Structural Analyses and Dynamics of Soluble and Cell Wall-bound Phenolics in a Broad Spectrum Resistance to the Powdery Mildew Fungus in Barley*

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High pressure liquid chromatography profiles of barley leaf epidermal soluble and cell wall-bound phenolics were analyzed in response to challenge with the fungal pathogen Erysiphe graminis f. sp. hordei. Only one soluble phenolic was found to accumulate differentially in a broad spectrum resistance reaction controlled by mlo resistance alleles in comparison to susceptible near isogenic Mlo lines. Structural analysis identified this compound as a novel phenolic conjugate, p-coumaroyl-hydroxyagmatine (p-CHA). p-CHA but not the nonhydroxylated derivative p-coumaroylagmatine exhibited antifungal activity both in vitro and in vivo. The accumulation of p-CHA in epidermal tissue correlated tightly with fungal penetration attempts of attacked host cells. Furthermore, upon penetration, epidermal cell wall-bound phenolics became resistant to saponification at sites of attempted fungal ingress (papilla), indicating a change in, or the addition of, different chemical bonding types. The switch in saponification sensitivity occurred at least 2 h earlier in the mlo-incompatible than in the Mlo-compatible interaction. Our results suggest that p-CHA and the speed of papillae compaction play important roles in non-race-specific powdery mildew defense.

Low molecular weight compounds have disparate functions in plant-microbe interactions. They can act as signal compounds to initiate a mutually beneficial interaction between plant and microbe. For example, the plant flavonoid luteolin attracts the bacterium Rhiobium mellotri to root hairs and triggers a cascade of events leading to root nodule formation, which provides plants with an efficient nitrogen uptake system (1).

Low molecular weight compounds can also act as part of a defense repertoire in resistance reactions against phytopathogenic microorganisms. The avenaecins are effective in oat against Gaumannomyces graminis var. tritici, and the structurally related α-tomatine of tomato inhibits Fusarium solani (reviewed in Ref. 2). Plant secondary metabolites for which complementary biochemical and genetic data are consistent with their function in pathogen defense are the stilbene-type resveratol in Z. mays (3, 4), the cyclic hydroxamic acid 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one in Zea mays (3, 4).

Although the response of barley to attack from powdery mildew has been investigated at cellular, biochemical, and genetic levels, no documented example of a host secondary metabolite has been reported to determine the outcome of the interaction. Powdery mildew, Erysiphe graminis f. sp. hordei, exclusively attacks the barley leaf epidermal cell layer. The fungus invades the plant cell mechanically and enzymatically through the cell wall with a penetration peg. This structure emerges from the appressorium, the thickened tip of the appressorial germ tube. Successful penetration is accomplished by subsequent development of the fungal feeding organ, the haustorium (5).

At least two genetically distinct pathways determine resistance in barley to powdery mildew (6). The first one is dependent on the presence of dominantly acting resistance genes and confers incompatibility only to those fungal isolates carrying corresponding avirulence genes. In most cases, this race-specific resistance is associated with a host cell death reaction at attempted infection sites (hypersensitive reaction; HR). The second pathway confers broad spectrum resistance to all tested isolates of the fungus and is stimulated by the absence of the Mlo wild type gene, a putative negative regulator of pathogen defense (7). The function of this pathway is also dependent on at least two further genes, Ror1 and Ror2 (8). Incompatibility mediated by recessive mlo resistance alleles is generally not associated with the occurrence of HR. The only visible cellular response is the formation of a subcellular cell wall apposition (CWA), termed papilla, directly subtending the fungal appressorium (9). Fungal penetration attempts are almost invariably arrested in CWAs in mlo-controlled resistance.

The potential role of pathogen-induced CWAs in resistance has been of considerable interest. In papillae, induced in different grass species (Gramineae), callose, cellulose, lignin, polyphenolics, reactive oxygen species, peroxidases, and silica were identified (10–15). In the barley-mildew interaction, two of these substances have been reported to contribute to fungal arrest in CWAs: callose deposition (16, 17) and cell wall-bound phenolics (17, 18).

Biological functions of phenolics range from cell wall reinforcement, antimicrobial activity, plant hormones, or defense signaling compounds to scavengers of reactive oxygen species (19, 20). Phenolics can be conjugated to various substances, for example carbohydrates, proteins, or polyamines. Polyamines have themselves been implicated in resistance responses as well as regulation of DNA replication, gene transcription, organel development, leaf senescence, and tuber dormancy (21, 22). In barley, well examined phenolic polyamine conjugates are the

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p-coumaroylagmatine (p-CA) derivatives. Dimers of p-CA, the hordatines, are implicated in defense responses and have been proposed as antifungal factors (23). Their formation appears to be restricted to the genus *Hordeum*. The synthesis of these phenolic polyamine conjugates combines the phenylpropanoid and polyamine pathway (Scheme 1).

**Scheme I. Pathway of p-coumaroyl-agmatine metabolism in barley.** p-Coumarate is conjugated by 4-coumarate: CoA ligase (4CL) to coenzyme A, leading to the thioester p-coumaroyl-CoA. Arginine is decarboxylated by arginine decarboxylase (ADC) to form agmatine. Agmatine coumaroyltransferase (ACT) conjugates p-coumaroyl-CoA and agmatine to p-coumaroylagmatine, which can be dimerized to hordatine A.

Phenolics in CWAs have been characterized mainly by histochemical analyses (10). These could not address the individual components of this compound class. In this study, we developed a detection system to monitor soluble and cell wall-bound phenolics in the target tissue of the pathogen. We used a combined genetic and biochemical approach to show their differential accumulation in compatible and incompatible interactions of the mlo-mediated powdery mildew resistance in barley.

**Experimental Procedures**

**Plant Material**—The susceptible *Mlo* barley genotypes used were cultivar (cv) Ingrid and cv Pallas. The *mlo*-resistant plants used were the back-cross lines (BC) BC Ingrid *mlo*-3, BC Ingrid *mlo*-5, and BC Pallas *mlo*-5 (24, 25). The examined *ror*1 mutants A89 (*ror*1–2) and C69 (*ror*1–4) and the *ror*2 mutant A44 have been described by Freialdenhoven *et al.* (8). 50 seeds of each cultivar were sown per pot in a peat-clay mixture and grown in a glasshouse at 15 °C, 75% humidity, and a photoperiod of 16 h with a light intensity of 100 microeinsteins.

**Pathogen Inoculation Conditions**—Powdery mildew inoculations were performed with the *E. graminis hordei* isolate K1 (24), propagated on the barley cv Golden Promise *Mlo*. Six days after sowing, both adaxial and abaxial surfaces of the primary leaves were inoculated in a spore settling tower (26), resulting in a density of approximately one spore/epidermal host cell.

**Analysis of Free Phenolics**—Fungal germlings were removed from leaf surfaces with a wet cotton ball. The efficiency of this procedure was checked both microscopically and macroscopically by testing the absence of colony growth on susceptible *Mlo* cultivars 7 days after inoculation. Abaxial epidermal peels were ground in liquid nitrogen and lyophilized. Samples of 2–5 mg were extracted four times with 500 ml 70% methanol (aqueous). The phenolics in the combined methanolic phase were separated and quantified by reverse phase HPLC using a Prodigy 5 ODS-2 column (Phenomenex; 5 μm, 250 × 460 mm) with gradient elution employing increasing methanol-acetonitrile (1:1 (v/v)) levels (solvent A) in 10 mM ammonium formate buffer, pH 3 (solvent B). The following gradient elution conditions were used: time 0 min/10% A; time 50 min/50% A; time = 60 min/100% A; flow rate = 1 ml/min; injection loop = 100 μl.

For separating p-coumaroyl-hydroxyagmatine and its oxidative product, a Hypersil 5 μm BDS C8 column (Hypersil; 5 μm, 250 × 460 mm) was used with a gradient of solvent A in 10 mM ammonium formate buffer, pH 8 (solvent C). The following gradient elution conditions were used: time = 0 min/10% A; time = 20 min/18% A; time = 50 min/50% A; time = 55 min/95% A; time = 60 min/100% A; flow rate = 1 ml/min; injection loop = 100 μl.

A dual wavelength detector was used for monitoring the phenolic profiles at 306 and 280 nm, and the peak area was quantified at 306 nm. Unless otherwise stated, all solvents were of HPLC grade purity. Absorbance spectra were performed with a diode array detector by scanning from 230 to 360 nm.

**Analysis of Cell Wall-bound Phenolics**—The residue of the methanolic extraction of epidermal peels was saponified in 2 ml of 1 N NaOH at 70 °C for 24 h, before neutralizing with 9 N HCl to pH 6 and analyzed by
reverse phase HPLC using a Prodigy 5 ODS-2 column under the conditions described above. For saponification of whole leaf tissue, squares of 1 cm were floated in 10 ml of 1 N NaOH at 70 °C for 24 h before washing with distilled water until the wash solution reached a pH of 6.

**Mass Spectrometry**—The molecular weight (Mm) and the structures of p-coumaroylammatine, p-coumaroyl-hydroxyammatine, and its oxidative product p-coumaroyl-hydroxydehydroammatine were determined by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) (27). The HPLC conditions used were as follows: column, Nucleosil 100–10 C18 (Varioprep; 10 μm, 250 × 40 mm); solvent, acetonitrile/H2O (containing 0.2% acetic acid) 40/60; flow rate = 0.2 ml/min; injection volume = 1 μl; injected amount, approximately 100 ng.

The ESI-MS/MS was performed on a Finnigan TSQ 7000 (Bremen, Germany). Conditions for the ESI-MS/MS were as follows: collision energy = −20 eV; collision gas, argon; collision pressure = 1.7 × 10−4 torr (27). The collision-induced dissociation was performed on the [M + H]+ ions formed by ESI. Data were acquired in the m/z range from 50 to 300.

Electrospray mass spectrometry was used for determining the Mm of the hordatines. Data were obtained on a Micromass Platform (Manchester, UK). The following conditions were applied: carrier solvent, acetonitrile/water (1:1 (v/v)); flow rate = 0.5 ml/min; injection volume = 1 μl; injected amount, approximately 100 ng.

The ESI-MS/MS was performed on a Finnigan TSQ 7000 (Bremen, Germany). Conditions for the ESI-MS/MS were as follows: collision energy = −20 eV; collision gas, argon; collision pressure = 1.7 × 10−4 torr (27). The collision-induced dissociation was performed on the [M + H]+ ions formed by ESI. Data were acquired in the m/z range from 50 to 300.

**Microscopic Analysis**—To quantify the frequency of haustoria formation, barley primary leaves were harvested at the indicated time points and fixed in alcoholic lactophenol (ethanol-lactophenol 2:1 (v/v)). Leaves were cleared by boiling for 5 min before staining for 5 s in Coomassie Blue (0, 6% (w/v) Coomassie Brilliant Blue R 250 (Sigma) in methanol) and rinsed in distilled water. Fungal structures were observed by bright field microscopy (Axiophot, Zeiss, Carl Zeiss, Oberkochem, Germany). The presence of fluorescent phenolics in cell wall appositions was examined by incident light fluorescence microscopy (Axiophot, HBO 50-watt mercury lamp), using a 365-nm exciter filter, 395-nm dichroic mirror, and >397-nm barrier filter.

**Test for Antifungal Activity in Vivo**—6-day-old seedlings of cv Ingrid Mlo were inoculated with E. graminis at a density of approximately one fungal spore/epidermal host cell. After 10 h, leaves were infiltrated with 50 μl of the aqueous test solutions through adaxial stomata using a blunt-ended syringe (29). This incubation scheme was chosen, since previous studies showed movement of low molecular weight substances within 2 h through the host cell wall into the cytoplasm (30). Because in our experimental conditions, accumulation of fraction A started 12 h after fungal spore inoculation, the injected test solutions were expected to mimic the observed timing of p-CHA accumulation. The area covering the injected test solutions was marked with a solvent-free correction fluid. Fungal germings in this area were microscopically scored on adaxial epidermal surfaces for haustoria formation rates 32 h after inoculation.

**Differential Accumulation of Fraction A in Response to Pathogen Attack**—The susceptible barley cv Ingrid Mlo and its near isogenic resistant BC line BC Ingrid mlo-5 were inoculated with E. graminis spores, and after 22 h epidermal peals of the primary leaves were harvested. The soluble phenolics of the two genotypes were resolved by HPLC (Fig. 1). Only peak 1 showed a significantly different accumulation in the comparison of the susceptible and resistant barley lines. This peak was named fraction A, and time course analyses were performed for its accumulation in both lines (Fig. 2A). In the resistant line, there was an approximately 25-fold increase in fraction A accumulation from the level of the noninoculated control plants with a maximum at 22 h after inoculation. The accumulation maximum in the susceptible line was about 2-fold lower and delayed by 2 h. To show that the differential accumulation patterns in the incompatible interaction were not dependent on a specific mlo allele, an additional resistant cv BC Ingrid mlo-3 was analyzed. No major differences were detectable in the accumulation profile of the plants carrying the different mlo resistance alleles (Fig. 2A).

In parallel to the characterization of the phenolic profiles, a quantitative cytological analysis of plant-fungus interaction sites was performed at each time point (Fig. 2A, bottom). The maximum penetration frequency of the fungus in the resistant cv BC Ingrid mlo-5 was less than 0.1%, and in the susceptible cv Ingrid Mlo it was about 60%, similar to previous reports (8). These data...
revealed a strong correlation between fraction A accumulation and the onset of host cell penetration in the susceptible interaction. We investigated whether the differential accumulation of fraction A was not only independent of different mlo resistance alleles but also of the genetic backgrounds. Both tested resistant lines, BC Ingrid and BC Pallas, carrying the mlo-5 allele, showed similar accumulation profiles with about a 30-fold increase in comparison with control plants. In the susceptible cv Pallas Mlo, the maximum accumulation is 2–3-fold lower and 2 h later than in the resistant line (data not shown).

To further investigate where and when fraction A might play an important role in the mildew resistance, we analyzed genotypes carrying mutations in the mlo-resistant pathway, Ror1 and Ror2 (Fig. 2B; Ref. 8). The mutants showed a fungal penetration frequency of about 20% for the ror1 mutants and about 10% for the ror2 mutant; each exhibits partial susceptibility (8). Fraction A was measured over time in the ror1-2 mutant (A89) and the ror2 mutant (A44) in comparison with the resistant line. No significant difference could be observed between the ror1 mutant and the resistant genotype. This finding was supported by similar results of an additional ror1 mutant ror1-4 (C69) (data not shown). However, the accumulation profile of fraction A in the ror2 mutant (A44) could be clearly discriminated from the resistant line, with its reduced and delayed accumulation maximum, similar to the susceptible cv Ingrid Mlo (Fig. 2, compare A and B). Since A44 was not back-crossed after mutagenesis of the parent line BC Ingrid mlo-5, the altered concentration of fraction A could also be due to second mutations in genes unrelated to the resistance response. Susceptible F3 plants homozygous for the ror2 mutation revealed results comparable with those obtained with the original A44 mutant (data not shown).

Structural Characterization of Fraction A—Since fraction A was the only detectable phenolic to show different accumulation in response to pathogen attack in the tested compatible and incompatible interactions, we purified the substance to determine its structure. In addition to peak 1 (fraction A), four other peaks of the soluble phenolic profile (peaks 2–5 in Fig. 1) were characterized to obtain a more complete picture of the soluble phenolics in barley epidermal cells. Absorbance spectra of the five peaks indicated that peaks 1–4 (Amax = 291, 298, 321, and 300 nm) belonged to the same family of compounds, the phenolic acid-polyamine conjugates (Fig. 3).

The identity of peak 2 was revealed by LC/ESI-MS/MS to be p-CA (Fig. 4). This was indicated by a [M + H]+ peak at m/z 277 and fragment ions at m/z 217, 147, and 131, as well as by comparison of authentic material (Fig. 4A). Peaks 3 and 4 were identified by ESI-MS as hordatine B and A, respectively (data not shown). The [M + H]+ peaks of m/z 581 and 551 and the [M + 2H]2+ peaks of m/z 291 and 276 indicate that hordatine A is a dimer of two molecules of p-CA and that hordatine B is a dimer of p-CA and feruloylagmatine. Both substances, p-CA and the hordatines have been previously characterized in young barley plants (31).

The LC/ESI-MS/MS analysis of fraction A (peak 1) showed a [M + H]+ value of m/z 293 and fragment ions at m/z 275, 233, 147, 130, and 129 (Fig. 4B), indicating a p-coumaroylagmatine with an additional hydroxy function, a p-coumaroyl-hydroxyagmatine (p-CHA). The ion fragments at m/z 233 and m/z 129 suggest that the hydroxy function is positioned in the agmatine moiety (Fig. 4C). MS analyses of a synthetic N-hydroxyarginine showed no loss of 18 mass units representing the loss of a water molecule (data not shown). Since a loss of 18 mass units is observed with p-CHA (fragment ion m/z 275), this provided strong evidence that the hydroxy group is located at the carbon
skeleton of the agmatine residue and not at the guanidino function. The higher electronegativity of the guanidino group suggested γ-(3)-C or δ-(4)-C for the position of the hydroxy function on the agmatine fragment. This assumption is also supported by data on δ-(4)-hydroxyarginine (32), γ-(3)-hydroxyarginine (33–35), and γ-(3)-hydroxyagmatine (36). Until now, only γ-(3)-hydroxylated arginine derivatives were found in the plant kingdom. However, any direct evidence for the position of the hydroxy group on the agmatine residue of the p-coumaroyl-agmatine cannot be derived from the MS data.

The MS analysis also revealed that peak 1 overlapped with a minor component, showing a [M + H]^+ value of m/z 291. The two substances could be separated on the HPLC and were named compound A1 (molecular mass of 292 Da, p-coumaroyl-hydroxyagmatine) and A2 (molecular mass of 290 Da). Both compounds A1 and A2 had identical absorbance spectra (Fig. 3, panel 1) and were found in a stable ratio of 7:3 (A1/A2) in the time course analysis. During the purification step of the two substances, the ratio changed in favor of A2, indicating that A2 might be an oxidative product of A1. LC/ESI-MS/MS data of compound A2 showing fragment ions of m/z 273, 255, 216, 147, and 127 supported this possibility, suggesting a p-coumaroyl-hydroxydehydroagmatine structure (p-coumaroyl-hydroxydehydroagmatine). To our knowledge, both substances have not been previously identified in plants until now.

In contrast to the polyamine conjugates of peak 1–4, peak 5 showed a characteristic absorbance spectrum of flavonoids (Fig. 3, panel 5). The λ_max of 270 and 337 nm and the molecular mass of 594 Da, determined by ESI-MS, suggested that it was 2-O-glycosylisovitexin. This substance has already been described in young barley shoots (37).

**Antifungal Activity of Fraction A in Vitro**—The p-coumaroyl-agmatine derivatives belong to the same compound family as the hordatines, which have been implicated as antifungal factors in barley (31). We therefore tested the influence of fraction A on the appressoria formation frequency of *E. graminis* (Fig. 5A; Ref. 28). Fraction A at both concentrations showed with about 25% reduction of appressoria formation frequency a comparable antifungal activity to the fungicide benzimidazol, used as positive control. No effect was achieved by applying the HPLC buffer mixture or p-CA. To investigate any dose dependence of the p-coumaroyl-agmatine derivatives, the purified compounds A1 and A2 were tested in three different concentrations (Fig. 5B). Both compounds showed a clear dose effect, with no detectable reduction of appressoria formation frequency at 0.1 nM and a slightly higher antifungal activity of the oxidative product A2. In summary, fraction A and its purified compounds have a significant antifungal activity in the tested range of 0.1–100 μM.

**Test for Antifungal Activity in Vivo**—To test whether fraction A and its purified compounds had also an antifungal activity in vivo, young, preinoculated barley seedlings were infiltrated with test solutions and scored for haustoria formation. The HPLC buffer mixture and p-CA showed no effect on the haustoria formation frequency, whereas benzimidazol reduced haustoria formation frequency about 90%. A significant antifungal activity in the range of 30–40% reduction could be observed for fraction A, compounds A1 and A2 (Fig. 5C).
Resistance of Cell Wall Appositions to Enzymatic Degradation—HPLC analysis revealed no qualitative differences in the cell wall-bound phenolics between samples of inoculated and noninoculated control plants of the resistant and susceptible cultivars. Profiles showed a typical phenolic acid ratio for a member of the Gramineae family (38). Ferulic acid was found to be the dominating compound with a minor quantity of p-coumaric acid. Quantitative analysis was not reliable, since sample amounts were too small and showed partial degradation of the phenolics during the saponification step.

Because of these limitations, we characterized the reaction of the bound phenolics against enzymatic degradation and saponification directly in the cell wall appositions. Tissue fragments of the cv BC Ingrid mlo-3, harvested 24 h after inoculation, were treated with a cellulase solution. In contrast to noninfected epidermal cell walls, papillae and neighbor hooded, altered lateral walls in barley appeared to be resistant to the enzymatic treatment (Fig. 6, A and B). Additionally, autofluorescence of the papillae was retained after the treatment (Fig. 6C), indicating the presence of phenolics. The digestion of epidermal fragments of the susceptible cv Ingrid Mlo gave similar results (data not shown).

Resistance of Cell Wall Appositions to Saponification—Initial experiments showed that the autofluorescence in cell wall appositions, harvested 24 h after inoculation, were not released by alkali extraction, a treatment that eliminates the background fluorescence of noninfected cell walls and stomata. We wanted to test whether the resistance to saponification might depend on harvesting time points (age of the cell wall apposition) and on different genotypes. Leaf samples of the resistant genotype BC Pallas mlo-5 and the susceptible cv Pallas Mlo were harvested 12, 14, and 16 h after inoculation. The identical interaction sites were examined before and after saponification.

In the susceptible genotype, only the autofluorescence in the cell wall appositions derived from 16 h after inoculation was retained. At the earlier time points, the phenolics appeared to be completely released from the papillae (Fig. 7). In contrast, in the incompatible cultivar autofluorescence was only eliminated at 12 h after inoculation, indicating that the phenolics in the cell wall of the incompatible genotype are resistant to alkali treatment at least 2 h earlier as in the susceptible genotype (Fig. 7). Similar results were also found in the genetic background of Ingrid (data not shown).

**DISCUSSION**

Changes in phenolic acid composition and in phenolic polymers have been found in all plant pathogen interactions investigated. Low molecular weight phenolics are known for their antimicrobial activity, for example ochrinal in Orchis militaris or pisatin in Pisum sativum (39, 40). Phenolic polymers like lignin are components of structural barriers such as CWAs or HR-mediated cell death responses (19). In the mlo-controlled incompatible interaction in barley, hypersensitive cell death generally does not occur. The bright autofluorescing CWA is the only microscopically visible cell response to pathogen challenge, suggesting the presence of increased amounts of phenolics. During early time points after pathogen inoculation, transcript levels and enzyme activity of phenylalanine ammonia lyase rise (41). Phenylalanine ammonia lyase is a key enzyme of the phenylpropanoid pathway leading to the synthesis of phenolic compounds. Therefore, to determine whether phenolics play a role in the powdery mildew resistance, we measured soluble and cell wall-bound phenolics in epidermal barley cells after inoculation with the pathogen E. graminis. More specifically, we were interested whether phenolics might play a role in the mlo-controlled incompatible interaction, which is not associated with a HR.

This study has characterized a novel compound, p-CHA, that shows a differential accumulation in resistant and susceptible plants after pathogen challenge. Its identity was confirmed by absorbance spectra and MS analysis. Previously described, highly related compounds p-CA and its dimers hordatine A and B were also detectable in the soluble phenolic fraction (31).

The formation of p-CHA involves the polyamine and phenylpropanoid pathway. The hydroxy function could be introduced at three feasible steps in these pathways: at the p-coumaroyl-agaratine, the agmatine, or arginine. p-CA did not show clear differences in its accumulation profile in resistant versus susceptible barley plants. However, p-CA concentration increased about 2-fold, and elevated levels fell 2 h earlier than those of p-CHA in both compatible and incompatible interactions (data not shown). Although these correlations could be explained by direct conversion of p-CA to p-CHA, both compounds could also be derived from two unrelated substance pools.

Labeling studies of a p-CHA-related substance in wheat, p-coumaroyl-2-hydroxyputrescine, suggests that hydroxylation occurs directly at the amino acid level (42). These data support hydroxylation of the arginine for p-CHA formation. A γ-hydroxyarginine was identified in various families of legumes (34, 35), and it was shown that the arginine decarboxylase of Escherichia coli accepts γ-hydroxyarginine as a substrate for decarboxylation (43). Additional radioactive labeling studies can answer the question of at which step the key enzyme for the p-CHA formation might introduce the hydroxy function.

p-CHA belongs to the family of hydroxyaminic acid amides. The accumulation of such substances correlates with pathogen invasion in a number of species. N. tabacum cv Xanthi nc forms large amounts of mono- and dihydroxylutrescine after infection with the tobacco mosaic virus (44). As described previously, p-coumaroyl-2-hydroxyputrescine accumulates in wheat challenged with rust (42). The hordatines in barley increase up to 6-fold 13 days after inoculation with powdery mildew (45). In contrast to the timing of p-CHA accumulation reported in this study, the hordatine induction is too late to be effective against powdery mildew attack.

The hydroxylated p-coumaroyl-agaratine showed significant accumulation after pathogen challenge in the resistant compared with the susceptible barley line. This differential accumulation was induced as early as 16 h after inoculation with the resistant line showing a higher and 2 h earlier maximum. These differences were also observed with two different mlo resistance alleles and with two different genetic backgrounds. The accumulation of p-CHA correlates with the onset of fungal penetration in the epidermal host cells of the susceptible genotype (Fig. 2). Therefore, p-CHA accumulation appears to form one of the early responses of the plant cell to attempted fungal host cell penetration and correlates with the earliest microscopically detectable time point at which a differential phenotype between compatible and incompatible interaction can be identified. To further investigate this correlation, we analyzed barley ror1 and ror2 mutants, carrying mutations in the mlo resistance pathway (8). p-CHA accumulation in the ror2 mutant resembles the accumulation profile of the susceptible gen-

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**Fig. 4.** LC/ESI-MS/MS spectra of p-coumaroyl-agaratine and p-coumaroyl-hydroxyagaratine. A, LC/ESI-MS/MS spectrum of authentic p-coumaroyl-agaratine. B, LC/ESI-MS/MS spectrum of p-coumaroyl-hydroxyagaratine purified from barley leaf epidermal tissue. Structural deductions from observed fragment ions of both spectra are shown in panel C, the left section referring to the spectrum in A and the right section referring to the spectrum in B, respectively.
otypes with a delayed and reduced accumulation maximum. Since the results of the *ror1* mutants were similar to the resistant genotype, it may indicate that *Ror1* and *Ror2* are not acting in a simple linear pathway but might represent components of a branched pathway in which only the one involving *Ror2* affects p-CHA accumulation.

Mutants have been described in *Arabidopsis thaliana* that either abolish or reduce the accumulation of the antimicrobial compound camalexin in response to pathogen attack (46, 47). These *pad* mutants do not compromise race-specific resistance reactions against the phytopathogenic bacterium *Pseudomonas syringae pv maculicola* but lead to increased growth of a moderately virulent *Pseudomonas* strain (47). Similar to the *Ror* mutants, the *pad4* mutant shifts infection phenotypes in interactions with the fungus *Peronospora parasitica* from incomparability to partial susceptibility (48).

A function of p-CHA in the defense response suggests that it would accumulate near to the interaction site, the cell wall apposition. This would involve trafficking of p-CHA in vesicles to the papillae, resulting in a locally higher concentration of p-CHA. In support of this, vesicle transport to the site of attempted penetration is well documented (49, 50). Additionally, cell wall appositions contain compounds possessing a guanidino function as shown by the histochemical Sakaguchi test (51), and their presence in CWAs is correlated with resistance to fungal penetration. The agmatine derivative p-CHA belongs to the family of guanidines, and hydroxyagmatine can be stained by the Sakaguchi reagent (43). Also, compounds with similar size and polarity as p-CHA have been reported to diffuse into papillae (30). Thus, p-CHA could accumulate close to the fungal infection structure. However, it will be a major technical challenge to examine directly the proposed subcellular accumulation of the compound in CWAs.

The polyamine conjugate p-CHA and its oxidative product p-coumaroyl-hydroxydehydroagmatine showed significant antifungal activity *in vitro* and *in vivo* against *E. graminis*. They also reduced germination of the biotrophic wheat pathogen *P. graminis* (40% reduction of at a concentration of 2 mM; data not shown). Both compounds are produced de novo in living host tissue, and they are not detectable after infection if whole leaf tissue is used for extraction. This is an indication that the defense reaction is confined to the tissue attacked by the fungus. The basic response of both compounds in resistant and susceptible barley lines is the same, the basis of differentiation between the hosts being the speed and amount of formation of the substances. Regarding these aspects and the definition of phytoalexins as postulated by Müller and Börger (52), we propose that we have identified a new phytoalexin in barley. The inhibitory effect of p-CHA on appressoria and haustoria formation of *E. graminis* is in the tested range of 0.1–200 μM and is comparable with known antimicrobial compounds such as camalexin in *A. thaliana*, pisatin in *P. sativum*, tomatine in *Solanum lycopersicum*, and avenacin in *Avena sativa* (53–56). However, in the applied concentrations of 200 μM, p-CHA did not seem to reduce mycelial growth on leaves after 7 days. A possible explanation could be a fungistatic rather than a fungitoxic effect of p-CHA on the early stage of fungal development such as the haustoria formation. This could also explain why 100% reduction of appressoria formation is never achieved and why no increase in antifungal activity can be found between the applied concentrations of 10 μM and 1 mM p-CHA.

Similar to the observation reported here, the nonhydroxylated p-CA, in contrast to p-CHA, had no effect on growth of the fungus *Monilinia fructicola* (23). Similar changes of antifungal activity with the introduction of keto or hydroxy functions and double bonds could be observed in related polyamines. The
polyamine putrescine is required for fungal differentiation. In contrast, ketoputrescine was effective in control of several fungi-like *E. graminis* or *Puccinia hordei* and interfered with germ tube formation in Mucorales (57, 58). Ketoputrescine and (E)-1,4-diaminobut-2-ene, a putrescine derivative, reduced enzyme activities of ornithine decarboxylase, S-adenosylmethionine decarboxylase, and diamino oxidase, leading to perturbation of putrescine and spermidine levels in the fungus (57–59). Since agmatine is known to be a substrate for diamino oxidase activity (60), p-CHA itself might interact with diamino oxidase, which could lead to perturbation of putrescine levels and a fungistatic effect. It is noteworthy that a potent fungicide, guazitine, perturbs polyamine metabolism by inhibiting polyamine oxidases, which are involved in spermidine catabolism (60, 61). Since p-CHA and guazitine are both members of the guanidine family, the fungal polyamine oxidases might be a further target for p-CHA. Experimental studies will be necessary to test the actual targets of p-CHA.

Attempted penetration of barley leaves by *E. graminis* is accompanied by the formation of CWAs. Penetration is achieved by mechanical pressure and chemical dissolution, facilitated by hydrolytic enzymes (62). We could show that CWAs of both compatible and incompatible interaction types (24 h after inoculation) were resistant to cell wall-degrading enzymes and that enzymatic treatment did not release phenolics. Similarly, CWAs in wheat are resistant against hydrolytic enzymes as soon as they become microscopically visible (10–12 h after inoculation); subsequent chemical delignification of the papilla allows the CWA to be enzymatically digested (10). This clearly shows that CWAs represent structural barriers against fungal ingress.

The effectiveness of papillae as a defense mechanism has been proposed to be related to the ability of the epidermal cell to fully complete papilla deposition and cross-linking (63). We have shown that phenolics in the cell wall of the *mlo*-incompatible interaction are at least 2 h earlier resistant to alkali treatment than in the *Mlo*-susceptible genotype. Under the applied saponification conditions, ester bridges are broken, whereas phenolic ether bonds are unaffected (64). This suggests a change in bonding types or the addition of different bonding types during CWA maturation. Bayles *et al.* (16) and Skou *et al.* (65) concluded that small differences in the timing

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**FIG. 6.** Resistance of cell wall appositions to cell wall-degrading enzymes. Light micrographs of a leaf epidermal tissue fragment containing a CWA before (A) and after (B) 7-h enzymatic digestion with cellulase (150 units/ml) are shown. C, epifluorescence micrograph of the same CWA as presented in B after enzymatic incubation. Bar, 7.5 μm. The fungal germling was removed mechanically from the epidermal surface prior to enzymatic digestion.

**FIG. 7.** Differential saponification sensitivity of CWA-bound phenolics. Epifluorescence micrographs of susceptible (*Mlo*) and resistant (*mlo-5*) near isogenic barley lines challenged with the powdery mildew fungus are shown. Fungal germlings were removed mechanically from the epidermal surface before saponification at the indicated time points (12, 14, and 16 h) after inoculation. Epifluorescence micrographs of identical interaction sites of both genotypes were taken before (left) and after (right) saponification. The arrows indicate sites of fluorescing CWAs.
of papillae formation determine the frequency of haustoria formation in mlo-controlled resistance. Our data suggest that mlo-resistant genotypes exhibit either an earlier onset or an accelerated speed of CWA compaction. Since the time point at which CWA-bound phenolics become insensitive to alkali treatment tightly correlates with the onset of haustorium formation in the compatible interaction, enhanced speed of CWA compaction may play an important role in determining incompatibility in mlo-resistant lines. This would be consistent with genetic data indicating a negative regulatory function of the Mlo wild type protein in pathogen defense (7).

Our HPLC analyses of epidermal cell wall-bound phenolics have shown that the cross-linked phenolics in barley consist mainly of derivatives of ferulic and p-coumaric acid, typical of Gramineae (38). Peroxidases are involved in lignification and cross-linking of p-CHA has in addition to its antimicrobial activity a role in -CHA seems to correlate with the observed change of chemical bonding types in the CWA (Fig. 7). It is therefore feasible that -CHA in addition to its antimicrobial activity a role in the cross-linking processes of papillae. One possibility could be cross-linking of -CHA to proteins, carbohydrates and other cell wall-bound phenolics by H2O2. Future experiments will have to test the proposed subcellular accumulation of -CHA in CWAs. Larger amounts of chemically synthesized p-CHA should also enable us to test its possible biological activity against a wider spectrum of plant pathogens.

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