Silencing of *RBOHF2* Causes Leaf Age–Dependent Accelerated Senescence, Salicylic Acid Accumulation, and Powdery Mildew Resistance in Barley

Denise Pereira Torres, Reinhard K. Proels, Harald Schempp, and Ralph Hükelhoven[†]

Lehrstuhl für Phytopathologie, Technische Universität München Emil-Ramann-Straße 2, D-85354 Freising-Weihenstephan, Germany

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Plant RBOH (RESPIRATORY BURST OXIDASE HOMOLOGS)-type NADPH oxidases produce superoxide radical anions and have a function in developmental processes and in response to environmental challenges. Barley *RBOHF2* has diverse reported functions in interaction with the biotrophic powdery mildew fungus *Blumeria graminis* f. sp. *hordei*. Here, we analyzed, in detail, plant leaf level- and age-specific susceptibility of stably *RBOHF2*-silenced barley plants. This revealed enhanced susceptibility to fungal penetration of young *RBOHF2*-silenced leaf tissue but strongly reduced susceptibility of older leaves when compared with controls. Loss of susceptibility in old *RBOHF2*-silenced leaves was associated with spontaneous leaf-tip necrosis and constitutively elevated levels of free and conjugated salicylic acid. Additionally, these leaves more strongly expressed pathogenesis-related genes, both constitutively and during interaction with *B. graminis* f. sp. *hordei*. Together, this supports the idea that barley *RBOHF2* contributes to basal resistance to powdery mildew infection in young leaf tissue but is required to control leaf cell death, salicylic acid accumulation, and defense gene expression in older leaves, explaining leaf age-specific resistance of *RBOHF2*-silenced barley plants.

When attacked by microbial pathogens, both susceptible and resistant plants are able to react to attempted infection. Thereby, the speed and intensity at which the immune responses are activated determine the outcome of the plant-pathogen interactions (Yang et al. 1997). A resistant plant can prevent disease or limit the extent of infection exploiting a large variety of preformed and inducible defense mechanisms. Inducible defense responses involve specific recognition of nonspecific pathogen elicitors (microbe-associated molecular patterns [MAMPs]) by plant cell surface-localized pattern recognition receptors (PRRs) or of specific effector proteins (avirulence factors) from the pathogen by corresponding resistance (R) proteins (Fu and Dong 2013; Thomma et al. 2011). Accordingly, pathogen recognition is pivotal to two major forms of plant immunity, pattern-triggered immunity and effector-triggered immunity. Additionally, plants show pathogen-induced acquired resistance in either attacked or systemic plant organs. The most prominent form of acquired resistance is called systemic acquired resistance. It occurs in

healthy tissues distant from the initial infection site. Systemic acquired resistance involves a systemic salicylic acid (SA)-dependent expression of PATHOGENESIS-RELATED (PR) genes that results in an incremental increase in the resistance against secondary infection in the whole plant (Hammerschmidt 1999). Besides this, plants can achieve resistance to one or several pathogens with the aging of the plant or an organ through a little understood mechanism known as developmental resistance, adult plant resistance (APR), or age-related resistance.

Reactive oxygen species (ROS) are important signaling molecules that are naturally produced as a consequence of the aerobic metabolism or are deliberately generated by enzymatic complexes as part of plant responses to environmental constraints. Plasma membrane-localized NADPH oxidases (RESPIRATORY BURST OXIDASE HOMOLOGS [RBOHs]) constitute a protein family and RBOHs are prominent sources of ROS during plant development and in several responses to the environment (Mittler et al. 2011; Torres et al. 1998). RBOHs have a prominent role during plant-pathogen interactions. *AtRBOHF* is a crucial modulator of defense-related metabolism upon pathogen infection and is involved, together with *AtRBOHD*, in the regulation of basal resistance and the hypersensitive response (HR) (Chaouch et al. 2012; Morales et al. 2016; Siddique et al. 2014; Torres et al. 2002). *AtRBOHD* is the major ROS-generating enzyme that operates in pattern-triggered immunity and in systemic stress signaling (Dubiella et al. 2013; Miller et al. 2009). *AtRBOHD* functions in long-distance signaling in response to stimuli such as wounding, heat, cold, and salinity (Miller et al. 2009). *OsRBOHA* is required for the induction of HR in rice, and both *OsRBOHA* and *OsRBOHE* are involved in the production of ROS in response to a specific elicitor of an avirulent strain of the bacterial pathogen *Acidovorax avenae* (Yoshie et al. 2005). Levels of the wheat *TaRBOHD* and *TaRBOHF* genes are up-regulated upon challenge with the rust fungus *Puccinia triticina*, and, in the backcross line *TcLr26*, the resistance-related oxidative burst response is sensitive to a chemical inhibitor of NADPH oxidases (Dmochowska-Boguta et al. 2013). The barley gene *HvRBOHF2*, a close homolog of *Arabidopsis AtRBOHF* and rice *OsRBOHA*, is involved in defense responses against phytopathogens. The expression of *HvRBOHF2* is enhanced in response to infection by both necrotrophic and biotrophic fungi (Lightfoot et al. 2008; Trujillo et al. 2006).

Powdery mildew fungi are worldwide-distributed ascomycete fungi of the *Erysiphales* order that cause disease in many economically relevant crops and ornamentals plants. These plant-pathogenic fungi are obligate biotrophs that obtain nutrients from living host cells through specialized feeding structures called haustoria (Glawe 2008). Among those, *Blumeria graminis* is

[†]Corresponding author: R. Hükelhoven; E-mail: hueckelhoven@wzw.tum.de

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unique due to the ability to infect grasses, such as wheat and barley, which provides a well-established model system to study the interaction of obligate biotrophic fungi with crop plants (Dean et al. 2012; Hückelhoven and Panstruga 2011).

Detached first leaves of barley showed reduced susceptibility to penetration by the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* at the single-cell level when *HvRBOHF2* (previously called *HvRBOHA*) was silenced by transient induced gene silencing after biolistic transformation (Trujillo et al. 2006). Proels et al. (2010) later reported on generation of stable transgenic *HvRBOHF2* knock down (KD) barley plants that showed an overall reduction of more than 80% in the *HvRBOHF2* transcripts level in the lines tested. These plants also exhibited decreased expression of *HvRBOHF1*, a closely related isoform that shares 91% amino acid identity with *HvRBOHF2*, but unaffected expression of the more divergent isoforms *HvRBOHE* and *HvRBOHJ*. Contrary to the outcome after transient silencing in detached leaves, stable *HvRBOHF2* KD plants displayed enhanced susceptibility to penetration by *B. graminis* f. sp. *hordei*. Additionally, *HvRBOHF2* KD plants showed a runaway cell-death phenotype when wounded and spontaneous cell death in old leaves (Proels et al. 2010).

Here, we studied in detail a transgene-induced leaf age-related resistance phenomenon to *B. graminis* f. sp. *hordei* in barley. Based on the observation that *HvRBOHF2* KD lines become less susceptible to *B. graminis* f. sp. *hordei* with the progression of leaf age, we analyzed the susceptibility to *B. graminis* f. sp. *hordei* and the progression of the powdery mildew disease in different leaves from 11- and 17-day-old barley plants silenced for *HvRBOHF2*. Our study suggests that leaf age-related resistance against *B. graminis* f. sp. *hordei* in *HvRBOHF2* KD lines is a consequence of mis-regulated senescence-associated cell death, SA accumulation, and PR gene expression.

RESULTS

HvRBOHF genes modulate susceptibility to *B. graminis* f. sp. *hordei* in a manner dependent on leaf age and leaf section.

In previous work, we showed that young leaves of barley transgenic plants, which were silenced for *HvRBOHF2* and cosilenced for *HvRBOHF1*, showed enhanced susceptibility to penetration by the barley powdery mildew fungus *B. graminis* f. sp. *hordei* (Proels et al. 2010). Following up, we observed that the susceptibility to penetration by *B. graminis* f. sp. *hordei* differed between different leaves of *RBOHF2* KD lines. Thereby, physiological older, first leaves were less susceptible than physiological younger leaves. In order to test whether the susceptibility to *B. graminis* f. sp. *hordei* differs in differently old parts of the leaf blade, we also separately evaluated the outcome of the interaction in the tip and in the base half sections of first three leaf levels (Fig. 1A and C). We quantified the frequency of successful cell-wall penetration as the percentage of cells with fungal haustoria 48 h after inoculation (hai) with *B. graminis* f. sp. *hordei*. In whole leaves, when we analyzed the tip and base leaf sections together, we noticed a reduction in the overall haustoria frequency in the first leaf of the 11-day-old *HvRBOHF2* KD line when compared with azygous controls (i.e., siblings that had lost the *HvRBOHF2* silencing cassette due to segregation). On the other hand, in the second, later developing and physiologically younger leaf, the frequency of haustoria formation was slightly but not significantly enhanced in *HvRBOHF2* KD plants (Fig. 1B). This phenotype was similarly documented in 17-day-old plants. In this case, the first and second leaves of the *HvRBOHF2* KD line showed reduced susceptibility, whereas the physiologically younger third leaf showed a tendency of enhanced susceptibility to *B. graminis* f. sp. *hordei* when compared with the azygous

control (Fig. 1D). Thus, in the progress of leaf development (between day 11 and day 17), the susceptibility of the second leaves of the *HvRBOHF2* KD line strongly dropped from about a 60% penetration rate to less than 35%. In parallel, second leaves of azygous controls retained susceptibility to fungal penetration at a frequency of around 50 to 55% (Fig. 1B and D). These data support the idea that leaf age but not leaf type was responsible for this phenotype. In general, susceptibility was higher in physiologically younger leaves of the *HvRBOHF2* KD line (50 to 60% penetration on second leaves of 11-day-old plants and third leaves of 17-day-old plants) when compared with physiologically older leaves (25 to 35% penetration on first leaves of 11-day-old plants and first and second leaves of 17-day-old plants). At the same time, azygous plants did not show a clear leaf age-dependent susceptibility (Fig. 1B and D).

Growth of grass leaves is limited to the growth zone at the basal meristematic part of the leaf. When entering the elongation zone, cells elongate without further cell division until they reach their final length (Kavanová et al. 2006). Therefore, the basal part of the leaf represents the physiologically younger part of the leaf, whereas the distal and tip part represents the physiologically older part of the leaf. When we considered the susceptibility of tip and base leaf sections separately, we observed leaf section-dependent susceptibility phenotypes in *HvRBOHF2* KD lines. In particular, in 17-day-old *HvRBOHF2* KD plants, the bases of young, third leaves were significantly more susceptible than in azygous controls, whereas the tips of the same leaves were similar to azygous controls. At the same time, older, first and second leaves were less susceptible than the controls, and this was statistically significant in the tip of the leaves (Fig. 1D). These phenotypes were reproduced using a second, independent *HvRBOHF2* KD line (Supplementary Fig. S1).

ROS production and pathogen-induced cell death in *HvRBOHF2* KD plants.

In compatible barley-powdery mildew interaction, non-penetrated cells usually show formation of papillae (cell-wall appositions) beneath the site of fungal penetration attempts. Alternatively, nonpenetrated cells can show a cell death (HR-like single cell death) even in compatible interactions, albeit to lower frequencies than papilla formation. We next analyzed the pathogen-triggered cellular ROS production that is associated with papilla formation, HR, or haustorium formation. To address this, we performed 3,3-diaminobenzidine (DAB) staining of leaves detached from 17-day-old plants at 48 hai with *B. graminis* f. sp. *hordei*. As documented in previous studies, DAB-staining events were associated with the cell-wall appositions in nonpenetrated cells. DAB staining in penetrated haustoria-containing cells was mainly associated with anticlinal cell walls facing neighboring epidermal cells. HR-related cell death was evident from whole-cell DAB staining (Hückelhoven et al. 1999, 2000a; Proels et al. 2010; Thordal-Christensen et al. 1997). To see whether silencing of *HvRBOHF2* influences the ability of barley to carry out localized oxidative defense or HR, we counted local cell wall-associated DAB-staining and whole-cell DAB-staining events (Fig. 2A and B). First leaves of *HvRBOHF2* KD plants showed, overall, 50% fewer DAB-staining events than azygous controls, but third leaves showed just 8% fewer events. Thereby, in agreement with Proels et al. (2010), the frequency of *B. graminis* f. sp. *hordei*-induced epidermal cell death (whole-cell DAB staining) was not affected in any leaves of *HvRBOHF2* KD plants. Instead, the percentage of *HvRBOHF2* KD cells with local cell wall-associated DAB staining in papilla or anticlinal cell walls was reduced, but only in the older first leaf (Fig. 2A). Taken together, cellular ROS production as evidenced

by DAB staining was reduced in older leaves of *HvRBOHF2* KD plants.

We next measured the oxidative burst triggered by different MAMPs in leaf discs from *HvRBOHF2* KD plants, by the use

of luminol derivates L-012 (Scheler et al. 2016). As elicitors, we used the bacteria-related flagellin peptide flg22, the fungus-related elicitor chitin, and laminarin, a β -glucan elicitor from the marine brown algae *Laminaria digitate*, recently shown to

Fig. 1. Interaction phenotype of *Blumeria graminis* f. sp. *hordei* with different leaves of either the *HvRBOHF2* knock down (KD) line 30/2E08 or the azygous control in two growth stages. **A**, Schematic representation of barley leaf development of 11-day-old plants (Zadoks growth scale 12). The first leaf (through coleoptile) and second leaf are completely unfolded. **B**, Frequency of successful cell-wall penetration with formation of visible haustoria at interaction sites in the whole leaf, older meristem-distal (tip), or younger meristem-proximal (base) sections of the leaf in 11-day-old plants. **C**, Schematic representation of barley leaf development of 17-day-old plants (Zadoks scale 13). The first three leaves are completely unfolded and the forth leaf is emerging. **D**, Frequency of successful cell-wall penetration with formation of visible haustoria at interaction sites in the whole leaf, tip, or base sections of the leaf in 17-day-old plants. Leaves were fixed 48 h after inoculation with *B. graminis* f. sp. *hordei*, and plant and fungal structures were analyzed by bright-field microscopy. Data represents average of 110 to 160 interaction sites per leaf of four leaves per line of one representative experiment. Error bars show standard errors of the mean. Lowercase letters show the grouping of data sets relative to successful penetration in the whole leaf and uppercase letters in the leaf sections, according to Tukey's test at $P = 0.05$. Data are representative for three independent biological repetitions.

act as an ROS elicitor in barley (Wawra et al. 2016). The kinetics and intensity of ROS production differed between the elicitors used (Supplementary Fig. S2). All three elicitors triggered typical transient elevations in ROS production, as measured by distinct kinetics of L-012 luminescence. For each elicitor, the kinetics of ROS accumulation was similar in discs from different leaves (first, second, or third leaves). However, the amount of ROS was strongly reduced in all leaf discs from the *HvRBOHF2* KD plants, after treatment with these elicitors. However, less oxidative defense could not explain higher penetration resistance of older leaves from *HvRBOHF2* KD plants. Therefore, we assumed that other defense mechanisms enhanced resistance in older *HvRBOHF2* KD leaves as detailed below.

Progression of the powdery mildew disease is affected in *HvRBOHF2* KD lines.

To get a better understanding of the role of *HvRBOHF2* in the postpenetration stages of *B. graminis* f. sp. *hordei* infection, we further followed powdery mildew disease progression by counting colony numbers per leaf area and by measuring the length of *B. graminis* f. sp. *hordei* colonies 4 days after inoculation of 17-day-old plants. When we first looked at green tissue, we observed a great reduction in the number of colonies per leaf area in older first leaves of *HvRBOHF2* KD in comparison with azygous plants, but no differences regarding younger third leaves (Fig. 3A; Supplementary Fig. S3A to D). We then fixed and destained these very same leaves, stained the fungal mycelium with blue ink, and again counted the number of colonies, now including microcolonies (elongated secondary hyphae without sporulation). In this evaluation, the differences in colony number in first leaves of *HvRBOHF2* KD compared with azygous control became smaller (Fig. 3B). This difference between the counts in green versus fixed tissue could be explained by the fact that the *B. graminis* f. sp. *hordei* colonies on *HvRBOHF2* leaves were much smaller, had fewer or no conidiophores and fewer hyphal branches compared with azygous controls. We therefore measured the size of the *B. graminis* f. sp. *hordei* colonies and found that the colony length was reduced in all tested leaves of *HvRBOHF2* KD plants compared with azygous controls (Fig. 3C). Moreover, the length of the *B. graminis* f. sp. *hordei* colonies decreased from the third to the first leaf in *HvRBOHF2* KD plants but stayed the same in all tested leaves of azygous controls.

In conclusion, there were two mechanisms that contributed to the observed *B. graminis* f. sp. *hordei*-related phenotypes: i) early in the interaction, a reduction in the frequency of penetration and haustorium formation in older leaves of *HvRBOHF2* KD plants (Fig. 1) and ii) a retarded development of the pathogen at the leaf surface. Eventually, silencing of *HvRBOHF2* resulted in a delay of postpenetration powdery mildew disease progression on all leaf levels. Similar results were obtained using a second, independent *HvRBOHF2* KD line (Supplementary Fig. S4).

HvRBOHF2 KD leads to premature leaf senescence and cell death.

Previously, we showed that *HvRBOHF2* KD plants are characterized by age-dependent pleiotropic effects, such as spontaneous mesophyll cell death and a failure to contain wound-induced cell death (Proels et al. 2010). To get a better understanding of the physiological status of 17-day-old *HvRBOHF2* KD plants, we analyzed corresponding leaves for the appearance of cell death. Necrotic lesions were visible at the leaf tip of first leaves of *HvRBOHF2* KD plants around 12 to 15 days after sowing. These lesions, usually followed by chlorosis, spread quickly throughout the leaf tip and further developed downward to the leaf base. The appearance of necrotic lesions was

Fig. 2. Pathogen-triggered accumulation of hydrogen peroxide in *HvRBOHF2* knock down (KD) plants. **A**, Frequency of local cell wall (CW)-associated diaminobenzidine (DAB) staining and whole-cell DAB staining events in the first and **B**, third of *HvRBOHF2* KD and azygous control plants. Seventeen-day-old barley plants were inoculated with *Blumeria graminis* f. sp. *hordei* and, 42 h after inoculation, the leaves were excised and placed with the cut face in a 1 mg ml⁻¹ DAB solution for 6 h. Then, the leaves were fixed and fungal structures were analyzed by bright-field microscopy. Data represent the means of one representative experiment with five to seven leaves per line. Error bars show standard errors of the mean and asterisks indicate significant differences between the genotypes (Student's *t* test: three asterisks, *P* < 0.001). Data are representative for three independent biological repetitions.

age-dependent and started first in oldest first leaves and, then, following the order of the leaf age. Necrotic lesions were prominent in the tip of first and second leaves of 17-day-old plants but not noticed in the young third leaf (Fig. 4A). When these leaves were fixed and observed under the stereo microscope, we saw a high level of necrotic spots throughout the leaf tip in the first and second leaves but little cell death in the tip of the third leaf. Azygous control plants, however, showed only a slight induction of cell death at the very tip of all the leaves (Supplementary Fig. S5).

In order to determine if the spontaneous cell death observed in the leaf tip of *HvRBOHF2* KD plants is associated with premature senescence of the leaves, chlorophyll content, which is known to decrease with leaf senescence, was measured in 17-day-old leaves. As necrotic lesions started to manifest at the tip of older leaves, we took samples of both leaf sections. We measured

no significant difference in chlorophyll content of third leaves between azygous controls and *HvRBOHF2* KD plants. First and second leaves, however, were characterized by significantly reduced chlorophyll content in tip and base compared with azygous controls. Different from azygous controls, *HvRBOHF2* KD plants showed a continuous decrease in chlorophyll content from the youngest third, over the second, to the physiologically oldest first leaf (Fig. 4B). This reduced chlorophyll content in older leaves, indicative of an early leaf-senescence phenotype, thus coincided with the appearance of spontaneous necrotic lesions in *HvRBOHF2* KD plants.

HvRBOHF2 expression is dependent on the leaf age.

We next studied the level of *HvRBOHF2* expression in different leaves of barley plants 48 hai with *B. graminis* f. sp. *hordei*, by reverse transcription quantitative real-time polymerase chain

Fig. 3. Effect of the silencing of *HvRBOHF* genes on the progression of the powdery mildew disease. Number of developing colonies per leaf area in 17 day-old-barley *HvRBOHF2* knock down (KD) line 30/2E08 and the azygous controls. **A**, Colonies were quantified in green tissue or **B**, in fixed leaves under a stereomicroscope. Plants were infected with *Blumeria graminis* f. sp. *hordei*, were incubated for 96 h in a climate chamber, and were individually harvested. Data represent the means of one representative experiment with five leaves per line. Error bars show standard errors of the mean and letters the grouping of data sets according to Tukey's test at $P = 0.05$. **C**, Length of *B. graminis* f. sp. *hordei* colonies on the surface of different leaves. Colony length was sized using the software Leica LAS v4.5. A total of 80 pustules per line and leaf type were evaluated. Box plots represent the median (horizontal line) and the interquartile range (box). Whiskers show the minimum and maximum measured values and letters indicate the grouping of data sets according to Games-Howell test at $P = 0.05$. Data are representative for three independent biological repetitions.

reaction (RT-qPCR). We could not get high quality RNA from first and second leaves of 17-day-old *HvRBOHF2* KD plants, most likely due to the necrosis shown by these leaves. Therefore, 11-day-old plants were investigated. As shown in Figure 5, we confirmed a robust reduction in the amount of *HvRBOHF2* transcripts in *HvRBOHF2* KD plants in all leaf samples tested, when compared with corresponding azygous controls. The basal expression of *HvRBOHF2* was higher in second, physiologically younger leaves of *HvRBOHF2* KD and the relative degree of silencing of *HvRBOHF2* was stronger in the first, compared with the second, leaf. Together, physiological younger second leaves of *HvRBOHF2* KD plants had a stronger residual expression of *HvRBOHF2*, compared with older, less-susceptible first leaves.

The *HvRBOHF2* KD plants showed reduced transcript amounts of *HvRBOHF2* at 48 hai with *B. graminis* f. sp. *hordei* in the second leaf when compared with noninoculated *HvRBOHF2* KD plants. The first leaves of these *HvRBOHF2* KD plants did not show a *B. graminis* f. sp. *hordei*-induced effect on *HvRBOHF2* expression, probably due to the already low level of constitutive *HvRBOHF2* expression (Fig. 5). For the azygous line, we did not observe any significant *B. graminis* f. sp. *hordei*-induced differential expression of *HvRBOHF2* at 48 hai.

PR gene expression is enhanced in *HvRBOHF2* KD lines.

PR proteins are involved in defense responses during plant-pathogen interactions and possibly in leaf senescence-associated cell death, as accumulation of these proteins at advanced stages of development have been reported for some plant species, including barley (Lim et al. 2007; Tamás et al. 1998). To check whether the pathogenesis phenotypes of *HvRBOHF2* KD plants

are related to a differential expression of *PR* genes, we investigated the expression level of the *PR* genes *HvPR1b*, *HvPR3*, *HvPR5*, and *HvPR10* in first and second leaves of 11-day-old barley plants in noninoculated controls and *B. graminis* f. sp. *hordei*-inoculated plants 48 hai, by RT-qPCR. In the first leaf of noninoculated *HvRBOHF2* KD plants, we observed a 10- to 100-fold constitutive overexpression of the tested *PR* genes (Fig. 6). This constitutive *PR* gene expression was much less prominent in second leaves of *HvRBOHF2* KD plants, indicating an increase in the *PR* gene expression with the progression of leaf age. Following inoculation with *B. graminis* f. sp. *hordei*, the expression of *HvPR3*, *HvPR5*, and *HvPR10* was strongly induced in both genotypes and leaves tested. In general, *B. graminis* f. sp. *hordei*-inoculated *HvRBOHF2* KD plants showed much higher levels of *PR* gene expression compared with corresponding azygous plants. In the first leaves of *HvRBOHF2* KD lines, the high level of constitutive *PR* gene expression was not further elevated by *B. graminis* f. sp. *hordei*-inoculation, except for *HvPR3*. Interestingly, no differences were detectable in the expression of *HvPR1b* and *HvPR3* between first and second leaves within each of the genotypes in *B. graminis* f. sp. *hordei*-infected plants. Hence, the strong *B. graminis* f. sp. *hordei*-related stimulus overrode developmental differences in *PR* gene expression.

Leaves of *HvRBOHF2* KD plants contain higher SA contents.

To determine whether the constitutive expression of *PR* genes and the spontaneous cell-death phenotype in older leaves of *HvRBOHF2* KD lines are associated with changes in the content of SA, we analyzed the SA content in different leaves of

Fig. 4. *HvRBOHF2* knock down (KD) results in premature leaf senescence. **A**, First, second, or third leaves of 17-day-old *HvRBOHF2* KD (left) and the azygous control (right) lines were harvested and pictures from the tip leaf section were taken. First and second leaves of *HvRBOHF2* KD plants displayed early senescence phenotype as evidenced by spontaneous necrotic lesions and chlorosis in the tip of the leaf. **B**, Chlorophyll content in tip and base leaf sections of 17-day-old barley *HvRBOHF2* (KD) and the azygous control lines. Data represent the means of five independent experiments. In each measurement, a pool of 16 leaf discs from four leaves per treatment were used. Error bars show standard errors of the mean and letters the grouping of data sets according to Tukey's test at $P = 0.05$.

17-day-old plants. We noticed only a modest, nonsignificant, increase in the levels of both free and conjugated SA (FSA and CSA, respectively) forms in young (third) leaves of *HvRBOHF2* KD plants compared with azygous controls (Fig. 7A and B). However, the SA content in older (first) leaves of *HvRBOHF2* KD plants was about nine- and 48-fold higher for FSA and CSA, respectively, compared with azygous controls. Additionally, SA content was higher in older leaves than in younger leaves of *HvRBOHF2* KD plants, while it was similar between azygous leaf levels. *B. graminis* f. sp. *hordei* infection did not trigger significant SA accumulation in any of the tested leaves or genotypes. These results show a coincidence of higher SA content in older leaves of *HvRBOHF2* KD barley lines with early senescence-associated cell death, *PR* gene expression, and higher resistance to *B. graminis* f. sp. *hordei*.

DISCUSSION

HvRBOHF2 limits or supports penetration success of *B. graminis* f. sp. *hordei* depending on leaf age.

In this study, we report on the role of the barley RBOHF-clade NADPH oxidases in the leaf age-dependent modulation of the susceptibility to barley powdery mildew. We noticed a reduction of susceptibility to *B. graminis* f. sp. *hordei* penetration in the progression of leaf age in *HvRBOHF2* KD lines, which are cosilenced for *HvRBOHF1* (Proels et al. 2010). This developmental resistance is reminiscent of APR, described by plant breeders in cereals, in which a shift from susceptibility to resistance to various diseases, such as bacterial leaf blight, rusts, and powdery mildews, is observed with the progression of the plant or leaf age (Century et al. 1999; Douglas et al. 1984; Ellis et al. 2014; Sunderwirth and Roelfs 1980). APR to *B. graminis* f. sp. *hordei* has been identified in some barley cultivars in both

laboratory and field conditions (Gupta et al. 2015; Lin and Edwards 1974; Wright and Heale 1984). Here, the leaf age-dependent phenotype we observed in *HvRBOHF2* KD lines was also associated with a senescence-like process evidenced by a decrease in chlorophyll content (Fig. 4), upregulated expression of *PR* genes (Fig. 6), and SA accumulation (Fig. 7) in older leaves. Noteworthy, this leaf age-specific degree of susceptibility of *HvRBOHF2* KD lines was related to the level of residual *HvRBOHF2* expression (Figs. 1 and 5). Although no data exist that would support a direct function of *HvRBOHF2* in regulation of naturally occurring APR, it might be worth measuring *RBOHF* expression levels in APR of *Triticaceae* spp.

The fact that the leaf age and physiological stage are critical parameters in the interaction of *HvRBOHF2* KD plants with *B. graminis* f. sp. *hordei* could also explain previous contrasting results regarding a lower susceptibility to *B. graminis* f. sp. *hordei* following a transient silencing of *HvRBOHF2* (formerly called *HvRBOHA*) (Trujillo et al. 2006) and a higher susceptibility to *B. graminis* f. sp. *hordei* in young leaves of stable transgenic *HvRBOHF2* KD lines (Proels et al. 2010). The resistant phenotype in transiently silenced leaf explants (Trujillo et al. 2006) could have resulted from the fact that, in this assay, first leaves are cut off the plant, are transiently transformed via particle bombardment, are left on agar plates for 24 h before *B. graminis* f. sp. *hordei* inoculation, and are evaluated for resistance 40 h thereafter. In the course of this experiment, the leaf explants most likely changed to a physiological older status, resulting in higher resistance to *B. graminis* f. sp. *hordei* when *HvRBOHF2* is silenced. However, enhanced susceptibility of young *HvRBOHF2* KD leaves is difficult to explain on the basis of our data, since no reduction in DAB staining or *PR* gene expression was observed in younger leaves. Yet, all leaf levels of *HvRBOHF2* KD leaves showed a reduced ROS production in response to diverse MAMPs. Although these data have to be interpreted with care (discussed below), one might speculate that a reduced ability to mount an early oxidative burst could have contributed to enhanced susceptibility of young *HvRBOHF2* KD leaves. This effect might have then been overcompensated for in older leaves by alternative defense responses associated with functions of *PR* proteins.

The oxidative burst in response to pathogens is affected in *HvRBOHF2* KD plants.

A prominent decrease in the frequency of cell wall-associated hydrogen peroxide formation in papilla and in anticlinal cell walls of cells adjacent to penetrated cells was observed in first leaves of *HvRBOHF2* KD plants. According to previous studies (Hückelhoven et al. 1999; 2000a; 2000b), nonpenetrated papilla are usually associated with hydrogen peroxide formation, whereas penetrated cells are often free of detectable H_2O_2 or show H_2O_2 formation in anticlinal cell walls facing neighboring cells. The reduced DAB staining in older resistant leaves of *HvRBOHF2* KD plants hence seems to indicate a contribution of the *HvRBOHF2* oxidase to H_2O_2 formation in interaction with *B. graminis* f. sp. *hordei*. However, transient silencing of *HvRBOHF2* (Trujillo et al. 2006) or silencing in young leaves (Proels et al. 2010) did not support this, because no consistent reduction of H_2O_2 formation was indicated. Taken together, we speculate that progressive leaf senescence rather than the direct lack of *HvRBOHF2* protein was responsible for reduced DAB staining in older leaves of *HvRBOHF2* KD plants. This is supported by the fact that younger third leaves of *HvRBOHF2* KD plants did not show differences in DAB staining (Fig. 2).

Our data also suggest a role of the *HvRBOHF* clade genes in the oxidative burst triggered by MAMPs. Originally, we did not observe changes in the flg22-triggered oxidative burst between *HvRBOHF2* KD and azygous leaves in a chemiluminescence

Fig. 5. *HvRBOHF2* expression decreases with leaf age. Relative expression levels of barley *HvRBOHF2* in first and second leaves of *HvRBOHF2* knock-down (KD) line 30/2E08 and the azygous control. Total RNA was extracted from a pool of three to four leaves of 11-day-old barley plants 48 h after inoculation from noninoculated or *Blumeria graminis* f. sp. *hordei*-inoculated leaves. The cDNA was synthesized and, afterward, was subjected to real-time polymerase chain reaction analysis. Expression of *HvRBOHF2* was measured relative to the expression of the reference gene (barley MONOUBIQUITIN-LONG-TAIL FUSION 1 [*HvMub1*]). Data represent the means of three technical repetitions from one representative experiment out of three independent biological experiments with similar results. Error bars show confidence intervals of the means ($n = 3$) at $P = 0.05$.

assay, based on oxidation of luminol, using a photomultiplier (Proels et al. 2010). In contrast, when we used the much more sensitive luminol derivative L-012, we measured a clear reduction in the ROS burst triggered by three distinct elicitors in the *HvRBOHF2* KD. However, because the current methods to determine the ROS burst rely on cut leaf discs, wound-related

phenotypes of *HvRBOHF2* KD lines (Proels et al. 2010) might have interfered with the ability of leaf discs to respond to MAMPs. Taken together, we suggest careful interpretation of the data, because the direct contribution of *HvRBOHF2* to the MAMP- and *B. graminis* f. sp. *hordei*-triggered ROS formation may be less prominent than data suggest. The ROS burst

Fig. 6. PATHOGENESIS-RELATED (*PR*) genes expression is enhanced in *HvRBOHF2* KD knock down (KD) lines. Relative expression levels of barley **A**, *HvPR1b*, **B**, *HvPR3*, **C**, *HvPR5*, and **D**, *HvPR10* in first and second leaves of *HvRBOHF2* KD line 30/2E08 and the azygous control. Total RNA was extracted from a pool of three to four leaves of 11-day-old barley plants 48 h after inoculation from noninoculated or *Blumeria graminis* f. sp. *hordei*-inoculated leaves. The cDNA was synthesized and, afterward, was subjected to real-time polymerase chain reaction analysis. Expression of the target genes was measured relative to the expression of the reference gene (barley MONOUBIQUITIN-LONG-TAIL FUSION 1 [*HvMub1*]) and normalized to the values of the first noninoculated leaf. Data represent the means of three technical repetitions from a representative experiment out of three independent experiments. Error bars show confidence intervals of the means ($n = 3$) at $P = 0.05$.

triggered by the bacterial elicitor flg22 was partly dependent on a functional *AtRBOHF* gene in a study performed by Zhang et al. (2007). Hydrogen peroxide accumulation in response to avirulent strains of *Pseudomonas syringae* pv. *tomato* and the necrotrophic fungi *Alternaria brassicicola* and *Sclerotinia*

sclerotiorum is not affected in the *atrbohF* mutant but is partially reduced after inoculation with the *Arabidopsis* powdery mildew fungus *Golovinomyces cichoracearum* (Berrocal-Lobo et al. 2010; Ma et al. 2016; Perchev et al. 2010; Pogány et al. 2009; Torres et al. 2002). Silencing of the *HvRBOHF2*-related *Nicotiana benthamiana* NADPH oxidase *NbRBOHA* completely abolishes the *Phytophthora infestans*-triggered hydrogen peroxide accumulation and also the silencing of the *HvRBOHF2*-related rice gene *OsRBOHA* affects a pathogen-associated molecular pattern (PAMP)-induced oxidative burst (Yoshie et al. 2005; Yoshioka et al. 2003).

Silencing of *HvRBOHF* clade NADPH oxidases affects programmed cell death (PCD) in leaves.

PCD is an organized and controlled dismantling of cells that occurs during plant development and in response to stresses (Greenberg 1996; Lockshin and Zakeri 2004). For instance, processes such as the development of tracheary elements in the xylem of vascular plants, root cap cell formation, plant senescence, and HR are classic examples of PCD (Pennell and Lamb 1997). Interestingly, mis-regulation of PCD processes, such as HR, senescence, or alterations in metabolic pathways, can result in so-called lesion-mimic mutants (LMM) that display spontaneous cell death or HR-like lesions in the absence of a pathogen (Dangl et al. 1996). Barley and wheat LMM often show constitutive expression of *PR* genes and constitutive or APR under conditions that promote lesion formation (McGrann et al. 2015; Wang et al. 2016). Interestingly, ectopic expression of the wheat APR gene *LR34* in barley leads to LMM-like leaf-tip necrosis, constitutive *PR* gene expression, and resistance to *B. graminis* f. sp. *hordei* and other pathogens in the seedling stage. Moreover, resistance in *LR34*-transgenic barley was associated with enhanced levels of SA and the defense hormone jasmonic acid (Chauhan et al. 2015; Risk et al. 2013). Considering that both initiation and suppression of cell death are involved in the regulation of PCD, two classes of LMM have been suggested, namely, initiation mutants and propagation mutants, respectively (Lorrain et al. 2003). *HvRBOHF2* KD lines show both classes of LMM phenotypes. Fully developed leaves of old *HvRBOHF2* KD plants show spontaneous mesophyll cell death and a runaway cell-death phenotype (cell-death propagation) appears when leaves are wounded by cutting the leaf blade (Proels et al. 2010). However, the number of cells undergoing *B. graminis* f. sp. *hordei*-induced local cell death were not different between the leaves of the *HvRBOHF2* KD plants and azygous controls (Fig. 2) (Proels et al. 2010). Therefore, *HvRBOHF2*, unlike its close homologs *AtRBOHF* and *OsRBOHA* (Torres et al. 2002; Yoshie et al. 2005), might not be involved in the regulation of pathogen-triggered cell death. However, since virulent *B. graminis* f. sp. *hordei* is not a strong trigger of cell death, further studies are necessary to finally conclude this.

HvRBOHF2 KD plants are characterized by an increased SA level and a strong overexpression of *PR* genes in older leaves.

Other than the relatively well-understood role of SA during the tobacco and *Arabidopsis* immune responses, its role in the barley-*B. graminis* f. sp. *hordei* pathosystem is still obscure. Exogenous application of SA or synthetic analogs of SA induces disease resistance against *B. graminis* f. sp. *hordei* (Beßer et al. 2000; Dey et al. 2014; Kogel et al. 1994). Moreover, barley transgenic plants silenced for the gene NON-EXPRESSOR OF PATHOGENESIS RELATED GENES1 (*HvNPR1*), a critical SA signal transducer, displayed enhanced susceptibility to *B. graminis* f. sp. *hordei*, suggesting a role of *HvNPR1* in barley immunity (Dey et al. 2014). However, although *B. graminis* f. sp. *hordei* inoculation triggered typical defense responses such as hydrogen

Fig. 7. Effect of the silencing of *HvRBOHF2* KD on the endogenous levels of salicylic acid (SA) in different leaves of the *HvRBOHF2* knock-down (KD) line 30/2E08 and the azygous control. **A**, Free and **B**, conjugated levels of SA in the *HvRBOHF2* KD line 30/2E08 and the azygous control. SA was extracted from the first and third leaves of 17-day-old plants 24 h after mock- or *Blumeria graminis* f. sp. *hordei* inoculation. SA content was determined by high-pressure liquid chromatography and fluorescence detection. Data represent the means of three independent experiments. Error bars show standard errors of the mean and letters indicate the grouping of data sets according to Tukey's test at $P = 0.05$. FW = fresh weight.

peroxide accumulation, HR, and induction of *PR* gene expression in 6- to 7-day-old barley plants, these responses were not associated with an increase in the SA content (Hückelhoven et al. 1999; Vallélian-Bindschedler et al. 1998). Likewise, we did not observe any statistically significant increase in the SA content after *B. graminis* f. sp. *hordei* inoculation (Fig. 7). On the other hand, we found strongly increased constitutive FSA and CSA levels in the first, older leaf of *HvRBOHF2* KD plants.

In dicots, SA accumulates during HR and SA plays a role in the regulation of leaf senescence (Bartsch et al. 2010; Morris et al. 2000; Zhang et al. 2013; Zhao et al. 2016). The relevance of SA and senescence during developmental resistance response and defense gene expression of dicots is well-established (Hugot et al. 1999, 2004; Shibata et al. 2010; Wyatt et al. 1991). In the monocot wheat, *PR* and other defense gene transcripts accumulate during different types of APR (Chen et al. 2013; Hao et al. 2016; Huang et al. 2013). *PR* proteins also accumulate in the intercellular space of barley primary leaves during leaf senescence (Tamás et al. 1998), and this gradual *PR* protein accumulation possibly correlates with the barley APR against *B. graminis* f. sp. *hordei*. Here, we showed that constitutive *PR* gene expression in the first, highly resistant leaf in 11-day-old *HvRBOHF2* KD plants is considerably higher when compared with azygous controls and reaches levels usually measured after *B. graminis* f. sp. *hordei* infection (Fig. 6). We also observed a *B. graminis* f. sp. *hordei*-triggered increase in *PR* gene transcripts in leaves of both *HvRBOHF2* KD and azygous plants, with a much higher total expression in *HvRBOHF2* KD plants.

SA has been described as an in-vitro antimicrobial agent against a broad range of pathogens (Amborabé et al. 2002; Cameron and Zaton 2004; Georgiou et al. 2000; Martín et al. 2009; Prithiviraj et al. 1997). Besides the reduction in haustoria formation at 48 hai, we also observed a reduced development of *B. graminis* f. sp. *hordei* colonies on the leaf surface of *HvRBOHF2* KD lines at 96 hai (Fig. 3). Thereby, the effect of a reduced colony length in *HvRBOHF2* KD lines was more pronounced in older leaves, which might be additionally caused by a reduced metabolic activity in those tissues, characterized by premature senescence. Since *B. graminis* f. sp. *hordei* follows an obligate biotrophic lifestyle, accelerated senescence and cell death should strongly limit its development. In addition, considering the high content of FSA found in older leaves of *HvRBOHF2* KD plants, the arrest in *B. graminis* f. sp. *hordei* growth could also be a result of antifungal activity of SA or of SA-induced defense compounds. Hence, resistance might be established from a partially preformed defense status in old leaves of *HvRBOHF2* KD plants.

Interestingly, there are further reports about RBOH functions in support of disease progression. Torres and coworkers (2002) reported that the reproductive success of *Hyaloperonospora arabidopsidis* isolate Emco5 was reduced on *AtrbohF* mutant leaves and plants showed enhanced HR. In interaction with cyst nematodes, *AtrbohD/AtrbohF*-double mutants showed enhanced cell death in response to nematode infection and feeding-site formation was limited on the mutants. Further genetic evidence showed that SA is involved in the expression of the resistant phenotype (Siddique et al. 2014). Thus, NADPH oxidases modulate the amount of cell death and control SA-induced defense responses in diverse plant tissues and under diverse physiological situations.

MATERIALS AND METHODS

Plants, pathogens, and inoculation.

Seeds of the transgenic barley *HvRBOHF2* KD (*Hordeum vulgare* L. cv. Golden Promise) lines 30/2E06 and 30/2E08 (Proels et al. 2010) were germinated on filter paper soaked with

tap water for 3 days in the dark. Subsequently, we transferred the germinated seeds to soil. Plants were grown in a growth chamber at 18°C with 60% relative humidity and a photoperiod of 16 h with 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photon flux density for a further 8 or 14 days. Inoculation experiments were conducted using the barley powdery mildew fungus *Blumeria graminis* (DC) Speer f. sp. *hordei* Marchal, race A6 maintained on the cultivar Golden Promise under the above-described conditions. Inoculations were performed in the adaxial side of intact barley plants (Zadoks growth stage 12 to 13) (Zadoks et al. 1974) by air current dispersion in an inoculation chamber. A density of 20 to 50 conidia per square millimeter of leaf surface was used for microscopic evaluation and gene expression analysis. For the evaluation of disease symptom progression and SA quantification, the plants were inoculated with 10 to 20 conidia per square millimeter and 50 to 60 conidia per square millimeter, respectively. Azygous siblings, offspring that lost the transgene due to segregation and behaved like the wild-type parent Golden Promise plants (Proels et al. 2010), were used as controls in all experiments.

Cytological analysis.

The outcome of the interaction of barley plants with barley powdery mildew was evaluated in leaf samples harvested 48 hai. The leaf segments were destained in an ethanol/chloroform solution (6:1 [vol/vol]), and the fungal mycelium was stained with acetic ink (25% [vol/vol] acetic acid/blue ink [Pelikan, Hanover, Germany], 9:1 [vol/vol]). The frequency of spore germination, penetration attempts, and successful cell-wall penetration was determined by detection of appressoria, papilla, and haustoria formation, respectively. The leaves were cut transversally into two sections of equal size and, in each section, meristem-distal (tip) and meristem-proximal (base), 50 to 75 interaction sites were evaluated. Four leaves per line and experiment were assessed and the experiment was performed three times. In each experiment, a total of 100 to 150 interaction sites per genotype and leaf were scored. Detection of hydrogen peroxide accumulation and hypersensitive cells using DAB staining was performed as described by Hückelhoven and colleagues (1999). Because leaves of 17-day-old-plants were quite long and to make sure of leaf uptake of the DAB-staining solution, we evaluated just the base of leaves. Only short cells (cell type A and B) were included in the evaluation, as the rate of fungal penetration into different types of epidermal cells varies (Koga et al. 1990).

Oxidative burst measurement.

ROS burst elicited by the MAMPs flg22 (250 nM), chitin (100 $\mu\text{g ml}^{-1}$), and laminarin (100 $\mu\text{g ml}^{-1}$) was measured in different leaves of 19-day-old plants (Zadoks stage 14) in a chemiluminescence assay based on oxidation of luminol derivative L-012 (Wako Chemicals GmbH, Neuss, Germany). Briefly, leaf discs of 5 mm diameter were cut from the middle section of leaves, to avoid the necrotic lesions in the tip of *HvRBOHF2* KD plants, and were floated overnight in 200 μl of water per well overnight in a 96-well microtiter plate. Then the water was replaced by 150 μl of measurement mixture containing 1 $\mu\text{g ml}^{-1}$ horseradish peroxidase and 5 μM L-012, and the plate was preincubated in the dark for 15 min. After the addition of the elicitors (50 μl pf elicitor diluted in measurement mixture), chemiluminescence was measured at 1-min intervals with a Tecan Reader (Infinite M200, Tecan, Männedorf, Switzerland) for 75 min.

Chlorophyll content estimation.

Leaves of 17-day-old plants (Zadoks stage 13) were harvested and were cut transversally into two sections of equal size

and, in each section, meristem-distal (tip) and meristem-proximal (base), four leaf discs of 4 mm diameter were taken. The leaf discs from a pool of four leaves of each line were homogenized in liquid nitrogen and the pigments were extracted into 2 ml of 96% [vol/vol] ethanol solution. After centrifugation, the absorbance spectrum of the supernatant was measured at 1-nm intervals between 300 and 700 nm. Total chlorophyll content was estimated from the absorption spectra according to the equations of Lichtenthaler and Buschmann (2001).

Assessment of disease progression.

The disease progression was assessed by measuring the number and length of colonies of *B. graminis* f. sp. *hordei* on different leaves of 17-day-old barley plants. Whole plants were infected with *B. graminis* f. sp. *hordei* and were incubated for 96 h in a climate chamber. The leaves were harvested separately and the number of colonies per square centimeter of leaf area was quantified under the stereomicroscope. Next, the leaf segments were destained in ethanol acetic acid solution (6:1 [vol/vol]), the fungal structures were stained with acetic ink (25% [vol/vol] acetic acid/blue ink, 9/1 [vol/vol]), and the density of colonies was quantified again. For the measurement of the length of the colony, pictures of interaction sites were taken with a Leica MC170 HD digital camera on a stereomicroscope Leica MDG41, and the length of oval colonies along the longitudinal leaf axis was measured. Eighty pustules per treatment were manually sized, using the software Leica LAS v4.5. The experiment was performed three times with five to six leaves per barley line in each experiment.

Gene expression analysis.

Total RNA from first and second leaves of 11-day-old (Zadoks stage 12) plants was extracted from a pool of three to four plants of each line. RNA integrity and amount were evaluated by both gel electrophoresis and spectrophotometry. The cDNA was synthesized, using the Quantitect reverse transcription reaction (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Primer efficiencies of gene-specific primers pairs (Supplementary Table S1) were evaluated on a standard curve, generated using a two-fold dilution series of a pooled sample over at least four points (5, 2.5, 1.25, and 0.625 ng per microliter of cDNA). RT-qPCR was performed in a Mx3005P Cyclor (Stratagene, La Jolla, CA, U.S.A.). Individual PCR reactions contained 100 ng of cDNA, 10 µl Maxima SYBR Green qPCR master mix (2×) (Thermo Fisher Scientific, Waltham, MA, U.S.A.), and 300 nM of a gene-specific forward and reverse primer, in a total volume of 20 µl. The program consisted of an initial step at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The specificity of amplification reactions was verified by a melting curve analysis performed at 95°C for 30 s, 60°C for 30 s, and, again, to 95°C for 30 s. Relative expression for genes of interest in each sample was calculated using primer efficiency correction, as suggested by Pfaffl (2001), and the barley MONOUBIQUITIN-LONG-TAIL FUSION 1 (*Mub1*) gene as normalizer (Ovesná et al. 2012; Pennington et al. 2016). The error propagation throughout all calculation steps were taken into account, as recommended by Hellemans and colleagues (2007). In all experiments, three biological replicates of each sample and three technical (PCR) replicates were performed.

SA quantification.

FSA and CSA from the first and third leaves of 17- to 18-day-old plants (Zadoks stage 14) were extracted from a pool of four to five plants of each line and were quantified as described by Meuwly and Métraux (1993), with some modifications. Ground leaf powder, corresponding to 0.2 to 0.4 g of fresh weight of

first leaves and 0.7 to 1 g of fresh weight of third leaves, was extracted in 9 ml of 100% methanol and 0.2 ml of internal standard solution (500 µM of o-anisic acid and 250 µM of p-hydroxybenzoic acid in 100% methanol). The methanol leaf powder suspensions were shaken for 1 min (2,500 rpm) and were centrifuged at 30,000 × g for 15 min at 6°C. The supernatant was dried under vacuum, was resuspended in 2 ml of 5% (wt/vol) trichloroacetic acid, and was then extracted twice for SA, with 5 ml of ethyl acetate and cyclohexane (1:1, vol/vol). The organic SA-containing phase was collected after centrifugation at 3,300 × g for 5 min. HCl was added (to 3.4 M) to the remaining aqueous trichloroacetic acid phase, was again supplemented with 200 µl of internal standard, and was kept at 80°C for 1 h, for acid hydrolysis. The phenolic compounds were resuspended in 1 ml of the mobile phase (citrate acetate buffer: 30 mM sodium citrate and 27 mM sodium acetate at pH 5). Samples were separated on 125-4 Merck Lichrospher100 RP-8 (5 µm) cartridge column with included precolumn (4-4) and were maintained at 35°C. Excitation and emission wavelengths were 298 and 400 nm, respectively. Elution was carried out with citrate acetate buffer for 15 min, followed by a linear 2-min gradient to methanol (100%), isocratic methanol (100%) for 7 min, linear 2-min gradient to citrate acetate buffer, and equilibration for 7 min before the next run.

Data analyses.

All the statistical analyses were performed using the SPSS 21.0 software package (SPSS Inc., Chicago). Data were subjected to the one-way analyses of variance (ANOVA) and the means were compared using Tukey's honest significant difference test at $P = 0.05$. Data on the length of *B. graminis* f. sp. *hordei* colonies did not meet the assumptions of homogeneity of variance and were subjected to Welch ANOVA, followed by a Games-Howell post hoc test at $P = 0.05$.

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