ARTICLE ADDENDUM

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Barley stripe mosaic virus-induced gene silencing (BSMV-IGS) as a tool for functional analysis of barley genes potentially involved in nonhost resistance

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Barley is an alternative host for the rice blast fungus *Magnaporthe oryzae* but is resistant to Magnaporthe species associated with the grass genera Pennisetum and Digitaria. The latter cases are examples for nonhost resistance which confers effective and durable protection to plants against a broad spectrum of pathogens. Comparative transcript profiling of host and nonhost interaction revealed an early and pronounced change in gene expression in epidermal tissue of barley infected with a Magnaporthe nonhost isolate. Interestingly, this set of genes did not overlap considerably with the transcriptional response of barley against nonhost rust or powdery mildew isolates. For a functional testing of candidate genes a combined approach of virus-induced gene silencing (VIGS) and subsequent pathogen challenge was established. As anticipated, VIGS-mediated downregulation of *Mlo*-transcripts led to higher resistance against *Blumeria graminis* f.sp. *hordei* and enhanced susceptibility against *M. oryzae*.

Key words: *Blumeria graminis*, Magnaporthe, macroarray, *mlo*, nonhost resistance, VIGS

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Nonhost resistance (NHR) of a plant species operates against all races of a given pathogen species for which the plant is not considered a host.[1] Sustainability and broad-spectrum resistance under field conditions make NHR a promising resource for crop improvement.[2,3] Interrogating for a common mechanism of NHR in barley against different pathogens, we analyzed the transcriptional response of one particular barley genotype against three pairs of adapted and non-adapted Magnaporthe, Blumeria and Puccinia isolates, respectively.[4] The study showed that NHR of barley against each pathogen is associated with the regulation of distinct sets of genes which, however, are involved in similar metabolic or signaling pathways. We chose the interaction between barley and fungi of the genus Magnaporthe as a model to study the mechanisms underlying NHR in more detail.[5] Isolates of the species *M. oryzae*, best-known as the causal agent of "rice blast," are pathogenic on rice and other cultivated grasses, such as millet, wheat and barley while other Magnaporthe species isolated from Digitaria or Pennisetum are not able to infect barley.[5,6] Mechanistically this nonhost type of resistance appears to be based on a more efficient execution of different defense strategies, i.e., formation of papillae and onset of the hypersensitive response, also known from attacked epidermal cells in the host interaction.[7,8] Here, we summarize our efforts to characterize the NHR of barley against Magnaporthe at the molecular level using transcriptome profiling and VIGS.

Transcriptional Response of Barley against Magnaporthe

To elucidate determinants of the barley NHR repertoire a global transcript profiling approach was conducted comparing barley plants inoculated with either host or nonhost Magnaporthe species (**Fig. 1A**). The analysis was restricted to the epidermis because this tissue primarily gets attacked by the pathogen

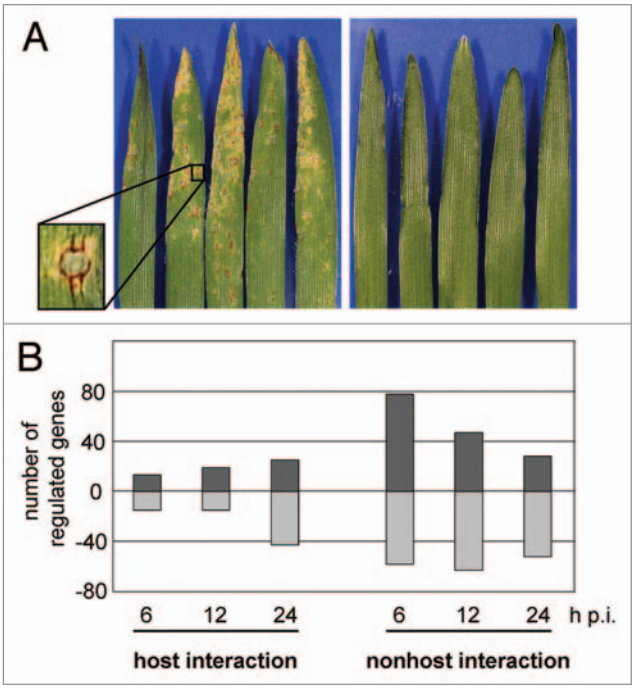

Figure 1. Characterization of host and nonhost interactions between barley and different Magnaporthe isolates at the macroscopical and transcriptional level. (A) Primary leaves of barley 5 days after inoculation with *Magnaporthe oryzae* isolate TH6772 (host interaction, left side) and a Magnaporthe species isolated from Pennisetum (CD180, nonhost interaction, right side). A typical *M. oryzae* disease symptom on barley is shown in larger scale (inset). (B) Number of genes up (positive values) or downregulated (negative values) in barley/Magnaporthe interactions was identified using the barleyPGRC1 macroarray. Only genes with a ≥2-fold differential regulation relative to the control treatment (FDR ≤5%) in four independent experiments are considered.

and in case of a nonhost interaction the pathogen gets locked in this tissue. Therefore RNA was isolated from peeled epidermis of barley harvested 6, 12 or 24 h post inoculation (h p.i.) and analyzed using the barleyPGRC1 macroarray at IPK Gatersleben.[4] As a result 250 genes could be identified, which were either up or downregulated during the nonhost interaction. Expression level of 180 of these genes was not altered during the host interaction. Looking at the kinetics of transcriptional changes it was remarkable that they arose as early as 6 h p.i. during the nonhost interaction (**Fig. 1B**). In contrast, generally fewer genes were regulated during the host interaction and the detected changes peaked rather late at 24 h p.i. This confirms the hypothesis, that defense reactions against Magnaporthe are triggered faster in the nonhost situation than in the host situation and therefore operate more efficiently.[5,9] This is in agreement with results of time-course analyses of the barley transcriptome during host and nonhost interactions with powdery mildew.[10] Among the genes that were specifically upregulated in the Magnaporthe nonhost interaction and therefore might play a crucial role in NHR, several lipid transfer proteins, a cytochrome P450 and an ascorbate peroxidase were listed.[4] Functional characterization of these genes could be achieved by generating stable RNAi transformants, however, this is difficult and time-consuming in barley. To circumvent this drawback we decided to adopt a VIGS approach using the rod-shaped hordeivirus BSMV as a vector which was the first to be used among monocotyledonous plants.[11] Gene fragments of interest can be placed into the viral γ-subunit of the tripartite BSMV genome using a multiple cloning site (MCS).[12] After infecting plants with the transformed BSMV the plant's natural antiviral defense system leads to a transient knockdown of the corresponding plant gene (reviewed in ref. 13).

Validation of BSMV-IGS using *Mlo*-Silencing as a Case Study

Prior to an analysis of candidate genes, the BSMV-IGS system was validated in our lab using *Mlo* as a test gene. Barley plants carrying a loss of function mutation at the *Mlo* locus are completely resistant to all known isolates of *Bgh* but behave hypersusceptible to *M. oryzae* and *Bipolaris sorokiniana*.[14-16] It has already been shown that silencing of *Mlo* using transient-induced gene silencing based on biolistic transgene delivery phenocopied the resistance of *mlo*-mutant plants against *Bgh*.[17] But to our knowledge this approach hasn't been addressed for barley using VIGS so far. A 251 bp gene fragment of the barley *Mlo* gene was amplified by PCR using primers Mlofor: GCA TTT TGT GTG GAC AGT GG and Mlorev: CCG TGT CTC GGA CTT TCT TC and cloned in antisense-orientation into *Bam*HI restriction site of pT7-BSMV-γMCS to form pT7-BSMV-γMlo. Inoculation of barley plants *cv.* Morex with viral RNAs was done as described in reference 12. Infection of barley plants with BSMV containing the *Mlo* silencing construct against the *Mlo* gene resulted in transcriptionally downregulation of the target gene as confirmed by qPCR (data not shown). However, we found an upregulation of *Mlo* transcripts in response to inoculation with the unmodified virus which is in accordance with the known responsiveness of *Mlo* to biotic and abiotic stresses.[18] This BSMV-related increase in *Mlo* transcript abundance was reduced by 60% in average due to the presence of the *Mlo* silencing construct in the modified BSMV-γMlo (data not shown). Plants from this experiment showing viral disease symptoms on secondary leaves were selected and inoculated on detached third leaves with *Bgh*. This resulted in heavily infected control *Mlo*-plants whereas *mlo11*-plants showed no mildew symptoms, thus confirming the suitability of the assay (**Fig. 2A**). Microscopic inspection of infection sites verified, that fungal penetration in the *mlo11* genotype was counterattacked to an extent of 100% by the formation of cell wall appositions (papillae), which couldn't be penetrated by *Bgh* (**Fig. 2B** and see also ref. 19). Plants inoculated with

unmodified BSMV showed more disease symptoms as compared to untreated *Mlo*-plants (**Fig. 2A**) which is in agreement with higher *Mlo*-transcript abundance detected in these plants. Plants infected with BSMV-γMlo showed less *Bgh*-pustules and, at the microscopic level, a higher frequency of effective papillae compared to control *Mlo*-plants and BSMV-γMCS infected plants (**Fig. 2A and B**). First results in an analogous experiment but with *M. oryzae* as challenging pathogen indicate the anticipated higher susceptibility of *Mlo*-silenced plants (data not shown). In sum our results confirmed that BSMV-mediated silencing in combination with Blumeria or Magnaporthe infections as a reliable system in barley to test candidate genes for their involvement in NHR.

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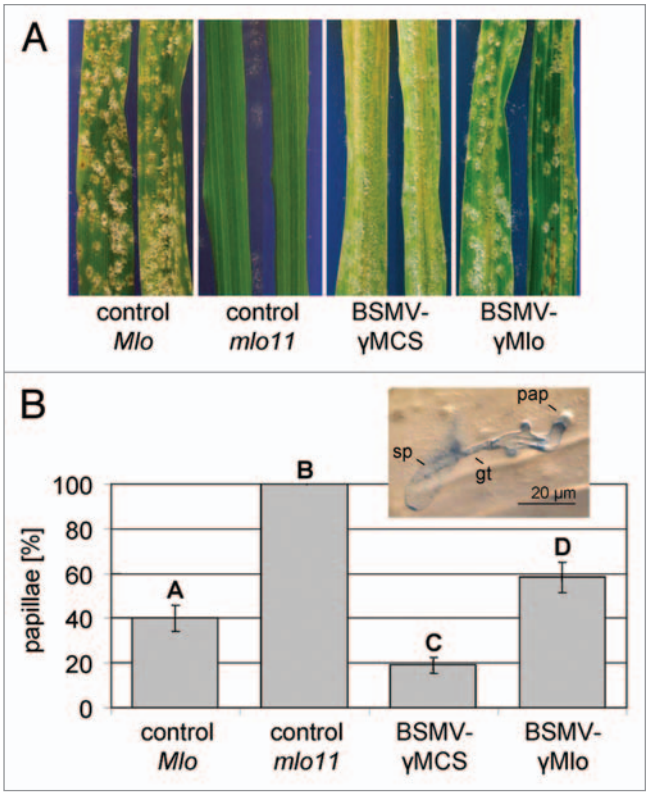

Figure 2. Macroscopical and microscopical analysis of *Bgh* inoculated barley leaves after BSMV-IGS of the *Mlo* gene. Third leaves of barley cv. Morex (control *Mlo*), Grannenlose Zweizeilige (*mlo11*), Morex infected with unmodified BSMV (BSMV-γMCS) and Morex infected with BSMV carrying a *Mlo* silencing construct (BSMV-γMlo), respectively, were inoculated with *Bgh*. (A) Powdery mildew disease symptoms 8 days after inoculation. (B) For quantitative cytological analysis leaves were harvested at 48 h p.i., cleared and stained with blue ink. Only sites with a non-penetrated papilla beneath the appressorium were counted. The micrograph shows an example of these interaction sites (sp = spore, gt = germ tube, pap = papilla). Results presented in the bar chart are means and standard errors from 4 leaves with 100 interaction sites inspected per leaf. Significant differences (α = 5%) were determined using OneWayAnova and indicated by different letters. The experiment was repeated twice with similar results.

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