Research Papers

Histopathological aspects of resistance in wheat to *Puccinia triticina*, induced by *Pseudomonas protegens* CHA0 and β-aminobutyric acid

FARES BELLAMECHE1, MOHAMMED ABBAS JASIM1,§, BRIGITTE MAUCH-MANI1, FABIO MASCHER2,*

1 University of Neuchâtel, Laboratory of Molecular and Cell Biology, rue Emile-Argand 11, 2000 Neuchâtel, Switzerland
2 Agroscope, Crop Plant Breeding and Genetic Resources, Route de Duillier 50, 1260 Nyon, Switzerland
§ Present address: University of Anbar, Biology department, Al Ramadi 31001, Iraq
*Corresponding author. E-mail: fabio.mascher@agroscope.admin.ch

Summary. After perception of specific biotic or abiotic stimuli, such as root coloniza-
tion by rhizobacteria or selected chemicals, plants can enhance their basal resistance
against pathogens. Due to its likely sustainability, this induced resistance will be val-
uable for disease management in agriculture. This study examined resistance against
wheat leaf rust (*Puccinia triticina*) induced by *Pseudomonas protegens* CHA0 (CHA0)
and β-aminobutyric acid (BABA). Seed dressing with CHA0 reduced the number
of sporulating pustules on leaves, and expression of resistance was visible as necrotic or
chlorotic leaf flecks. Beneficial effect of CHA0 on wheat seedlings growth was observed
in when they were challenged or not with leaf rust. BABA was tested at 10, 15 or 20
mM, and a dose-dependent reduction of leaf rust infections was observed with great-
est protection at 20 mM. However, BABA treatment repressed plant growth at 20 mM.
Balancing the BABA impact on plant growth and its protective capacity, 15 mM of the
compound was selected as suitable to protect wheat seedlings against leaf rust, with
the least impact on vegetative host growth. Histological aspects of the pathogen infection
process was studied to understand mechanisms of behind the observed resistance. The
pre-entry process was not affected by the two resistance inducers, but both treatments
reduced fungus penetration and haustorium formation. Timing and amplitude of the
resistance reactions were different after bacterial or chemical induction, leading to dif-
ferent levels of resistance. During fungal colonization of host tissues, high deposition
of callose and accumulation of H₂O₂ in both CHA0- and BABA-treated plants indicated
important contributions to resistance.

Keywords. Leaf rust, callose deposition, hydrogen peroxide (H₂O₂), plant resistance
inducers.

INTRODUCTION

Plants use several layers of defense mechanisms to prevent pathogen
attack. The first layer includes preformed physical and chemical barriers that
impede pathogen penetration to initiate infections (Ferreira et al., 2006). Once pathogen presence has been detected, host plants activate further chemical and physical barriers that delay or block the attack (second layer; Jones and Dangl, 2006). Defense success depends on host readiness to detect the pathogen. For the interaction between wheat and the leaf rust pathogen \( P\). \textit{triticina} \( \text{(Puccinia triticina)} \), the plant can detect specific fungal avirulence factors (effectors) with leaf rust resistance \( \text{(Lr)} \) genes. This gene-for-gene interaction is a very rapid recognition-reaction event leading to elevated resistance against the disease. However, the avirulence patterns can change and the pathogen may become undetectable by the plant. This wheat resistance breakdown has been reported for yellow rust (Hovmøller et al., 2010) and stem rust (Singh et al., 2011).

In non-specific pathogen recognition, plants can still contain pathogen development, but with reduced and variable degrees of infection severity (Jones and Dangl, 2006). The degree of this quantitative resistance is linked to the readiness of plant defenses and depends on a series of genetic and environmental factors. Beside the pathogen, biological and abiotic stimuli, and some chemicals, can enhance plant resistance (Mauch-Mani et al., 2017). Induced resistance can be limited to the site of the inducing treatment, but it can also be systemic and thereby effective in parts of the plant distant from the site of induction (Van Loon, 1997). For example, some root-associated bacteria \( \text{(e.g. the biocontrol strain Pseudomonas protegens CHA0, formerly P. fluorescens CHA0)} \) induce systemic resistance against virus and fungus diseases in various dicot (Maurhofer et al., 1994; Haas and Keel, 2003; Iavicoli et al., 2003) and monocot hosts (Sari et al., 2008; Henkes et al., 2011). Particular chemical compounds can also induce disease resistance in plants, including the non-protein amino-acid \( \beta\)-amino-n-butryic acid (BABA). Root colonizing bacteria and BABA root treatments reduce severity of infection by the oomycete \textit{Hyaloperonospora arabidopsis} on \textit{Arabidopsis thaliana}, and the induced state is regulated by different defense signalling pathways, depending on the inducing agent and the challenging pathogen (Van der Ent et al., 2009).

The aim of the present study was to examine mechanisms underlying resistance induced by CHA0 and BABA in wheat against \( P. \) \textit{triticina}. Sharifi-Tehrani et al. (2009) showed that root colonization by \textit{Pseudomonas protegens} strain CHA0 reduced the number of leaf rust uredia on susceptible wheat seedlings. The enhanced resistance was possibly due to a resistance priming event by induction of systemic resistance (ISR). This priming enables hosts to cope with pathogens at early stages of infection. The present study followed the interaction between \( P. \) \textit{triticina} and wheat at the microscopic level (De Vleesschauwer et al., 2008).

The leaf rust infection process is well known (Bolton et al., 2008). After adhesion of each urediospore on the leaf surface, germination, directed germ tube growth on the plant surface towards a stoma, and recognition of host guard cell lips take place. A small appressorium is formed over the stomatal opening, and a penetration hypha then enters through the stomatal pore. Following penetration, a substomatal vesicle and haustorium develop (Bolton et al., 2008).

Primed plants recognize the pathogen, and then produce reactive oxygen species (ROS) and deposit callose at the infection sites (Balmer et al., 2015). This rapid local oxidative burst generates, among others, hydrogen peroxide \( \text{(H}_2\text{O}_2) \) during pre-haustorial resistance against wheat leaf rust caused by \( P. \) \textit{triticina} (Wesp-Guterres et al., 2013; Serfling et al., 2016). Callose is an effective barrier that sometimes is induced at the sites of pathogen attack during the early stages invasion (Luna et al., 2011). Strong deposition of callose has been reported for the wheat near-isogenic line Thatcher, which carries the leaf rust resistance gene \textit{Lr9} (Wang et al., 2013).

Few studies have investigated rhizobacteria- and BABA-induced resistance against wheat leaf rust. In the present study, mechanisms involved in CHA0-ISR and BABA-IR were compared during interaction between the pathogen and host. Development of fungal structures were examined microscopically, along with occurrence of callose deposition, and hydrogen peroxide accumulation in leaf tissues.

**MATERIALS AND METHODS**

**Induced resistance assay**

Plant material and growth conditions

Experiments were carried out with the leaf rust-susceptible bread wheat cultivar Arina (Agroscope/DSP). Surface sterilized seeds were used in all experiments. Seeds were rinsed in 70% ethanol, incubated for 5 min in 5% sodium hypochlorite solution (Fisher Chemical), and then washed three times in sterile distilled water. The sterilized seeds were germinated on moist filter paper (Filterkrepp Papier braun, E. Weber & Cie AG) in plastic bags maintained in the dark at room temperature. Three to 4 d later, resulting seedlings at similar growth states and morphology were selected and planted in 120 mL capacity polypropylene tubes (Semadeni) filled with a standard seedling growth medium.
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Effects of CHA0 and BABA on plant development

In a first step, the effects of CHA0 and BABA treatments on wheat plants were assessed. To measure root colonization by CHA0, 0.1 g each of inoculated or control roots were each shaken in 10 mL of sterilized distilled water for 1 min on a benchtop vortex mixer, followed by 1 min of sonication. The resulting root extract was serially diluted and plated on solid King’s medium B supplemented with 100 μg mL⁻¹ of rifampicin. The plates were then incubated at 28°C in the dark, and the numbers of CFUs were determined after 24 to 36 h.

To investigate possible effects of CHA0 and BABA treatments on plant growth, the dry mass of the shoots of pre-treated seedlings was measured at 12 d after inoculation with *P. triticina*. Shoot length was defined as the upper part of each cut at the residue of the seed. Shoot fresh weights were measured, and they were then each placed on coffee filter paper and dried in an oven at 65°C until sample weight remained constant (shoot dry weight).

Inoculations with *Puccinia triticina*

Inoculations of wheat seedlings with *P. triticina* were carried out at the two-leaf stage (BBCH 12; Meyer, 1997), using freshly harvested urediniospores of *P. triticina* isolate Pr2271 (Agroscope, Changins, Switzerland). In a differential test, this isolate showed the following avirulence/virulence properties; *Lr1, Lr2a, Lr2b, Lr2c, Lr3, Lr3bg, Lr9, Lr10, Lr14a, Lr26/Lr3ka, Lr11, Lr12, Lr14b, Lr18, Lr25* (unpublished results). In a preliminary test, the isolate showed high aggressiveness towards the wheat cultivar Arina. Urediniospores were produced on leaves of cv. Arina. For inoculations, fresh urediniospores were mixed with talcum powder in a 1:9 w/w ratio, and were then rubbed gently on seedling leaf surfaces. Inoculated plants were placed in a dew box in the dark at 18 to 22°C for 24 h to promote infection. The plants were then placed in the growth chamber as described above. After 12 d or when symptoms were sufficiently developed on control plants, the leaf rust infection types were assessed using the 0 to 4 scale (Table S1) described by Stakman *et al.* (1962).

Histochemical assessments of leaf rust infections in presence of CHA0 and BABA

Assessments of fungal growth and development

Leaf rust growth was observed on 2 cm leaf segments from the centres of the second seedling leaves at

Bacterium inoculum

The bacterium inoculum was the biocontrol agent *P. protegens* strain CHA0-Rif (Natsch *et al.*, 1994) (hereafter referred to as CHA0), which is a spontaneous rifampicin resistant strain of *P. protegens* CHA0 (Stutz *et al.*, 1986; Ramette *et al.*, 2011). Both strains have similar growth rates, production of antimicrobial compounds (Natsch *et al.*, 1994) and capacities to induce resistance in wheat (Sharifi-Tehrani *et al.*, 2009). Routinely, strain CHA0-Rif was grown at 25°C in the dark for 3 d on solid King’s medium B (Pseudomonas agar F, Merck KGaA) supplemented with 50 μg mL⁻¹ rifampicin. For long-term storage, 1 mL of a freshly grown bacterial suspension in King’s liquid medium B (30 g proteose-peptone, 1.5 g K₂HPO₄, 2.46 g MgSO₄, 1.5 g glycerol in 1 L distilled water) was mixed with 1 mL glycerol (87%) and stored at -80°C. For inoculum production, a single colony from a freshly grown culture was transferred to a 300 mL capacity Erlenmeyer flask containing 100 mL of King’s liquid medium B (Pseudomonas agar F, Merck KGaA) supplemented with 50 μg mL⁻¹ rifampicin. For long-term storage, 1 mL of a freshly grown bacterial suspension in King’s liquid medium B (30 g proteose-peptone, 1.5 g K₂HPO₄, 2.46 g MgSO₄, 1.5 g glycerol in 1 L distilled water) was mixed with 1 mL glycerol (87%) and stored at -80°C. For inoculum production, a single colony from a freshly grown culture was transferred to a 300 mL capacity Erlenmeyer flask containing 100 mL of King’s liquid medium B supplemented with 50 μg mL⁻¹ rifampicin. After 12 h incubation at 28°C with continuous shaking at 150 rpm, the resulting culture was centrifuged at 3700 rpm and washed twice with sterile 10 mM MgSO₄ solution. The cell pellet was re-suspended in 20 mL of sterile distilled water and adjusted to an OD₆₀₀ of 0.1 corresponding to approx. 10⁶ CFU mL⁻¹, and was then used for seed inoculation. For this, surface sterilized wheat seeds were immersed in the bacterial suspension for 6 h with shaking at 35–40 rpm at room temperature. Inoculated seeds were then pre-germinated (as above). Control seeds were soaked in distilled water for 6 h before pre-germination.

*Treatments with β-aminobutyric acid*

The resistance inducer BABA was obtained from Sigma-Aldrich (Buchs SG, Switzerland). Dilutions of 10, 15 and 20 mM of BABA in distilled water were used as soil drenches. For each of these, 10 mL of BABA solution were added to the soil containing wheat seedlings at the two leaf stage, 48 hours before inoculation with *P. tritici.* Control plants were treated with the same amount of distilled water.
0, 6, 12, 24, 48, 72 and 96 h after inoculation (hai). Leaf segments were immersed in 96% ethanol for 2-3 d to remove chlorophyll. The leaf segments were then washed in an ethanol/water (1:2 v/v) solution, and then incubated in 0.5M sodium hydroxide for 15 min with slight shaking. The leaf segments were incubated for 15 min in distilled water and then immersed 2 h in 0.1 M Tris–HCl buffer (pH 8.5). Fungal structures were then stained with a 0.2% Calcofluor White solution in water (Sigma-Aldrich) for 5 min. After four washings in distilled water, the samples were stored in 50% (v/v) glycerol for microscopic observation.

The preparations were examined with an epifluorescence microscope (Model E800; Nikon Instruments) using excitation at 365 nm in combination with a 450 nm barrier filter and a dichroic mirror at 400 nm. This lighting set-up allowed determination of positions and number of all fungal structures on and in the leaves, including germinated and non-germinated urediniospores, appressoria, sub-stomatal vesicles and haustoria.

Identification and quantification of callose deposition

Assessments of callose deposition were carried out for on segments from the centres of the second leaves of seedlings at 0, 24, 48 and 72 hai with P. triticina, using the methods of Scalschi et al. (2015). Leaf tissues were decoloured for 48 h in 96% ethanol until transparent. Leaf tissues were then rehydrated in 0.07 M phosphate buffer (pH = 9) for 30 min, and then incubated for 15 min 0.05% aniline blue (Sigma) prepared in 0.07 M phosphate buffer, and finally stained overnight in 0.5% aniline blue. Microscopic observations were carried out with the epifluorescence microscope using a UV filter, as described above.

The presence and quantity of deposited callose was determined from digital photographs (supplementary Figure 1S) by counting the white pixels (representing callose deposits) in 20 infection sites for each experimental replicate, using the GNU Image Manipulation Program (GIMP 2.10.10) software. Contrast settings of the photographs were adjusted to obtain an optimal separation of the callose signals from the background signals. Callose was automatically identified using the “Color Range” tool and callose-corresponding pixels were recorded as the area covered by the total number of selected pixels (Scalschi et al. 2015).

Accumulation of H₂O₂ at the infection sites

Detections of H₂O₂ were carried out using 3,3-diaminobenzidine (DAB; Sigma-Aldrich) staining, as described by Thordal-Christensen et al. (1997). The second fully expanded seedling leaves were cut at 0, 24, 48 and 72 hai and immediately immersed in a solution containing 1 mg mL⁻¹ DAB dissolved in HCl acidified distilled water (pH = 3.8). Leaves were then incubated in the dark for 8 h to allow DAB uptake and reaction with H₂O₂. Subsequently, leaves were cleared in saturated chloral hydrate and scanned at 1.200 dpi (Epson perfection, V370 PHOTO).

In presence of H₂O₂, DAB is reduced to a dark brown deposit that can be easily visualized in leaves. The H₂O₂ content of the seedling leaves was quantified by counting the number of dark-brown DAB pixels using GIMP 2.10.10 software, and the proportions of DAB stain were calculated in relation to total leaf area (Luna et al. 2011). The dark brown DAB pixels were selected using “Color selection” and the total areas of leaves were determined using the “Free Selection” tool in the image analysis software.

Experimental set up and statistical analyses

All experiments were carried out, then repeated twice. The induced resistance assay consisted of seven biological replicates. The fungal growth and the callose deposition assessments were carried out with three independent replicates, and the H₂O₂ quantification was measured for ten biological replicates.

Fungal development structures were identified and counted at 50 sites in each replicate. The proportion parameters were calculated as follows: percentage of germinated spores = (germinated spores/observed spores) × 100; percentage of stomatal appressoria = (stomatal appressoria/germinated spores) × 100; percentage of sub-stomatal vesicles = (sub-stomatal vesicles/stomatal appressoria) × 100; and percentage of haustoria = (haustoria/sub-stomatal vesicles × 100).

Data were collected and stored in spreadsheets (Microsoft Excel 2010). All statistical analyses were carried out with R (R Core Team, 2017).

In most experiments, statistically significant differences in responses to CHA0 and BABA treatments compared to controls were tested with Student’s T-test. The exception was in the experiment testing effects of CHA0 and BABA on plant development, where data were analyzed by two-way ANOVA with the factors; treatment (CHA0 and BABA) and rust inoculation (infected or not). The Tukey Honest Significant Differences (HSD) test was used for multiple comparisons. Significant differences were considered at P < 0.05.
RESULTS

Plant growth and biomass in presence of CHA0, BABA and following inoculation with Puccinia triticina

Twelve days after planting of the seedlings, an average of $5 \times 10^5$ CFU g$^{-1}$ of CHA0 were recovered on the fresh roots, demonstrating the capacity of the bacterium to successfully colonize the roots. In a preliminary experiment, the initial concentration of bacteria (either $10^4$, $10^6$ or $10^8$ CFU mL$^{-1}$) used for seed inoculation did not affect the number of bacteria on the roots.

The effects of CHA0 and BABA on plant length and biomass are illustrated presented in Figure 1. The results indicated that seedlings treated with CHA0 were longer and had more biomass than untreated seedlings. These parameters were not influenced by the presence of the pathogen (Figure 1A and B). In contrast, plants treated with BABA at 20 mM were shorter and lighter than the untreated seedlings. The seedlings treated with 10 or 15 mM of BABA were not different to the untreated controls. Infections with *P. triticina* did not affect plant growth or biomass, except for the treatment with BABA at 20 mM (Figure 1C and D).

Phenotypic reaction to leaf rust of wheat seedlings pre-treated with CHA0 or BABA

Twelve days after inoculation with *P. triticina*, uninoculated control plants were healthy (Figure 2a) while the inoculated plants were chlorotic and covered with uredia corresponding to infection type score 3 (high infection type (HI) (Stakman et al., 1962)) (Figure 2b). In plants treated with CHA0 (Figure 2c), the leaves showed overall less uredia compared to the inoculated controls. The symptoms were heterogeneous, including chlorotic flecks (score “,” low infection type (LI)), but also uredia without sporulation (score “2”, LI) and with sporulation and chlorotic halos (score “3”, HI).

The BABA treatments also resulted in a mix of chlorotic flecks (score “,”) and small to medium pustules with and without low sporulation, scored as “1” and “2”. Generally, all the BABA treatments gave low infection type symptoms. The scores were dose-dependent, since the greater the BABA concentration, the lower were the scores (Figure 2d, e and f).

Fungal infection structures

Calcofluor white staining was used to visualize the pathogen structures during the first 96 h after *P. triticina* infection (hai) in non-treated controls and on the plants pre-treated with CHA0 and BABA at 15mM. Within 6 hai, germ tubes started to elongate (Figure 3A, 1). Independent of the pre-treatments, about 90% of urediniospores had germinated within 6 hai, in all treatments (Figure 3B, 1). In the other treatments, the proportions of germinated spores remained constant.

Once germinated, the fungus forms appressoria at the
stomatal regions (Figure 3A, 2). The formation of appressoria started at 6 hai (data not shown). At 24 hai, 85–88% of the germinated spores had formed appressoria (Figure 3B, 2). This proportion varied only slightly between the controls and the bacterial and BABA treatments.

Through appressoria, the fungus penetrated into the cavities below leaf stomata, forming infection vesicles in the substomatal cavities (Figure 3A, 3). Formation of vesicles were observed at 12 hai (data not shown). On leaves of non-treated control plants, approx. 37% of appressoria had formed vesicles after 24 hai, with the proportions increasing to 50% after 48 hai. In plants inoculated with CHA0, approx. 29% of the appressoria had formed vesicles. At 48 hai, the mean proportion of vesicles decreased slightly to approx. 23% but this decrease was not statistically significant compared to the proportion of vesicles at 24 hai. In BABA (15 mM) treated plants, the proportions of formed vesicles was 10%, and these increased to 30% at 48 hai (Figure 3B, 3).

At 48 h, formation of haustoria out of vesicles was observed (Figure 3A, 4). At 72 hai, more than 80% of the sub-stomatal vesicles had formed haustoria in the untreated control plants, and this mean proportion did not change at 96 hai. In the CHA0 treated plants, the proportions of formed haustoria were not different to the control plants, at both time points (72 and 96 hai). However, the absolute number of haustoria was significantly less in the CHA0-treated plants compared to the

**Figure 2.** Leaf rust infections on seedling leaves of wheat cultivar Arina at 12 dpi. a, control plants non-infected; b, infected plants non-treated; c, infected plants pre-treated with CHA0; d, e, and f, infected plants treated with, respectively, 10, 15 or 20 mM of BABA. Images were obtained by scanning at 1,200 dpi a segment of 3 to 4 cm from the centre of the second leaf of each seedling.
controls. With BABA treatment, only about 50% of the vesicles formed haustoria, significantly less than haustorium formation in the controls. At 96 hai, haustorium formation increased in the BABA treatment to 70% and there was no significant difference from the other treatments. Also from the BABA treatment, the absolute number of haustoria was less compared to the control plants (Figure 3B, 4).

Callose deposition after Puccinia triticina inoculation

Callose deposition was quantified at 24, 48 and 72 hai after inoculations with *P. triticina* in the control, CHA0 and BABA 15mM treatments, using the aniline blue method (Supplementary Figure 1S). Callose deposition occurred in all treatments within the first 24 hai (Figure 4). However, in plants pre-treated with CHA0 and BABA, greater quantities of callose were detected compared to the controls. With CHA0, callose accumulated at the leaf guard cells and was greatest at 72 hai. In plants treated with BABA, greatest callose deposition was measured at 48 hai. Callose was observed in the guard cells of stomata) and eventually in leaf mesophyll cells, at 72 hai.

Figure 3. Microscopic observations and quantification of fungal structures *Puccinia triticina* in wheat seedlings. A, fungal structures stained with calcofluor white and visualized under the epifluorescence microscope. Bars = 20 µm. B, Mean percentages of fungal infection structures during infection of wheat by *P. triticina*: (1) spore germination, (2) appressoria, (3) sub-stomatal vesicles, and (4) haustoria. Treatments: CHA0, plants obtained from seeds inoculated with CHA0 (10⁶ CFU mL⁻¹); BABA, plants soil-drenched with BABA (15 mM) 48 h before *P. triticina* inoculation; Water, plants treated with sterile distilled water. Fungal structures: U, urediniospore; GT, germ tube; Ap, appressorium; H, Haustoria. Error bars indicate standard errors of the average values of three replicates at 50 infection sites for each replicate. Asterisks indicate statistically significant differences in response to CHA0 or BABA treatments (Student's t-test; *P < 0.05; ** P < 0.01; *** P < 0.001).

Figure 4. Callose deposition in wheat leaves in response to *Puccinia triticina* infection in treated and control plants at 24, 48 and 72 hai. Treatments: CHA0, plants obtained from seeds inoculated with CHA0 (10⁶ CFU mL⁻¹); BABA, plants soil-drenched with BABA (15 mM) 48 h before inoculation; Water, plants treated with sterile distilled water. Bars indicate standard errors of the average values for 20 infection sites for each of three replicates. Asterisks indicate statistically significant differences in response to CHA0 or BABA treatments (Student’s t-test; *P < 0.05; ** P < 0.01; *** P < 0.001).

Accumulation of *H₂O₂* after Puccinia triticina inoculation

Hydrogen peroxide released by plant tissue was measured between 0 and 72 hai with *P. triticina* in the control,
CHAO and BABA 15mM treatments. Hydrogen peroxide was monitored with the DAB staining that produces dark-brownish dots (Supplementary Figure 2S). Figure 5 shows the accumulation of H2O2 in leaves after the treatments. At 24 hai, H2O2 concentrations were greater in the CHAO and the BABA treated plants than in the controls. Similarly, at 48 hai, in CHAO and BABA treatments, the concentrations of released H2O2 were greater than in the controls. At 72 hai, accumulation of H2O2 in the CHAO treatment decreased to the level of the controls, while the BABA treatment increased at this time.

DISCUSSION

Induced resistance has been demonstrated as a potential complementary control strategy for protecting wheat plants from foliar diseases (Görlich et al., 1996; Sharifi-Tehrani et al., 2009). The present study has confirmed the efficacy of beneficial bacteria CHAO and BABA for induction of resistance in wheat to the leaf rust pathogen.

Effects of both resistance inducers were assessed on wheat growth. Efficient root colonization by a plant growth promoting bacterium is a prerequisite for successful biocontrol effects on host plants, either directly (e.g. disease suppression) or indirectly (e.g. ISR) (Lugtenberg and Kamilova, 2009; Beneduzi et al., 2012). In the present study, after seed inoculation, CHAO was colonized wheat roots, and more than 10^8 CFU g^-1 root fresh weight were recovered. Preliminary results showed that the initial concentrations used for seed inoculum (10^4, 10^6 or 10^8 CFU mL^-1) did not affect final root colonization. The bacterial titre in wheat roots was great enough for effective plant protection, as has been shown for soils suppressive to take-all of wheat and barley caused by Gaeumannomyces graminis var. tritici (Weller et al., 2007), Fusarium wilt of pea caused by Fusarium oxysporum f. sp. pisi (Landa et al., 2002), and black root rot of tobacco (Stutz et al., 1986). Additionally, the growth promotion capacity of CHAO was apparent with or without presence of P. triticina infections. In field experiments, positive effects of beneficial soil organism applications, including CHAO, on performance of wheat crops have been observed, especially when plants were under biotic stress (Imperiali et al., 2017). The observed plant growth promotion of CHAO could be from production of phytohormones and increased nutrient availability to plants, particularly phosphate. CHAO can solubilize mineral phosphate and improve plant growth in phosphate-limiting conditions (de Werra et al., 2009).

Thevenet et al. (2017) showed that BABA is a natural product in plants including wheat, but applications of BABA can reduce growth of some plants (Cohen et al., 2016). At the concentration of 20 mM, BABA induced resistance to P. triticina but reduced growth of wheat plants. The costs of induced resistance have also previously been linked to reductions in plant growth (van Hulten et al., 2006; Heil, 2007). Nevertheless, soil drenching with low concentrations of BABA (15 mM) did not affect plant growth and reduced infection types in wheat seedlings infected with leaf rust. This indicates the possibility to optimize the BABA dose rate for effective wheat protection against P. triticina with little impact on plant growth. Similarly, Luna et al. (2016) identified feasible BABA application methods by decreasing the concentration, which induced resistance in tomato against Botrytis cinerea without impacts on plant growth.

BABA is a well-recognized inducer of resistance against a broad spectrum of pathogens, including fungi, bacteria, viruses and nematodes (Baccelli and Mauch-Mani, 2016; Cohen et al., 2016). This compound has often been applied as soil drenches (Hodge et al., 2005; Luna et al., 2016). Several studies demonstrate that BABA was effective when applied 1-3 d post-infection against a spectrum of pathogens (Justyna and Ewa, 2013). In the present study, BABA was applied as a soil drench 2 d before inoculation with P. triticina. This treatment reduced leaf rust in wheat similarly to results obtained with other rust diseases on wheat (Amzalek and Cohen, 2007; Barilli et al., 2012). Inoculation with P. protegens strain CHAO led to a specific reaction to infection with P. triticina: while plants without CHAO inocu-
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Peroxide (H$_2$O$_2$). During fungal infection, callose can be were carried out of callose deposition and hydrogen peroxide (H$_2$O$_2$). During fungal infection, callose can be deposited at infection sites, which provides physical barriers preventing the pathogen penetration (Voigt, 2016). The present results showed that callose depositions were mainly detected in host guard cells. In support of these observations, Wang et al. (2015) demonstrated that the resistance response to *Puccinia graminis* f. sp. tritici was associated with callose deposition in the wheat guard cells. The increase of callose in BABA- and CHA0-treated plants could restrict penetration and development of *P. triticina*, correlating with the increase of resistance in wheat seedlings against the pathogen. This host defense mechanism is enhanced at the post-challenge primed stage after perception of a stimulus from beneficial bacteria and BABA (Mauch-Mani et al., 2017). In addition to guard cells, callose was observed in leaf mesophyll cells of BABA-treated plants. This could also explain the high resistance observed compared to plants inoculated with bacteria. A similar pattern was observed in defence mechanisms induced by *P. fluorescens* WCS417r and BABA against *Hyaloperonospora arabidopsis*. Both WCS417r and BABA prime for enhanced deposition of callose, although more callose accumulated in BABA-than WCS417r-treated plants (Van der Ent et al., 2009).

Reactive oxygen species (ROS), and especially H$_2$O$_2$, constitute a further important plant defense mechanism in interactions between plants and pathogens. H$_2$O$_2$ accumulation was investigated after infection with leaf rust in plants treated with BABA and CHA0. H$_2$O$_2$ accumulation was mostly detected in host guard cells. At these penetration sites, appressoria develop over the stomatal openings. During recognition or formation of appressoria, generation of H$_2$O$_2$ in guard cells is probably induced, possibly following secretion of rust effectors. Mechanical forces during adhesion of appressoria over stomata may also elicit H$_2$O$_2$ generation in guard cells. In *Arabidopsis*, it was reported that H$_2$O$_2$ accumulation in guard cells was involved in signal transduction during ABA-mediated stomatal closing (Sun et al., 2017). This could explain how the present study results showing accumulation of H$_2$O$_2$ in guard cells following recognition of leaf rust structures may be involved in stomatal closure (data no presented). This is supported by the measurements of increased accumulation of ABA in plants infected with leaf rust. It has been reported that appressorium formation of *P. triticina* also caused stoma closure in wheat leaves (Bolton et al., 2008). Other studies have shown correlations between H$_2$O$_2$ generation and hypersensitive reaction (HR) in resistance against wheat rust species (Wang et al., 2007; Orczyk et al., 2010; Serfling et al., 2016). In the present study, accumulation of H$_2$O$_2$ caused by resistance inducers was observed 24 hai, which corresponds to the begin-
ning of haustorium generation. This suggests that $H_2O_2$ initiated and HR defense mechanism. These results are similar to the observation of Serfling et al. (2016), where HR was observed in mesophyll cells that were in contact with fungal haustorium mother cells at 24 hai, and the observed pre-haustorial resistance in the resistant accession PI272560 was due to an early HR of the first infected mesophyll cells. Hypersensitive responses accompanied by $H_2O_2$ accumulation also occur in other interactions of plants with fungal parasites, and these caused non-host resistance to wheat stripe rust in broad bean (Cheng et al., 2012).

Plant-pathogen interactions can be modulated after induced resistance. We present a model for the wheat-

$P. triticina$ interaction (Figure 6), where infection of a host plant and growth of fungal structures have been interrupted at different phases in response to BABA- or rhizobacteria-induced resistance. In experimental condi-

Figure 6. Diagrams illustrating an overview of fungal development and determined defense reactions of wheat to $Puccinia triticina$ infection as affected by resistance inducers. A, compatible interaction between host and pathogen. In the untreated plant, $P. triticina$ overcomes the resistance mechanisms, and is able to complete the infection cycle producing urediniospores (left and right). B, enhanced defense reactions in plants treated with CHA0: left, fungus penetration is aborted after callose deposition in leaf guard cells; right, the fungus spreads partially but is stopped after $H_2O_2$ accumulation and activation of HR in some haustorium penetration sites. Formation of small uredia without or with low spore production. C, enhanced defense reactions in BABA-treated plants: left, fungus penetration is aborted after callose deposition in leaf guard and mesophyll cells; right, fungus growth is totally blocked after accumulation of elevated amounts of $H_2O_2$, and HR activation occurs in cells penetrated by rust haustoria. Fungal structures: U, urediniospore; GT, germ tube; SSV, substomatal vesicle; Ap, appressorium; H, Haustorium. Yellow dots represent callose depositions. Brown spots indicate $H_2O_2$ generation.
Here, H$_2$O$_2$ accumulation can be accompanied by the first barrier, callose deposition is no longer effective. However, when the fungus overcomes highly elevated leading to abortion of fungal penetration. Low generation of H$_2$O$_2$ is not able to initiate required mechanisms to prevent infection. In BABA- and CHA0-treated plants, pathogen spread is differently affected (Figure 6), with exception of the pre-entry process where spores germinate normally and appressoria are formed over stomatal openings in both cases. In CHA0-treated plants, callose deposition in guard cells is highly elevated leading to abortion of fungal penetration (Figure 6B, left). However, when the fungus overcomes the first barrier, callose deposition is no longer effective. Here, H$_2$O$_2$ accumulation can be accompanied by the activation of HR at some haustorium penetration sites which could partially stop fungal spread leading to formation of small uredia (Figure 6B, right). With BABA, in addition to what was observed after CHA0 treatment, an accumulation of callose occurs in leaf mesophyll cells. This could explain the high resistance observed after BABA treatment (Figure 6C, left). High accumulation of H$_2$O$_2$ initiates HR in cells penetrated by haustoria, and fungal spread is arrested without uredium formation (Figure 6B, right).

The present study has provided new insights into the histological basis of BABA- and rhizobacteria-induced resistance in wheat against leaf rust, showing the key roles of callose deposition and H$_2$O$_2$ generation in prevention of penetration and spread of leaf rust. Future studies will focus on expression analysis of some defense-related genes during the infection process, to underline differences and similarities in defense mechanisms induced by CHA0 and BABA.

**ACKNOWLEDGEMENTS**

Stefan Kellenberger, Agroscope Changins, Nyon, provided technical support advice for manipulating the leaf rust pathogen. FB gratefully acknowledges financial support from the Swiss Federal Commission for Scholarships for Foreign Students, and BMM acknowledges the financial support of the Swiss National Science Foundation, Grant No. 312 310030_160162.

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