Broad-Spectrum Acquired Resistance in Barley Induced by the *Pseudomonas* Pathosystem Shares Transcriptional Components with *Arabidopsis* Systemic Acquired Resistance

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Inducible resistance responses play a central role in the defense of plants against pathogen attack. Acquired resistance (AR) is induced alongside defense toward primary attack, providing broad-spectrum protection against subsequent pathogen challenge. The localization and molecular basis of AR in cereals is poorly understood, in contrast with the well-characterized systemic acquired resistance (SAR) response in *Arabidopsis*. Here, we use *Pseudomonas syringae* as a biological inducer of AR in barley, providing a clear frame of reference to the *Arabidopsis*–*P. syringae* pathosystem. Inoculation of barley leaf tissue with the nonadapted *P. syringae* pv. *tomato avrRpm1* (*PstavrRpm1*) induced an active local defense response. Furthermore, inoculation of barley with *PstavrRpm1* resulted in the induction of broad-spectrum AR at a distance from the local lesion, “adjacent” AR, effective against compatible isolates of *P. syringae* and *Magnaporthe oryzae*. Global transcriptional profiling of this adjacent AR revealed similarities with the transcriptional profile of SAR in *Arabidopsis*, as well as transcripts previously associated with chemically induced AR in cereals, suggesting that AR in barley and SAR in *Arabidopsis* may be mediated by analogous pathways.

Upon recognition of an invading pathogen, plants activate a suite of inducible defense responses. In directly attacked tissue, pathogen-associated molecular pattern (PAMP)- and effector-triggered immunity act to restrict primary infection (Jones and Dangl 2006). Acquired resistance (AR) is activated alongside these primary responses, and provides longer-lasting and broad-spectrum protection against subsequent pathogen challenge (Sticher et al. 1997; Van Loon 1997). The first systematic characterization of AR was in tobacco (Ross 1961a and b), where induction of a hypersensitive response (HR) by *Tobacco*

mosaic virus resulted in localized acquired resistance (LAR), a 5-mm zone of near-immunity immediately surrounding the lesion; and systemic acquired resistance (SAR), an enhancement of resistance beyond the LAR zone and throughout the plant. Both LAR and SAR conferred resistance to subsequent challenge with a range of viral strains (Ross 1961a and b). More recent work in *Arabidopsis* has shown that SAR can be triggered by an avirulent or virulent isolate of *Pseudomonas syringae* or by PAMPs (Mishina and Zeier 2007). The efficiency of SAR appears to be dependent on the strength of the locally induced, primary defense response, and cell death may not be required for induction as previously thought (Liu et al. 2010; Mishina and Zeier 2007).

In *Arabidopsis* and tobacco, the induction of SAR is dependent on the local production of mobile signals which trigger the systemic, de novo synthesis of salicylic acid (SA) (Chanda et al. 2011; Jung et al. 2009; Kiefer and Slusarenko 2003; Park et al. 2007; Vernooij et al. 1994; Vlot et al. 2008). Systemic SA accumulation triggers *nonexpressor of pathogenesis-related (PR) genes* (NPR1)-mediated induction of a characteristic subset of PR genes (Mou et al. 2003; Ryals et al. 1996; Zhou et al. 2000) and the establishment of a “primed” state whereby plants respond more strongly or rapidly to secondary challenge by a pathogen (Conrath 2011). Enhanced levels of PR proteins, as well as enhancement of structural defenses such as lignification and papillae formation, are all thought to contribute quantitatively to SAR (Hammerschmidt 1999; Sticher et al. 1997).

In contrast to SAR in dicots, there is only limited evidence for biologically induced SAR in the cereals (Kogel and Langen 2005; Smith and Métraux 1991). Studies in barley have concluded that AR induced by compatible and nonadapted *Blumeria graminis* isolates is strictly localized, being transmitted only to cells immediately adjacent to the site of attempted penetration by 25 h following inoculation (Kunoh 1988; Lyngkjær and Carver 1999; Ouchi et al. 1976). As such, there appears to be major differences in the spatial and temporal development of AR between cereals and dicots where, in the latter, LAR extends beyond a few cells and SAR generally develops in secondary leaves by 24 to 48 h after pathogen inoculation (Cameron et al. 1994; Kunoh 2002; Smith et al. 1991).

Further evidence of differential AR responses between cereals and dicots comes from studies of chemical induction of AR, seen in both groups of plants after application of SA analogues benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) and 2,6-dichloroisonicotinic acid (DCINA) (Friedrich et al. 1996; Uknes et al. 1992; Ward et al. 1991). In *Arabidopsis*

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and tobacco, chemical and biological inducers of SAR are believed to induce the same “SAR genes” (Ryals et al. 1996). In contrast, in wheat, barley, and rice, a set of chemically induced genes do not appear to be differentially expressed during biologically induced local AR, while some *PR* genes do not respond to chemical induction (Beßer et al. 2000; Görlach et al. 1996; Molina et al. 1999; Schaffrath et al. 1997; Schweizer et al. 1999). This has led to suggestions that biologically and chemically induced resistance are mediated by separate pathways in the cereals (Beßer et al. 2000; Kogel and Langen 2005; Schaffrath et al. 1997). However, other data indicate that barley chemically induced (*BCI*) genes *BCI-1* and *BCI-3* are responsive to inoculation with *Magnaporthe oryzae* (Jarosch et al. 2003), while microarray data from wheat suggest that defense-related genes are strongly upregulated after treatment with BTH (Pasquer et al. 2005). This indicates that there is at least some overlap between biologically and chemically induced defense in cereal species.

There is limited evidence for conservation in cereals of components of the dicot SAR response, with a functional homologue of the key *Arabidopsis* SAR regulator *NPR1* having been identified in rice (Chern et al. 2001; Fitzgerald et al. 2004; Yuan et al. 2007). However, *NPR1* does not function specifically in SAR (Cao et al. 1994; Feng et al. 2011; Pieterse et al. 1998) and may have other, non-SAR roles in rice. Nevertheless, recent work suggests that the *M. oryzae*-derived elicitor PemG1 induces SAR in rice (Peng et al. 2011), while another biological form of AR, induced systemic resistance (ISR), has also been demonstrated in rice (De Vleeschauwer et al. 2008) and barley (Waller et al. 2005, 2008). ISR has been well characterized in dicots (Pieterse et al. 1996), indicating that at least one form of systemic acquired immunity is conserved between dicots and cereals. It has also been reported that the application of petiole exudate from SAR-induced *Arabidopsis* seedlings to wheat spikelets led to enhanced resistance to *Fusarium graminearum* (Chaturvedi et al. 2008). However, the conflicting data available for cereals clearly show that the molecular basis of AR in cereals (and monocots more widely) is poorly understood, and suggests that, for AR, studies in *Arabidopsis* may not accurately predict responses in cereal crops.

Most studies of SAR in barley have used *B. graminis* (the causal agent of powdery mildew) as the inducer pathogen. However, we are not aware of any published evidence that this pathogen induces SAR in dicots. Other aspects of the interaction may also be at odds with characterized dicot SAR induction systems. Vallelian-Bindschedler and associates (1998) measured local accumulation of SA and SA conjugates in barley after inoculation with *B. graminis* and after *P. syringae* pv. *syringae* inoculation. Only *P. syringae* pv. *syringae* inoculation resulted in the local accumulation of SA in barley. The induction of SAR by *P. syringae* has been reported for several plant species and is particularly well characterized in *Arabidopsis* and cucumber, providing a clear frame of reference for this work (Cameron et al. 1994; Smith and Métraux 1991; Smith et al. 1991). In this study, *P. syringae* pv. *tomato* was used as a biological inducer of AR in barley that was effective against *P. syringae* pv. *syringae* and the hemibiotrophic fungus *M. oryzae*. A transcriptomics analysis also indicated that AR in barley shares transcriptional components with the well-characterized SAR response of dicotyledonous plants.

RESULTS

Interaction of *P. syringae* pathotypes with barley.

In order to establish whether the *P. syringae* pathosystem used in the study of AR in *Arabidopsis* could be transferred to barley, the growth of a selection of *P. syringae* strains was

monitored after inoculation into barley leaves. The *P. syringae* pv. *tomato* DC3000 was able to multiply in planta (Fig. 1). In contrast, for strain DC3000 containing the *avrRpm1* gene (*PstavrRpm1*), bacterial levels 2 to 3 days postinoculation (dpi) were similar to inoculation levels (day 0), suggesting that *avrRpm1* restricted growth of this strain in barley. DC3000 *hrcC*- (deficient in type III secretion) (Deng et al. 1998) failed to thrive in barley leaf tissue, suggesting that, as in *Arabidopsis*, effector secretion may play a role in enabling DC3000 to

Fig. 1. Interaction between barley and isolates of *Pseudomonas syringae*. CFU recovered from barley leaves after syringe inoculation with **A**, *PstavrRpm1* (*avr*), *P. syringae* pv. *tomato* DC3000 (DC3000), or *P. syringae* pv. *tomato* DC3000 *hrcC*- (*hrcC*) (10^8 CFU ml⁻¹); or **B**, *P. syringae* pv. *syringae* B64 (10^7 CFU ml⁻¹). Leaf discs were 0.5 cm in diameter and produced using a cork borer. Error bars indicate mean of three to four biological replicates $\pm$ standard error. Growth of each isolate was tested in at least two independent experiments, with similar results. **C**, Symptoms on inoculated leaves photographed 4 days postinoculation. Inoculations at densities indicated for **A** and **B**; W indicates water-infiltrated control. Black marks indicate limit of infiltration.

colonize host tissue (Collmer et al. 2000). Both DC3000 and *PstavrRpm1* inoculation induced a local necrotic response (similar to the necrotic lesions caused by *P. syringae* in *Arabidopsis*) (Katagiri et al. 2002), unlike DC3000 *hrcC*- (Fig. 1C). *P. syringae* pv. *syringae* B64, originally isolated from wheat (Hwang et al. 2005), was found to multiply rapidly in planta (Fig. 1B). The leaf area inoculated with strain B64 also became necrotic (Fig. 1C).

PstavrRpm1 was shown to induce strong local defense responses in barley by evaluating the expression of several *PR* genes, including the SAR marker gene *PR-1* (Cameron et al. 1999; Maleck et al. 2000), following *P. syringae* pv. *tomato* inoculation at a range of inoculum densities and over several time points (Colebrook 2010). The efficiency of SAR induction has previously been shown to correlate with the strength of the locally induced defense responses (Mishina and Zeier 2007); therefore, *PstavrRpm1* was used as the inducer strain and strain B64 as the challenger strain in subsequent analyses of AR.

Local inoculation of barley leaves with *P. syringae* induces acquired resistance in adjacent leaf tissue.

In order to examine the spatial and temporal localization of AR in barley, the distal third of the first leaf of 'Golden Promise' seedlings was syringe inoculated with *PstavrRpm1* (10^8 CFU ml⁻¹). This directly inoculated leaf region was termed the "local" leaf region (Fig. 2). The proximal third of each leaf, termed the "adjacent" leaf region, was challenged by inoculation with *P. syringae* B64 (Fig. 2). To confirm that *PstavrRpm1* remained confined to the inoculated leaf region, leaf disks were sampled from the adjacent leaf region 0, 6, and 24 h postinoculation (hpi) of the local leaf region. No CFU were recovered from the adjacent region (Colebrook 2010). This made it possible to accurately analyze the transfer of induced resistance to the adjacent leaf region.

At 24 h after local inoculation with the inducer strain *PstavrRpm1*, the adjacent region of each leaf was inoculated

Fig. 2. Local and adjacent leaf regions of the first leaf of barley seedlings. The local leaf region was defined as the distal third of the first true leaf of the seedling and the adjacent leaf region was the proximal third. Responses in water-infiltrated (control) leaves were compared with the equivalent regions in *PstavrRpm1*-inoculated leaves.

with the challenger strain B64. The levels of strain B64 within the adjacent region were then measured 24 h after challenger inoculation. Strain B64 levels were reduced 0.5-fold (on a log scale) in seedlings preinoculated locally with *PstavrRpm1*, relative to the water-infiltrated control (Fig. 3), indicating that *PstavrRpm1* induced AR in barley at a distance from the site of inoculation.

To determine whether *PstavrRpm1* induced broad-spectrum AR in the adjacent region of the barley leaf, Golden Promise seedlings were spray inoculated with a compatible strain of the blast fungus *M. oryzae* 24 h after local inoculation with *PstavrRpm1* (Fig. 4). The leaf area showing blast lesions was significantly reduced in the adjacent region of *PstavrRpm1*-induced leaves relative to water-infiltrated controls (Fig. 4). Therefore, the AR induced in adjacent leaf tissue following local inoculation with *PstavrRpm1* would appear to be effective against subsequent challenge by both a bacterial and a fungal pathogen of barley.

In order to determine whether the AR induced by *PstavrRpm1* was systemic, the second seedling leaf was challenged with strain B64 1 to 4 dpi of the first leaf with *PstavrRpm1*. No AR was seen in the second seedling leaf toward strain B64 (data not shown). Likewise, to test for *PstavrRpm1*-induced AR toward *M. oryzae* in secondary seedling leaves, whole seedlings were spray inoculated with *M. oryzae* 1, 2, 6, and 10 days after local *PstavrRpm1* inoculation. Diseased leaf area on second, third, and fourth leaves was scored and compared with that of water-infiltrated controls. No significant differences in diseased leaf area were seen in secondary leaves at any of the four challenge time points (Supplementary Fig. 1). Therefore, under the conditions tested, it would appear that the AR induced by *PstavrRpm1* in Golden Promise barley does not become systemic.

Transcriptome reprogramming in response to *PstavrRpm1* inoculation.

To further characterize the nature of the AR induced by *PstavrRpm1*, the Affymetrix Barley1 GeneChip (Close 2005) was used to profile the transcriptome of the local and adjacent leaf regions 24 h after local inoculation with *PstavrRpm1*. Comparisons were made between water-infiltrated and *PstavrRpm1*-inoculated local tissue, and between adjacent regions of water-infiltrated and *PstavrRpm1*-inoculated leaves, to identify differ-

Fig. 3. Resistance to *Pseudomonas syringae* pv. *syringae* in the adjacent leaf region 24 h after local inoculation with *PstavrRpm1*. The adjacent leaf region was challenged with *P. syringae* pv. *syringae* isolate B64 24 h after local inoculation with *PstavrRpm1* (Pst) or infiltration with water (W). Leaf disks were sampled from the adjacent region 24 h after *P. syringae* pv. *syringae* B64 challenge and CFU assayed. Bars show averages of pooled means from 10 independent experiments $\pm$ standard error. Differences between treatments are statistically significant at $P < 0.05$ (*t* test).

entially expressed transcripts in each leaf region. At this time point, the *PstavrRpm1*-inoculated local leaf region showed weak chlorosis (data not shown). Genes were considered differentially expressed if they were up- or downregulated by at least twofold, with a false discovery rate adjusted *P* value < 0.05. Gene lists were annotated using PLEXdb (Wise et al. 2007) and functionally categorized using the MIPS classification system (Ruepp et al. 2004). To ensure that local inoculation with *PstavrRpm1* had induced AR in the adjacent leaf region, a set of barley seedlings was retained for subsequent strain B64 challenge of the adjacent region. Results of this challenge inoculation were consistent with previous tests (Fig. 3), showing AR toward strain B64. Array data was validated by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis of a selection of probe sets on tissue from three to five independent experiments. Probe sets were selected to cover those transcripts differentially expressed in either the local or adjacent regions as well as transcripts that were regulated in both leaf regions (see below for further details). The majority of transcripts tested showed an expression pattern similar to that seen in the array, confirming the validity of the

array data (Supplementary Fig. 2). For a number of transcripts selected for validation, qRT-PCR detected differential expression where none had been identified by the array; this is likely due to the greater sensitivity of qRT-PCR or the detection of transcripts of other gene family members not detected by hybridization to the array chip (Cools et al. 2007; Coram and Pang 2006).

The transcriptional profile of local *PstavrRpm1*-inoculated barley leaf tissue.

Inoculation with *PstavrRpm1* resulted in the differential expression of 5,109 transcripts in the directly inoculated (local) leaf tissue 24 hpi, with 2,403 being upregulated and 2,706 downregulated relative to the water control (Fig. 5; Supplementary Table 1). Overall, functional classification indicated that similar categories of transcripts were up- and downregulated, although transcripts involved in defense (cell rescue, defense, and virulence category) were primarily upregulated, whereas transcripts associated with energy production (e.g., photosynthesis-related transcripts) were mainly downregulated, suggesting a shift from primary metabolism to defense (Supplementary Fig. 3).

Almost a third of the upregulated defense-related transcripts (cell rescue, defense, and virulence category) in the local leaf tissue were associated with H₂O₂ production or oxidative stress processes (e.g., blue copper binding protein-like, oxalate oxidase-like, germin-like, glutathione[S-] transferase-like, and peroxidase-like), suggesting that an oxidative burst is triggered by inoculation with *PstavrRpm1*. The local defense response also included the upregulation of transcripts with similarity to phenylalanine ammonia lyase (*PAL*) and many *PR* genes, including *PR-1*, *PR-2* (β -1-3-glucanase), *PR-4* (chitin-binding protein), *PR-5* (thaumatin-like), chitinases (*PR-3/-8/-11*), and *PR-10* ("ribonuclease-like") (Ferreira et al. 2007). The most strongly induced transcript was similar to the rice *PIR7B* esterase, transcripts of which are induced in rice by the *P. syringae* pv. *syringae*-derived elicitor syringolin (Reimann et al. 1995; Wäspi et al. 2001). Several transcripts with similarity to the *Arabidopsis* *Defective in induced resistance-1* (*Dir-1*) gene were also differentially expressed in the local region in response to *PstavrRpm1* inoculation. This may be significant given that, in *Arabidopsis*, the *DIR1* lipid transfer protein is required for the production or transmission of the mobile SAR signal from the primary inoculated leaf (Maldonado et al. 2002). As with local expression of *Dir-1* in *Arabidopsis* (Maldonado et al. 2002), these transcripts were all downregu-

Fig. 4. Resistance to *Magnaporthe oryzae* in the adjacent leaf region 24 h after local inoculation with *PstavrRpm1*. The adjacent leaf region was challenged with *M. oryzae* 24 h after local inoculation with *PstavrRpm1* (*PstavrRpm1* or *Pseudomonas syringae* pv. *tomato*) or infiltration with water (W). **A**, Representative symptoms 5 days after *M. oryzae* challenge. The region of the leaf above the black line defines the area of the local infiltration. **B**, Diseased leaf area of the adjacent leaf region was scored 5 days after *M. oryzae* challenge. Bars show means $\pm$ standard error ($n = 14$ to 15). Means are significantly different at $P < 0.05$ (*t* test). Similar results were obtained in at least two further independent experiments.

Fig. 5. Transcripts up- and downregulated in the local and adjacent regions of the first leaf of seedlings of the barley 'Golden Promise' following inoculation of the local region with *PstavrRpm1*. Transcripts identified by microarray analysis using the Affymetrix Barley1 GeneChip. The number of transcripts upregulated (red, upper line) and downregulated (green, lower line) are shown as specific to the local or adjacent region or common to both leaf regions.

lated. Together, these results suggest that the local response to *PstavrRpm1* in barley involves elements of defense responses previously characterized in *Arabidopsis*, barley, and rice.

The transcriptional profile of adjacent barley leaf tissue exhibiting acquired resistance induced by *PstavrRpm1*.

To further characterize the AR response in adjacent leaf tissue, the transcriptome of this leaf region was characterized. Adjacent leaf tissue was sampled 24 h after local *PstavrRpm1* inoculation and transcript levels relative to the equivalent leaf region of water-infiltrated controls determined. Of 343 transcripts differentially expressed in the adjacent leaf tissue (Supplementary Table 2), 326 were upregulated and 17 were downregulated (Fig. 5). In all, 64% (219 transcripts) of the differentially expressed transcripts were common to both the local and adjacent leaf tissue transcriptome, while 36% (124 transcripts) were specific to the adjacent region (Fig. 5). Of the 20 most highly upregulated transcripts in the adjacent region, 13 were also differentially expressed in the local response. Three of these transcripts showed the opposite direction of regulation between the local and adjacent regions, and a further eight were more strongly upregulated in the adjacent region than in the local leaf tissue. This suggests that the response in the adjacent leaf tissue is, to some extent, distinct from the local response, rather than a weaker version of locally induced responses.

To further investigate the relationship between the transcriptional responses of the local and adjacent leaf regions, the expression of selected defense-related transcripts was compared in the local and adjacent leaf regions 24 h after local inoculation with DC3000 *hrcC*- or *PstavrRpm1*. As noted above, DC3000 *hrcC*- is deficient in type III secretion and does not multiply or cause necrosis in barley leaf tissue (Fig. 1), in contrast to *PstavrRpm1*. This suggests that the nature of the interaction between these isolates and barley is different, with effector-recognition likely to play a role in the response of barley to *PstavrRpm1* but not DC3000 *hrcC*-. As such, comparison of adjacent responses induced by each isolate might provide some indication of what aspect of the local interaction triggers the adjacent response. qRT-PCR data indicated that local inoculation with DC3000 *hrcC*- did not induce expression in the adjacent region of several of the defense-related transcripts which were induced after local inoculation with *PstavrRpm1* (Supplementary Fig. 4). This further supports a model of active gene induction in the adjacent region in the barley-*PstavrRpm1* interaction.

As in the local leaf region, the largest functional categories of differentially expressed transcripts (aside from those with unknown function) were defense and metabolism. In terms of defense, transcripts with similarity to *PR* gene families were again well represented, with *PR-1*, *PR-2* (β -1-3-glucanase), *PR-5* (thaumatin-like), *PR-6* (proteinase-inhibitor), *PR-9* (peroxidase), *PR-13* (thionin), *PR-15* (oxalate oxidase), and chitinases upregulated in the adjacent leaf tissue. Metabolic responses included the upregulation of transcripts associated with phenylpropanoid biosynthesis (PAL and cinnamyl alcohol dehydrogenase), the shikimate pathway (prephenate dehydratase), oxylipin biosynthesis (lipoxygenase, allene-oxide synthase [AOS], and 12-oxophytodienoate reductase), and jasmonic acid (JA) responsiveness (putative polygalacturonase and putative cysteine protease), indicating a key role for secondary metabolism. In addition, transcriptional activation and signal transduction were also predominant responses, with all transcripts in these categories being upregulated, unlike in the local response, where similar numbers were up- and downregulated. Interestingly, transcripts with high similarity to five of nine *BCI* genes (*BCI-1*, 2, 3, 4, and 9) (Beßer et al. 2000) and

wheat chemically induced 5 (*WCI-5*) were upregulated in the adjacent leaf region. None of these transcripts showed common responses between the local and adjacent leaf regions (Supplementary Table 3).

Associations between the transcriptome of the adjacent leaf region and transcriptional responses to other biotic and abiotic stress treatments in barley were analyzed by performing hierarchical clustering with the 343 transcripts identified as differentially expressed in the adjacent leaf tissue (Fig. 6; Supplementary Table 4). Datasets were selected from those publicly available at PLEXdb (Wise et al. 2007). The analysis indicated that the transcript profile in the adjacent leaf tissue was highly similar to the transcript profiles seen in compatible and incompatible interactions between barley and *B. graminis* f. sp. *hordei* (Caldo et al. 2004), with many transcripts showing similar, strong upregulation. This is suggestive of similarities between the *PstavrRpm1*-induced adjacent AR responses and basal resistance to powdery mildew in barley. Interestingly, all *BCI* genes upregulated in the adjacent leaf tissue were present in a single cluster, which included transcripts with similarity to lipoxygenase, cysteine proteinase, AOS, and lipase. This transcript cluster was also upregulated during interactions between barley and the aphid *Rhopalosiphum padi* (Fig. 6) (Delp et al. 2009), further highlighting a possible link between the *BCI* genes and octadecanoid or JA biosynthesis and signaling (Kogel and Langen 2005).

DISCUSSION

Inoculation of barley leaf tissue with the *P. syringae* isolate *PstavrRpm1* induced broad-spectrum AR, at a distance from the local lesion, effective against bacterial and fungal pathogens. This is in contrast with previous studies in barley in which AR, induced by compatible or nonadapted isolates of *B. graminis*, appears to be strictly localized to the inoculated cell or transmitted only to cells immediately adjacent to the site of attempted penetration (Lyngkjær and Carver 1999; Ouchi et al. 1976). The reasons for the different spatial profile of AR induced in barley by *B. graminis* and *P. syringae* are currently not clear. Results from several dicot systems have indicated that, at least for SAR, the nature of the inducing interaction determines the strength and timing of the AR response. In *Arabidopsis*, the magnitude of SAR is correlated with the strength of the local defense responses triggered by the inducer isolate (Mishina and Zeier 2007). As such, the different spatial profile of AR induced in barley by *B. graminis* and *P. syringae* could be due to differences in the strength of the local responses induced by each pathogen. Local *PR* gene induction in barley was similar after inoculation with *P. syringae* pv. *syringae* and with virulent or nonadapted isolates of *B. graminis*; however, *B. graminis* isolates failed to induce the accumulation of SA (Vallée-Bindschedler et al. 1998). Because SA-mediated defense is a general feature of SAR (Vlot et al. 2008), this may be significant. Alternatively, differences in the spatial profile of AR induced by *B. graminis* and *P. syringae* may be due to differences in pathogen lifestyle. *B. graminis* interacts exclusively with epidermal cells while *P. syringae* attacks mesophyll cells; therefore, it is conceivable that signals from the latter might be more easily translocated. A further consideration is that, in studies of *B. graminis*-induced AR in barley, the challenger pathogen has been a virulent isolate of *B. graminis* (Kunoh et al. 1988; Lyngkjær and Carver 1999; Ouchi et al. 1976). The apparent failure of *B. graminis* to induce long-distance AR in these studies could be related to the ability of highly adapted, virulent isolates of *B. graminis* to overcome the AR response at a one- two-cell distance from the location of induction.

The AR induced by *PstavrRpm1* in barley is effective in the adjacent leaf region but did not become systemic under the conditions tested. This could also be related to the nature or strength of the inducing interaction (Mishina and Zeier 2007) or to environmental or developmental factors (Kiefer and Slusarenko 2003; Reimmann et al. 1995; Zeier 2005; Zeier et al. 2004). Systemic resistance can be induced in barley by the beneficial root colonizing fungus *Piriformospora indica* (Waller et al. 2005), which induces systemic resistance reminiscent of ISR in *Arabidopsis* (Stein et al. 2008). In barley, leaves of plants of which the roots had been colonized by *P. indica* showed enhanced resistance to virulent isolates of *B. graminis* f. sp. *hordei* (Waller et al. 2005). Only two genes, a heat-shock protein 70 precursor (Barley1_49394) and *HvPRI7b* (Barley1_00590), showed consistent differential expression in barley leaves following root induction with *P. indica* (Waller et al. 2008). This would suggest that *P. indica* induces a distinct, ISR-type response very different from SAR (Waller et al. 2008) and that the response is distinct from that induced by

PstavrRpm1 (because neither gene was identified here as differentially expressed in the adjacent leaf region). Although the interaction between barley and *P. indica* suggests that some aspects of systemic defense signaling pathways are conserved in barley, it still remains to be proven whether SAR can be biologically induced in cereal species (Kogel and Langen 2005).

Transcriptional profiling revealed extensive local transcriptional reprogramming in barley leaf tissue 24 hpi with *PstavrRpm1*. Transcripts associated with the oxidative burst were highly represented, as were transcripts with similarity to genes classically associated with defense against *Pseudomonas syringae* in *Arabidopsis*, such as *PR-1*, *PR-5*, and *PAL* (Cameron et al. 1999; Mishina and Zeier 2007; Scheideler et al. 2002). Caldo and associates (2004) identified a set of 22 Barley1 GeneChip probe sets that were differentially expressed between virulent and avirulent interactions of barley with *B. graminis* f. sp. *hordei* 16 hpi. Interestingly, 18 of these 22 transcripts were also differentially expressed in the local response to *PstavrRpm1* (Supplementary Table 5), indicating that com-

Fig. 6. Hierarchical clustering of probe sets differentially expressed in the adjacent leaf region with selected Barley1 GeneChip experiments. **A**, Clustering of selected experiments with probe sets differentially expressed in the adjacent leaf region in this study. Pst A and Pst L indicate adjacent and local datasets from this study, respectively (each compared with appropriate region of water-infiltrated control); Mla 6 and 9472 indicate avirulent and virulent interactions between barley and *Blumeria graminis* f. sp. *hordei*, respectively (compared with noninoculated controls); T indicates time point postinoculation (Meng et al. 2009) (PLEXdb exp BB10); 72 Fg indicates 72 h postinoculation of barley with *Fusarium graminearum* (compared with control) (Boddu et al. 2006) (PLEXdb exp BB9); Martin and Hs (*Hordeum spontaneum* 41-1) indicate drought-tolerant cultivars of barley compared with a drought-sensitive cultivar (PLEXdb exp BB84); Lina and Kara indicate barley cultivars susceptible to infestation with aphid *Rhopalosiphum padi*; and DH28:4 and Hsp5 are resistant (compared with a control treatment) (Delp et al. 2009) (PLEXdb exp BB71). **B**, Probe sets clustering with the barley chemically induced (BCI) genes.

mon defense pathways are activated during the local response of barley to both pathogens. Furthermore, none of these transcripts were differentially expressed in the adjacent leaf region, indicating that the local and adjacent responses were fundamentally different. The broad-spectrum adjacent AR induced by *PstavrRpm1* in barley developed outside of the classical LAR zone described for tobacco (Dorey et al. 1997; Ross 1961a and b) but, as noted, did not become systemic. However, the transcriptional profile of the adjacent leaf region exhibiting AR did suggest striking similarities with the transcriptional responses observed in *Arabidopsis* leaves in which SAR had been activated. The adjacent AR response included transcripts with similarity to genes of the *PR-1* regulon, considered to be marker genes for *Arabidopsis* SAR and SA-mediated defense (Maleck et al. 2000; Ryals et al. 1996). Transcripts with similarity to heat shock family proteins; bHLH, zinc finger, NAC domain, and WRKY transcription factors; cytochrome p450; EF-hand domain-containing proteins; and leucine-rich repeat receptor-like kinases were all common to both responses (Maleck et al. 2000; Truman et al. 2007). Transcripts with similarity to genes involved in octadecanoid biosynthesis and signaling were upregulated in the adjacent AR response, consistent with reports for early systemic transcriptional responses in *Arabidopsis* (Truman et al. 2007). These similarities suggest that SAR in *Arabidopsis* and adjacent AR in barley could be mediated by analogous pathways.

In addition to biological induction, SAR in *Arabidopsis* can be induced by the application of SA analogues DCINA and BTH which, in *Arabidopsis*, induce a set of SAR genes similar to biological inducers of SAR (Uknes et al. 1992). In barley, these chemicals induce a set of specific *BCI* genes (Beßer et al. 2000). These *BCI* genes were not induced by inoculation with *B. graminis* f. sp. *hordei*, leading to suggestions that biologically and chemically induced AR are mediated by separate pathways in barley (Beßer et al. 2000; Kogel and Langen 2005). Here, the activation of transcripts with similarity to *BCI* genes in adjacent leaf tissue suggests that there is at least some overlap in the transcriptome of biologically and chemically induced AR in barley. Hierarchical clustering revealed that all five *BCI* transcripts induced in the adjacent leaf region cluster together, along with a set of transcripts whose annotation suggests links to octadecanoid biosynthesis or signaling. Transcripts in this cluster were generally upregulated during interactions with the aphid *R. padi* (Fig. 6), with transcripts similar to *BCI-1* and *BCI-4* being specifically induced in barley lines resistant to aphid infestation (Delp et al. 2009). Although the functional roles of the *BCI* genes are currently unclear, data suggest that enhanced expression of *BCI-1* (*LOX2:Hv:1*) Vörös et al. 1998), *BCI-2*, and *BCI-3* is associated with susceptibility of barley to *B. graminis* and *M. oryzae* (Jarosch et al. 2003; Kogel and Langen 2005). This may also support the involvement of these transcripts in JA-mediated responses that act antagonistically to SA-mediated responses which promote resistance to biotrophs such as *B. graminis* (Kunkel and Brooks 2002).

Taken together, the data suggest that the transcriptional profile of the *PstavrRpm1*-induced adjacent AR response includes a set of transcripts relating to JA biosynthesis or signaling, acting alongside *PR* genes and transcripts involved in basal defense toward *B. graminis* (Fig. 6). This would be consistent with data from *Arabidopsis*, in which JA or octadecanoid signaling appears to play a role in SAR, although the nature of that role is currently a subject of intensive debate (Attaran et al. 2009; Truman et al. 2007; Vlot et al. 2008). Previous work in cereals, aimed at testing the role of JA in AR, has produced some intriguing results (Kogel and Langen 2005). Treatment with JA did not induce local resistance to *M. oryzae* in rice (Schweizer et al. 1997, 1998) or *B. graminis* in barley (Kogel

et al. 1995), although systemic resistance was induced in rice (Schweizer et al. 1998). However, treatment with JA prior to treatment with the SA analogue DCINA enhanced resistance in rice against *M. oryzae* (Schweizer et al. 1997). This may suggest that an SA- (or DCINA)-mediated signal is required for AR but that a JA- or octadecanoid-related response can also contribute to the resistance induced. Detailed metabolite profiling, together with functional analysis of the genes involved, will be important in elucidating the role of JA in AR in cereals.

In conclusion, AR induced in barley by *PstavrRpm1* appears broad spectrum in nature, being effective at a distance from the site of pathogen induction. Whether biologically induced AR in barley can become systemic remains to be determined. However, transcriptional profiling indicates similarities between the transcriptome of AR in the adjacent leaf region of barley and SAR in *Arabidopsis*. Therefore, the results presented further our understanding of AR in cereals and provide a basis for future dissection of the mechanisms involved.

MATERIALS AND METHODS

Plant materials and growth conditions.

Barley Golden Promise was used throughout. Seed were sown directly in barley mix (375 liters of Levington M3 compost [Scotts Professional, Ipswich, U.K.], 100 liters of Perlite, 200 liters of 4-mm grit, and 1.6 kg of Osmocote Plus [Scotts Professional]) in Plantpak p15 trays (Desch, Waalwijk, The Netherlands). Plants were grown in a controlled environment (CE) cabinet (Snijders Scientific, Tilburg, The Netherlands) with a 16-and-8-h light-and-dark cycle (18°C during light and 12°C during darkness). Watering was as required. Golden Promise seedlings were inoculated with *P. syringae* pv. *tomato* (inducer isolate) at the one- to two-leaf stage. After bacterial inoculation, seedlings were transferred to a second CE cabinet with identical settings, except for a constant 23°C temperature to facilitate bacterial growth. Seedlings were moved from the CE cabinet to the lab bench 1 h prior to bacterial inoculation.

P. syringae isolates and growth conditions.

Strains DC3000, *PstavrRpm1* (carrying pVSP61::avrRpm1), and DC3000 *hrcC*- were obtained from J. Fan (John Innes Centre, Norwich, U.K.). *P. syringae* pv. *syringae* isolate B64 was obtained from K. Sohn (The Sainsbury Laboratory, Norwich, U.K.) and originally described by Hwang and associates (2005). For *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* inoculations, overnight log phase cultures grown on King's B (KB) medium with the appropriate antibiotics were diluted to 10⁸ CFU ml⁻¹ (*P. syringae* pv. *tomato*, optical density at 600 nm [OD₆₀₀] = 0.2) or 10⁷ CFU ml⁻¹ (*P. syringae* pv. *syringae*, OD₆₀₀ = 0.02) in sterile water.

Magnaporthe isolates and growth conditions.

M. oryzae isolate *Guy11* was obtained from A. Sesma (John Innes Centre). *M. oryzae* was cultured as previously described (Tufan et al. 2009).

Measuring *P. syringae* bacterial growth in planta.

Leaves were syringe inoculated by pressure infiltration of bacterial suspensions through the abaxial surface of the first true barley leaf using a 1-ml needleless syringe. Pressure infiltration was via two small punctures created by piercing the epidermis once on either side of the midrib using a 200-μl pipette tip. The central third of the leaf was flooded, with the limit of inoculation marked using a marker pen. Control seedlings were infiltrated in the same way with sterile water. After the indicated time, 0.5-cm leaf discs were taken from the inoculated leaf area using a cork borer and homogenized in KB broth.

Appropriate serial dilutions were plated on KB media containing the appropriate antibiotics, and colonies counted after 24 h of incubation at 28°C to provide a measure of CFU.

Tests of AR in leaf tissue adjacent to local, *P. syringae*-inoculated leaf tissue.

For inoculation of the local leaf region (Fig. 2) a *P. syringae* pv. *tomato* suspension was pressure infiltrated, as described above, via a single puncture toward the distal end of the midrib of the first true leaf. The distal third of the leaf was flooded with bacterial suspension, with the limit of inoculation being defined using a marker pen (Figs. 1 and 2). Control seedlings were infiltrated with sterile water. To test for AR to *P. syringae* pv. *syringae*, the adjacent leaf region (Fig. 2) was inoculated with strain B64 24 h after local inoculation. Syringe inoculation was carried out as described above into the proximal third of the first true leaf (Fig. 2). Then, 24 h later, growth of strain B64 was measured as described above. To test for AR to *M. oryzae*, whole seedlings were mist inoculated with 2 to 4 ml of conidial suspension 24 h after local inoculation with *PstavrRpm1*. To test for AR to *M. oryzae* in secondary leaves, seed sowing and local inoculation with *PstavrRpm1* was staggered so that challenge with *M. oryzae* was carried out simultaneously for all time points. *M. oryzae* inoculation and subsequent plant growth conditions were as previously described (Tufan et al. 2009). Leaves were photographed 5 days later and diseased leaf area scored using ImageJ (Fig. 4) (Abramoff et al. 2004) or APS Assess (Lamari 2002).

Global transcriptional profiling.

Local inoculation with *PstavrRpm1* (and infiltration of water controls) was carried out as described above. At 24 h after local inoculation, tissue from the local and adjacent leaf regions (Fig. 2) was harvested. A number of seedlings were retained to verify symptom development and adjacent AR to *P. syringae* pv. *syringae* challenge. Three biological replicates of each leaf region from both *P. syringae* pv. *tomato*-inoculated and water control seedlings were taken, each made up of pooled tissue from three seedlings. Leaf tissue was ground under liquid nitrogen and RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). DNase I treatment was performed using TURBO DNase (Ambion, Austin, TX, U.S.A.). Global transcriptional profiling was performed using the Affymetrix Barley1 GeneChip (Affymetrix, Santa Clara, CA, U.S.A.). Assessment of RNA quality, cRNA preparation, and microarray hybridization were carried out by Cogenics (Morrisville, NC, U.S.A.), according to the standard Affymetrix protocol (two-cycle target labeling method). Data were analyzed as described previously (Tufan et al. 2009), with the additional application of a multiple testing correction (false discovery rate) (Benjamini and Hochberg 1995). Probe sets with a fold change of >2 relative to the control and a moderated *t* statistic of *P* < 0.05 were considered differentially expressed. Array data has been deposited in PLEXdb (Wise et al. 2007) as experiment BB92.

qRT-PCR.

Leaf tissue was sampled and RNA prepared as described above. cDNA was synthesized from 1 µg of RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, U.S.A.), primed with random hexamers. cDNA was diluted 1:10 in sterile water. qRT-PCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma, St. Louis). Primers were designed using Primer3 (Rozen and Skaletsky 2000) to the exemplar sequence for each probe set, or taken from the literature. A DNA engine Opticon2 Continuous Fluorescence Detector (Bio-Rad, Hercules, CA, U.S.A.)

was used for qRT-PCR with the following protocol: 95°C for 4 min; 40 cycles of 95°C for 30 s, 59 to 66°C for 30 s, and 72°C for 30 s; followed by melt-curve analysis (65 to 95°C). The average quantification cycle of two technical replicates was determined for each sample. Genorm v3.5 (Vandesompele et al. 2002) was used to identify three reference genes that were stable under our experimental conditions; the geometric mean of the two most stable reference genes in each experiment was then used to calculate normalized expression ratios and relative expression according to Pfaffl and associates (2001). Primer details are given in Supplementary Table 6.

Microarray meta-analysis.

Experiments used in meta-analysis were selected from PLEXdb (Wise et al. 2007). Experiments used were BB10 (Meng et al. 2009) (8, 20, and 32 hpi time points), BB9 (Boddu et al. 2006) (72 hpi time point), BB84 (M. Baum, *unpublished*), and BB71 (Delp et al. 2009). Data files were normalized using RMA (Irizarry et al. 2003) in R using the package AffyLMGUI (Wettenhall et al. 2006). Linear modeling and an Empirical Bayes moderated *t* statistic (Smyth 2004) were used to calculate differential expression based on contrasts for each experiment made between biotic or abiotic stress-treated samples and their respective controls. The expression profile of those probe sets differentially expressed in the adjacent leaf region (this study) was identified and the data exported into a tab-delimited file. Hierarchical clustering was conducted in Cluster 3 (Eisen et al. 1998) using the Euclidean distance matrix and complete linkage clustering technique. Treeview v1.0.13 was used to visualize the data.

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