. indica*, whereas glutamine, alanine, and the glutamine/glutamate ratio were elevated in leaves of *P. indica*-colonized plants. Interestingly, *P. indica* root colonization and *B. graminis* leaf infection appeared to additively influence the latter changes in leaf amino acid metabolism, supporting the idea that part of the redirection in metabolic flux initiated in primed leaves is further aggravated upon pathogen challenge. However, *B. graminis*-induced changes in oxoglutarate, glutamine, and alanine were attenuated at 96 hpi, whereas leaves of *P. indica*-colonized plants exhibited more persistent changes of these metabolites compared with controls.

Fig. 5. Systemic effect of *Piriformospora indica* on expression of *Blumeria graminis* f. sp. *hordei* (*B. graminis*)-induced genes. Expression pattern of **A**, pathogenesis-related (*PR*)1; **B**, *PR*2; **C**, *PR*5; and **D**, heat-shock protein (*HSP*)70 transcripts in barley leaves 0 to 24 h postinoculation (hpi) with *B. graminis*. Transcript levels determined by quantitative real-time polymerase chain reaction were calculated relative to the mean of three constitutively expressed genes. Shown are expression values relative to control plants at 0 hpi set as 1. Similar results were obtained in an independent experiment. C, control; P, *P. indica* colonized. *B. graminis* inoculation is indicated by a “+” while mock inoculation (control) is indicated by a “-” below the respective columns. The gray frame highlights the 12-hpi time point for which differential induction was originally detected in the GeneChip experiment.

***P. indica* root colonization affects the photosynthetic acclimation response in *B. graminis*-infected leaves.**

Although steady state electron transport rate and CO₂ assimilation rate were not significantly altered at ambient (280 μE m⁻² s⁻¹) and saturating photon flux density (PFD) (1,500 μE m⁻² s⁻¹), we observed transient differences in photosynthetic acclimation of *B. graminis*-infected leaves when plants were colonized by *P. indica* (Fig. 7). Although the initial establishment of photosystem II quantum efficiency (ΦPSII) was delayed in leaves of *P. indica*-colonized plants challenged with *B. graminis* nonregulated energy dissipation Y(NO) was established faster than in control plants inoculated only with *B. graminis* (Fig. 7B). It was apparent that ΦPSII and Y(NO) of both *B. graminis*-infected and noninfected regions of the assessed leaves were altered in *P. indica*-primed plants compared with control plants inoculated with *B. graminis* only (Fig. 7A).

DISCUSSION

Powdery mildew induces large-scale changes in barley leaf transcripts related to recognition and transcriptional regulation 12 hpi.

The barley leaf transcriptome at the prepenetration phase of a compatible powdery mildew (*B. graminis*) interaction 12 hpi was characterized by large-scale changes in transcript abundance (Fig. 2). Among 1,976 transcripts specifically regulated by *B. graminis* compared with mock-treated leaves, 400 upregulated transcripts with unknown functions represent a resource for further characterization of pathogen defense and fortification processes. Among upregulated transcripts with assigned functions, overrepresentation of the functional categories defense response, apoptosis, and “protein folding/ubiquitination” was detected (Fig. 2). Specifically, receptor kinases and transcription factors were affected. Whereas many transcripts assigned to transcriptional regulation were downregulated by *B. graminis*, several WRKY, Zn finger, AP2, and NAC family transcription factors were upregulated at 12 hpi, including HvNAC6, a previously identified positive regulator of powdery mildew penetration resistance (Jensen et al. 2007). Specific activation of these genes is probably in response to PAMPs presented by *B. graminis* during formation of the primary germ tube 1 to 2 hpi and the appressorial germ tube with a mature appressorium 10

to 12 hpi (Aist and Bushnell 1991; Ellingboe 1972). Genes coding for specific enzymes in secondary metabolism were also significantly induced at 12 hpi, with up to 100-fold-induction levels (e.g., transcripts related to enzymes catalyzing important and committed steps in the synthesis of hydroxycinnamic acid amide phytoalexins, phenylpropanoid and monolignol biosynthesis, arginine decarboxylase, anthranilate N-benzoyltransferase, and tyrosine decarboxylase).

Finally, PR genes were upregulated at 12 hpi. The induction of these genes precedes the formation of the haustorium initial at 16 to 24 hpi, which invaginates the plasma membrane of one epidermal host cell and forms the haustorium (Aist and Bushnell 1991; Ellingboe 1972). A detailed time-course analysis of PR1, PR2, and PR5 revealed the highest (25- to 90-fold) induction between 14 and 20 hpi in our experiments (Fig. 5). At later time points of the compatible interaction, transcript levels of early induced genes were dampened, indicating effective suppression of defense-associated genes upon haustorium establishment (e.g., by secreted fungal effectors) (Bindschedler et al. 2009; Eichmann and Hückelhoven 2008; Spanu et al. 2010). The repression of initially *B. graminis*-induced transcript levels has been also observed by Caldo and associates (2004) (e.g., for transcripts related to biosynthesis of phenylpropanoid phytoalexins and lignin).

Powdery mildew induces vesicular-localized barley transcripts and increases metabolic activity 24 and 96 hpi.

Enrichment analysis of GO terms revealed specific overrepresentation of vesicular-localized transcripts 24 hpi (Fig. 3). Vesicle transport is a process involved in secretion of PR proteins and also accompanies SAR (Wang et al. 2005) but timing in case of the barley-*B. graminis* interaction correlates with the formation of the fungal haustorium, which requires transport and a membrane system involved in supplying nutrients to the fungus (Aist and Bushnell 1991; Ellingboe 1972; Hückelhoven et al. 1999). A second pattern characteristic for 24 hpi was an elevated translational activity, because transcripts assigned to this functional category and to protein folding were specifically overrepresented among 24-hpi *B. graminis*-upregulated transcripts and in cluster 9, containing 24- and 96-hpi *B. graminis*-upregulated transcripts (Fig. 2). Both trends indicate

Fig. 6. Systemic effect of *Piriformospora indica* on expression of barley chemically induced 7 (BCI-7). Transcript levels of defense-associated BCI-7 in *Blumeria graminis* f. sp. *hordei* (*B. graminis*)-inoculated barley leaves 0 to 16 h postinoculation (hpi), determined by quantitative real-time polymerase chain reaction, were calculated relative to the mean of three constitutively expressed genes. Shown are expression values relative to *P. indica*-noninoculated, *B. graminis*-inoculated plants at 12 hpi set as 1. Shown are expression values of a typical experiment; similar results were obtained in an independent experiment. C, control; P, *P. indica* colonized; ND, no transcript detected.

higher metabolic activity of the leaf tissue nourishing the biotrophic powdery mildew fungus.

Carbon and nitrogen assimilates accumulate during the compatible *B. graminis*–barley interaction.

Because powdery mildew is a biotrophic fungus that nourishes from single penetrated epidermal host cells, we aimed at the identification of transcripts and metabolites specifically affected during the establishment of the compatible interaction. On the transcript level, gene set enrichment analysis revealed a significant overrepresentation of “photosynthesis/carbon fixation” among downregulated transcripts (Fig. 2), specifically for transcripts related to the light reactions of photosynthesis (Supplementary Fig. 5a to c). Consequently, the content of 3-PGA, the primary product of carbon assimilation, was significantly diminished compared with controls at 96 hpi (Fig. 4). Cell wall invertase (Contig6503) was 10-, 4.5- and 3.1-fold induced at 12, 24, and 96 hpi, respectively; therefore, expression of photosyn-

thesis-related genes might be repressed as a consequence of a high rate of apoplastic sucrose cleavage during sugar-mediated defense (Biemelt and Sonnewald 2006; Swarbrick et al. 2006). In addition, the sucrolytic enzyme sucrose synthase was induced 1.9-fold in *B. graminis*-infected leaves at 24 hpi. Consistently, we could determine an accumulation of Glc1P and UDP-Glc in *B. graminis*-infected leaves compared with controls (Fig. 4). An accumulation of these precursors in sucrose biosynthesis could be caused by increased sugar cycling in infected source leaves (Fig. 8). Furthermore, we observed a shift in carbon partitioning of *B. graminis*-infected leaves at 24 and 96 hpi in expense of starch and diminished contents of the substrate for starch biosynthesis, ADP-Glc (Fig. 4). Together, this indicates that restricted sucrose export does not result in elevated synthesis of starch in infected leaves but, rather, in elevated sucrose cycling or consumption by the fungus. In line with our results, it has been shown that susceptible barley cultivars accumulate substantially more sucrose upon *B. graminis* infection compared with

Fig. 7. Analysis of chlorophyll fluorescence induction. Induction of chlorophyll fluorescence parameters in the youngest fully expanded leaf of barley plants at 12 h to 18 h after inoculation with *Blumeria graminis* f. sp. *hordei* (*B. graminis*) were recorded with a combined gas exchange/chlorophyll imaging system at a photon flux density (PFD) of $350 \mu\text{E m}^{-2} \text{s}^{-1}$. **A**, Representative false color images representing photosystem II quantum efficiency (ΦPSII) yield in the light adapted state (ΦPSII , left column) and nonregulated energy dissipation ($\text{Y}(\text{NO})$, right column) of *B. graminis*-inoculated leaves of plants with (bottom row) or without (top row) *Piriformospora indica* root colonization. The false-color scale is indicated at the bottom of the figure. Arrows point to infection sites. **B**, Mean values of (top to bottom) maximum PSII quantum yield (F_v/F_m), ΦPSII , $\text{Y}(\text{NO})$, and nonphotochemical quenching (NPQ) from four replicates $\pm$ standard error at 100 s after light on (left column) and in the completely light-adapted steady state at 600 s after light on (right column) are given. Plants not colonized with *P. indica* (light gray bars) and *P. indica*-colonized plants (dark gray bars) are grouped based on the presence or absence of *B. graminis* infection as indicated at the bottom of the individual graphs. Values significantly different ($P < 0.05$) between *P. indica*-colonized and noncolonized plants were calculated with a Welch-Satterthwaite test embedded in the VANTED v1.71 program (Junker et al. 2006) and are indicated by asterisks; “inf”, infected leaf area; “rem”, leaf area remote from infection site.

mock-treated controls (Swarbrick et al. 2006). The transcriptional induction of the low C/N ratio indicator Asn synthase (Lam et al. 1994, 1998) could indicate carbon shortage in *B. graminis*-infected leaves, despite the retention of sucrose in the infected tissue, indicating that sucrose availability is low in the symplastic compartment.

This could serve the pathogen in two ways: first, HR induction is hampered by the scavenging of Pro and, second, Gln accumulation represents a valuable nutrient to the invader.

How does *P. indica* mycorrhization influence systemic gene expression and metabolism in the leaf?

Fig. 8. Model of leaf metabolism in *Blumeria graminis* f. sp. *hordei* infected leaves. Based on the results of the combined metabolome and transcriptome analysis, a model illustrating the major redirection of metabolism in *B. graminis*-infected barley leaves was developed that integrates the dynamics in central metabolism observed at 24 and 96 h postinoculation (hpi) (changes observed only at 96 hpi are depicted in gray). Yellow indicates higher compared with mock control and blue indicates lower compared with mock control. Arrow thickness correlates with the proposed metabolic flux relative to the other depicted metabolic pathways. Only significantly altered metabolites and transcripts with a fold change >2 relative to mock control are included in the model. Amino acids are abbreviated according to three letter code, other metabolites were abbreviated as follows: ADPglc (ADP-glucose), aro-aa (aromatic amino acids), aKG (α -ketoglutarate), bc-aa (branched-chain amino acid), Cit (citrate), Hex (hexoses), HexP (hexose phosphates), Icit (isocitrate), PEP (phosphoenol pyruvate), 3PGA (3-phosphoglycerate), Suc (sucrose), TP (triose phosphates), UDPglc (UDP-glucose). Transcripts depicted in the model were abbreviated as AsnS (asparagine synthetase), CS (chorismate synthase), cwINV (cell wall invertase), GDH (glutamate dehydrogenase), GS (glutamine synthetase), IPMS (isopropylmalate synthase), P5CDH (Δ^1 -pyrroline-5-carboxylate dehydrogenase), PGM (phosphoglycerate mutase), PPT (phosphoenolpyruvate / phosphate translocator), SuSv (sucrose synthase).

P. indica in the absence of *B. graminis* challenge. This is clearly different from SAR, which is accompanied by constitutive activation of SA and NPR1-regulated transcripts. Therefore, barley root mycorrhization is similar to ISR triggered by nonpathogenic root-colonizing rhizobacteria in *Arabidopsis*, which does not affect leaf transcripts in the absence of pathogen challenge (Verhagen et al. 2004).

Absence of constitutively elevated defense-related transcripts can also be regarded as one reason for the low metabolic cost of the enhanced resistance capacity of *P. indica*-colonized plants, which are characterized by enhanced growth and grain yield under outdoor conditions (Achatz et al. 2010; Waller et al. 2005). In line with this view, the root endophyte affected only a few leaf metabolites. Of these, the effects of *B. graminis* and *P. indica* were mostly additive (Fig. 4), indicating that, in *P. indica*-colonized plants, some of the metabolic rearrangements specific to *B. graminis*-infected leaves might be anticipated.

Photosynthetic activity of leaves from 3-week-old barley plants used in our study was not drastically different between *P. indica*-colonized and noncolonized plants. However, significant differences in the photosynthetic acclimation response during the course of *B. graminis* infection (Fig. 7) indicated that the light-dependent activation of the Calvin cycle in *P. indica*-colonized plants was impeded, which could be caused by defense priming. The reduction in photosynthetic efficiency was only observed very transiently during light acclimation of *B. graminis*-challenged *P. indica*-colonized plants but not during steady-state photosynthesis; therefore, this is unlikely to be a consequence of the diminished transcript abundance of photosynthesis-related genes at 24 and 96 h post-*B. graminis* infection in *P. indica*-colonized plants.

***P. indica* root colonization primes defense-related and other transcripts induced by *B. graminis* in the barley leaf.**

We detected and confirmed 22 barley transcripts regulated by *B. graminis* as differentially expressed in leaves of the more resistant *P. indica*-colonized plants. Among them, defense-related genes *PR1*, *PR2*, and *PR5* as well as *Hsp70* were expressed at higher levels 12 hpi and earlier in response to *B. graminis* (Fig. 5). Therefore, *P. indica* root mycorrhization leads to priming of PR and other transcripts of yet unknown functions.

The detected priming of PR genes might relate to their direct or indirect antifungal activity or be due to their role in signaling. PR1b as well as basic PR5 isoforms are thought to have direct antimicrobial activity (Hejgaard et al. 1991; Rauscher et al. 1999), and transient silencing of PR1b was shown to weaken barley penetration resistance to *B. graminis* (Schultheiss et al. 2003). The primed PR2 transcript has high similarity to a putatively extracellular localized β -1,3-endoglucosidase, which can hydrolyze (1-3,1-6) branched β -glucans of fungal cell walls (Roulin et al. 1997; Xu et al. 1992). Faster induction of these transcripts resulting in earlier and more abundant presence of antifungal activity could, therefore, slow down fungal penetration by destruction or weakening of the penetration peg formed approximately 15 hpi (Hückelhoven et al. 1999; Thordal-Christensen et al. 2000). This is in line with microscopic observations that *P. indica* enhances penetration resistance in the early phase of interaction with *B. graminis*, leading to reduced formation of haustoria (Waller et al. 2005) and a partial requirement of ROR1, which functions in penetration resistance processes (Achatz 2006; Collins et al. 2003).

In addition to PR genes, we confirmed priming of *Hsp70* and *Hsp17.9* (Fig. 5). Heat shock cognate proteins play a role in both compatibility and resistance processes (Byth et al. 2001; Gjetting et al. 2007; Hein et al. 2005) but, for the two barley genes *Hsp70* and *Hsp17.9*, no specific roles are known.

BCI-7 showed the strongest priming in *P. indica*-colonized plants at 10 and 12 hpi (Fig. 6). Interestingly, *BCI-7* (Contig 34s_at) is upregulated in a Rar1-dependent manner and was among 14 differentially expressed genes identified in a barley transcriptome analysis of Mla-specified Rar1-dependent versus Rar1-independent incompatible interactions of barley and powdery mildew (Caldo et al. 2004). Besser and associates (2000) detected elevated levels of this transcript after treatment with the SA analogue BTH, which enhances resistance against powdery mildew. Therefore, enhanced *BCI-7* expression not only correlates with genetically (Rar1-dependent) and chemically induced *B. graminis* resistance but also is elevated during mycorrhiza-induced enhanced plant resistance by *P. indica*. *BCI-7* has homology to serine proteinase inhibitors but its function has not been clarified. Transient expression of *BCI-7* in single barley epidermis cells did not enhance powdery mildew penetration resistance (S. von Rüden, unpublished), indicating that *BCI-7* alone is not sufficient to enhance resistance or that it does not act cell autonomously.

P. indica could also induce resistance by downregulating compatibility factors. Indeed, we detected weaker *B. graminis* induction of five transcripts in *P. indica*-colonized plants. Transient-induced gene silencing (Hückelhoven et al. 2003) of the transcript corresponding to probe set 23097 (unknown function) in barley epidermal cells had no effect on *B. graminis* penetration (data not shown). Interestingly, the same transcript is upregulated in roots 3 days after inoculation with *P. indica* (Schäfer et al. 2009). This could indicate a conserved, positive role of the gene product for enabling compatible plant-fungus interactions, although its function has yet to be established.

Taken together, we show that *P. indica* has only subtle systemic effects on steady-state transcript levels and on metabolites in the absence of a pathogen. This is in line with ISR mechanisms, which typically do not require additional host plant resources, and with the observed positive effect of *P. indica* on host plant growth. Upon powdery mildew infection, *P. indica* primes defense-associated genes at an early stage of the interaction. Because several of these transcripts code for antifungal proteins, this represents an important mechanism responsible for the observed reduction of *B. graminis* haustorium formation in leaves of *P. indica*-colonized plants. In addition, several previously uncharacterized (specifically, *B. graminis*- and *P. indica*-regulated) transcripts were identified, which are of particular interest to understand the plants' response to a compatible biotrophic pathogen and to identify new molecular players in pathogen defense and for reverse-genetic approaches to enhance pathogen resistance in crops.

MATERIALS AND METHODS

Plant material and growth conditions.

Barley (*Hordeum vulgare*) 'Golden Promise' seed were surface sterilized and germinated on filter paper soaked with 1 mM CaSO₄ in the dark for 3 days. Before transferring seedlings into a 2:1 Seramis/OilDri mixture (Masterfoods, Verden, Germany; Damolin, Mettmann, Germany), roots were either mock inoculated in water or inoculated with a suspension of crushed *P. indica* mycelium at 2.5 g per 20 ml for 2 h. Afterward, seedlings were transferred to pots (three plants per 300-g pot) and 20 ml of the fungal suspension was added. Plants were grown in controlled-environment growth chambers with a cycle of 16 h of light (4:1 Osram L30W/830 Lumilux Warmwhite and Osram L30 W77 Fluora fluorescent cool white; 300 μ mol/(m² s⁻¹)) and 8 h of darkness at 22 and 18°C, respectively, and 60 to 70% relative humidity for another 19 days. Plants were fertilized once after 10 days with 20 ml of 0.5% Wuxal top N solution (Scherling, N-P-K, 12-4-6) per pot.

Fungal materials, inoculation procedures, and bioassays.

P. indica was provided by R. Oelmüller, Jena (Peskan-Berghofer et al. 2004), and was propagated on a rotary shaker at 18 to 22°C in liquid '*Aspergillus a*' complex medium (Pham et al. 2004), with the modification that vitamin concentrations were 0.1 ppm (wt/vol). Mycelium from liquid culture was washed with water three times to completely remove medium, and crushed with 5 to 10 short pulses (5 s) of a blender (Waring Blender, VWR International, Darmstadt, Germany).

B. graminis isolate A6 (Wiberg 1974) was propagated on *Hordeum vulgare* Golden Promise at 18°C (16 h of light and 8 h of darkness). For gene expression studies and microarray experiments, plants were inoculated with 120 to 140 conidia/mm² 19 days after potting. Inoculation was performed by shaking densely *B. graminis*-infected barley plants (Golden Promise) approximately 1 m above the plants placed at the bottom of a settling tower. At 12, 24, and 96 hpi, the youngest, fully developed leaves (third leaves) were harvested into liquid nitrogen. Each sample consisted of 12 to 16 leaves, which were ground in liquid nitrogen with mortar and pestle to a fine powder. Samples were divided into two portions for transcriptome and metabolome analysis.

Macroscopic analysis of powdery mildew infections was done in a detached-leaf-segment assay as described in (Waller et al. 2005).

RNA isolation and expression analysis by QPCR.

Total RNA was isolated from plant material using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol, and 500 ng of RNA was transcribed to cDNA using the SuperScriptII cDNA Synthesis Kit (Invitrogen). Aliquots of 10 ng of first-strand cDNA were subsequently used as a template for QPCR with gene-specific primers. PCR amplifications were performed in 20 µl of SYBR Green JumpStart TaqReadyMix (Sigma-Aldrich Chemie, Munich) using a Stratagene Real Time PCR Cycler (Stratagene-Mx3000PQPCR SystemMx3000P; Stratagene Research, La Jolla, CA, U.S.A.) as described in (Stein et al. 2008). For normalization, the mean value of three constitutively expressed genes was used: a ubiquitin gene (Ubi60) (Deshmukh et al. 2006), a constitutively expressed polyubiquitin (HKG1), and an 18S rDNA-primer (18S).

DNA oligonucleotide primers used were:
HKG1 forward (fwd) 5'-GGCCTGTAAGTGCTTGGC-3' and reverse (rev) 5'-CGCTAGACCACAACACAGTAGC-3',
PR-1 fwd 5'-GGACTACGACTACGGCTCCA-3' and rev 5'-GGCTCGTAGTTGCAGGTGAT-3',
PR-2 fwd 5'-TACTTCGCGTACCGTGACAA-3' and rev 5'-GTGTAGGTCAGCCCGTTGTT-3',
PR-5 fwd 5'-GCCGACCAACTACTCAATGT-3' and rev 5'-AGGGCAGGTGAAGGTGCT-3',
Hsp70 fwd 5'-GGAGGTTGACTAAGCTGTTGC-3' and rev 5'-ACATGACACGACGACAAACG-3',
BCI-7 fwd 5'-ACTTCAGGAGCAACCGTGTC-3' and rev 5'-CGCGCACTAATTCATCCTTT-3',
Ubi60 fwd 5'-ACCCTCGCCGACTACAACAT-3' and rev 5'-CAGTAGTGCGGTCGAAGTG-3', and
18S fwd 5'-GCGAGCACCGACCTACTC-3' and rev 5'-GGACCGGAATCCTATGATGTT-3'.

Microarray analysis.

To analyze gene expression patterns in barley leaves, the Barley1 GeneChip probe array (Affymetrix, Santa Clara, CA, U.S.A.) was used (Close et al. 2004). For expression analysis, one sample consisted of 10 to 16 of the youngest, fully developed leaves (third leaves) from *P. indica*-colonized or noncolonized plants 12, 24, or 96 h after *B. graminis* inoculation. Ad-

ditionally, non-*B. graminis*-treated plants were collected at all three time points. Therefore, one experiment consisted of 12 samples. Three experiments were independently performed, requiring a total number of 36 Barley1 Gene Chips. Microarray data are available in the ArrayExpress database under accession number E-MEXP-3389.

Total RNA was isolated according to Logemann and associates (1987). Probe labeling was performed with 5 µg of total RNA using the Affymetrix One-Cycle Target labeling kit and hybridization was performed according to the GeneChip Expression Analysis Technical Manual (Affymetrix), with the modification that the clean-up step preceding probe fragmentation was performed with spin columns provided with the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany). Quality of cDNA, cRNA, and fragmented cRNA was verified at each step using an Agilent 2100 Bioanalyzer equipped with an RNA Nano LabChip (Agilent Technologies, Palo Alto, CA, U.S.A.).

Data analysis.

Data analysis was performed using the Bioconductor packages "affy", "gcRMA", and "limma" within the R environment. Data were preprocessed using the affy package (Gautier et al. 2004) and normalized by the gcRMA method (Wu et al. 2004). The limma package (Smyth 2004) was used for analysis of differentially expressed genes. The *P* values for the contrasts of interest were adjusted for multiple testing by estimation of the FDR using the method introduced by Benjamini and Hochberg (1995). Genes with an FDR < 0.05 that were at least twofold regulated were selected as differentially expressed.

Expression data were visualized using MapMan (Thimm et al. 2004; Usadel et al. 2005), which was also used to find over-represented functional categories. Clustering of expression data was done by the k-means clustering algorithm within the R environment, including all genes that were differentially expressed in at least one comparison. Enrichment analysis of gene clusters was done using the web-based platform AgriGO. Gene set enrichment analysis was done using the algorithm described by Subramanian and associates (2005).

Metabolite analysis.

Metabolite analysis was performed from the same pool of barley leaves harvested for microarray analysis (discussed above). Leaf sampling, pooling of sample material, and all metabolite analyses were conducted as described by Horst and associates (2010).

Imaging-PAM analysis of leaf photosynthesis.

Chlorophyll fluorescence imaging of the youngest fully expanded barley leaf per plant was conducted at a PFD of 350 µE m⁻² s⁻¹ using a combined gas exchange-chlorophyll imaging system (GFS-3000 and MINI-Imaging-PAM Chlorophyll Fluorometer; Walz, Effeltrich, Germany) at the indicated time points after *B. graminis* inoculation and *P. indica* pretreatment, as described previously (Horst et al. 2008). The measured plants originated from the same experiments used for combined transcriptome and metabolite analysis (discussed above).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

AgriGO database: bioinfo.cau.edu.cn/agriGO
 ArrayExpress database: www.ebi.ac.uk/arrayexpress
 BarleyBase database: www.plexdb.org/plex.php?database=Barley
 Bioconductor software: www.bioconductor.org