Suppression of Induced Resistance in Cucumber Through Disruption of the Flavonoid Pathway

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ABSTRACT

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In this study, cucumber plants (*Cucumis sativus*) expressing induced resistance against powdery mildew (caused by *Podosphaera xanthii*) were infiltrated with inhibitors of cinnamate 4-hydroxylase, 4-coumarate:CoA ligase (4CL), and chalcone synthase (CHS) to evaluate the role of flavonoid phytoalexin production in induced disease resistance. Light and transmission electron microscopy demonstrated ultrastructural changes in inhibited plants, and biochemical analyses determined levels of CHS and β -glucosidase enzyme activity and 4CL protein accumulation. Our results

showed that elicited plants displayed a high level of induced resistance. In contrast, down regulation of CHS, a key enzyme of the flavonoid pathway, resulted in nearly complete suppression of induced resistance, and microscopy confirmed the development of healthy fungal haustoria within these plants. Inhibition of 4CL ligase, an enzyme largely responsible for channeling phenylpropanoid metabolites into the lignin pathway, had little effect on induced disease resistance. Biochemical analyses revealed similar levels of 4CL protein accumulation for all treatments, suggesting no alterations of nontargeted functions within inhibited plants. Collectively, the results of this study support the idea that induced resistance in cucumber is largely correlated with rapid de novo biosynthesis of flavonoid phytoalexin compounds.

Cucumber (*Cucumis sativus* L., family Cucurbitaceae) and the powdery mildew fungus *Podosphaera xanthii* (syn. *Sphaerotheca fuliginea* auct. p.p.) are a convenient model for examining host-pathogen interactions. Accordingly, several elicitor-induced defense responses were reported for this pathosystem, including the lignification of cell walls (2,20), increased activities for pathogenesis-related (PR) proteins such as chitinases, peroxidases, and β -1,3-glucanases (11,22,29,36), and more recently, production of flavonoid phytoalexins (9,10,14,26,27,41). Milsana, a commercial preparation made from the leaves of the giant knotweed plant (*Reynoutria sachalinensis* L.), is an eliciting mixture containing proteins, glycoproteins, peptides, carbohydrates, and lipids that signals the presence of the pathogen and triggers defense responses in a non-cultivar-specific manner (9,10,22). In cucumber, Milsana induced resistance against *P. xanthii* and this resistance correlated with increased mRNA accumulation and greater extractable enzymatic activity for chalcone synthase (CHS) and chalcone isomerase, two key enzymes of the flavonoid pathway (17). Recently, it was found that rapidly synthesized C-glycosyl flavonoid phytoalexins, known as cucumerins, accumulated at the sites of fungal penetration within Milsana-elicited cucumber plants (26,27). Despite recent advances in the understanding of induced resistance for this species, the importance of the role played by phytoalexins, compared with that of other defense responses reported for cucumber, is poorly understood.

The phenylpropanoid pathway in higher plants is responsible for generating metabolic intermediates directed into several path-

ways, including the lignin and flavonoid pathways (12,25,40). 4-Coumarate:CoA ligase (4CL) plays a particularly important role in plant defense reactions because of its position joining the phenylpropanoid pathway with lignin and flavonoid branch pathways (Fig. 1). In fact, different isoforms of 4CL direct carbon flow to the diverse pathways of phenylpropanoid metabolism according to different substrate preferences. For example, 4CL3 (a class II 4CL) has a high affinity for 4-coumarate, while 4CL1 and 4CL2 have strong affinities for 4-caffeate (1,13,39). Thus, Ehrling et al. (13) and Stuible et al. (39) hypothesized that the primary function of class II 4CL is to channel 4-coumarate to the first enzyme of the flavonoid pathway, CHS.

Given the importance of the secondary metabolites produced by phenylpropanoid metabolism, potent chemical inhibitors have been developed for biosynthetic enzymes leading to lignin and flavonoid branch pathways. For instance, α -aminooxy- β -phenylpropionic acid is an inhibitor of phenylalanine ammonia-lyase (5,6,33). Piperonylic acid (PA) mimics cinnamic acid, the natural substrate of cinnamate 4-hydroxylase (C4H), and is a specific inhibitor of C4H (37,38). 3,4-Methylenedioxy cinnamic acid (MDCA) is a structural analogue of 4-hydroxy cinnamic acid and is a reported inhibitor of 4CL (19,30,37), and cycloheximide (CHX) is an efficient inhibitor of CHS transcription (7,23). Although the specificity of inhibitors has been debated (4,38), previous reports using enzyme inhibitors such as PA have demonstrated that its impact on nontargeted plant function was minimal (37). Moreover, addition of CHX was shown to decrease the accumulation of CHS transcripts, without affecting the transcriptional level of constitutive genes in *Arabidopsis thaliana* and parsley (*Petroselinum crispum*) (7,15,23).

The objective of this study was to evaluate the role of flavonoid phytoalexin production, lignification, and chitinases in disease resistance for cucumber. To this end, elicited cucumber plants ex-

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pressing induced resistance against powdery mildew were infiltrated with PA, MDCA, and CHX, reported inhibitors of C4H, 4CL, and the translation of the CHS transcript, respectively (7, 19,37). Disease incidence was closely monitored over a period of 4 weeks. Light and transmission electron microscope investigations coupled with cytochemical labeling of chitin were used to document ultrastructural changes in inhibited plants. In order to determine the effects of inhibitor treatments on cucumber plants, biochemical methods were used to analyze protein levels of 4CL and enzyme activity of CHS and β -glucosidase.

MATERIALS AND METHODS

Chemicals. PA, MDCA, and CHX were purchased from Sigma Chemical (St. Louis, MO), dissolved in H_2O , and diluted to 0.5 to 1, 3, and 0.5 mM, respectively.

Plant material. Cucumber plants (*C. sativus* cv. Corona) were grown as described by Wurms et al. (41). Briefly, seeds were planted in Pro-mix (Premier Horticulture, Rivière du Loup, Québec, Canada) and watered daily until emergence. Seedlings were then placed in a climate-controlled growth chamber maintained at 23°C, with a 16-h photoperiod ($145 \mu E m^{-2} s^{-1}$). Plants were then fertilized with a sterile nutrient solution (N:P:K, 7:11:27) starting at the one- to two-leaf stage (3 to 4 weeks). Inoculation of cucumber plants with *P. xanthii* (syn. *S. fuliginea*) was performed at the four- to six-leaf stage (approximately 6-week-old plants) and was done by shaking the leaves of infected plants over uninfected plants. Uninoculated *A. thaliana* (ecotype Colombia), poplar (*Populus tremuloides*), and cucumber plants were grown and fertilized in a separate climate-controlled growth chamber under the same conditions. Tissue harvested from healthy plants of these two species served as

positive controls for 4CL protein expression and β -glucosidase enzyme activity.

Inhibitor and elicitor treatments and disease assessment. Inhibitors were injected into the leaves of cucumber plants with a medical intravenous infusion system ($0.5 ml h^{-1}$ Multiday Infusor Syringe; Baxter, Deerfield, IL). As a preliminary test, injection of 5 ml of an aqueous blue copper sulfate solution ($CuSO_4$, $1 mg ml^{-1}$) established that the solution had translocated throughout the leaf tissue within 10 h.

Upon appearance of the first signs of infection covering approximately 2% of leaf area (4 to 6 days after inoculation), plants were segregated using Mira cloth tissue. Inoculated (I+) and uninoculated (I-) plants were sprayed until runoff with an aqueous solution of Milsana (0.5%, vol/vol) once a week for 4 weeks (the duration of the experiment) and were referred to as I+M+ and I-M+, respectively. Following the first Milsana treatment, I+M+ plants selected at random were syringe-injected through the petiole of the third leaf with 5 ml of 0.5 or 1 mM PA, 3 mM MDCA, or 0.5 mM CHX, and were designated I+M+(PA), I+M+(MDCA), and I+M+(CHX), respectively. As controls, selected inoculated Milsana-elicited plants were syringe-injected with 5 ml of pure water and were referred to as I+M+(H_2O). Disease assessment was carried out on a weekly basis following treatment with Milsana, or water, for 4 weeks according to the system developed by Nicot et al. (31). The experiment was repeated three times, and data were collected from individual injected leaves and entire plants.

Extraction of enzymes and proteins from bacterial and plant material. As source of enzymes and proteins, recombinant 4CL2 and 4CL3 constructs were propagated and expressed in *Escherichia coli* (strain M15) (Qiagen, Mississauga, Canada) according to standard practice (13,24,28). Briefly, transformed

E. coli cultures were first induced with 2 mM IPTG, an analogue of galactose, for 5 h followed by centrifugation (14,000 rpm, 4°C). The bacterial pellet was then sonicated in 200 mM Tris-HCl (pH 7.8), and clear lysates were supplemented with 30% glycerol and stored at -20°C.

In a similar manner, 4CL enzymes were extracted from cucumber, *A. thaliana*, and poplar tissues. Harvested plant tissues (200 mg) were first ground in liquid nitrogen, mixed in 1 ml of buffer containing 200 mM Tris-HCl (pH 7.8) and 15 mM 2-mercaptoethanol, and placed on a rotary shaker for 20 min in the presence of DOWEX 1× 2-200 resin (20 mg) (Aldrich Chemical, Sheboygan, WI) at 4°C to clear supernatant by fixing pigments. Following centrifugation (14,000 rpm, 4°C), the supernatant was supplemented with 30% glycerol and stored at -20°C.

Analysis of protein synthesis and CHS enzymatic activity.

To determine the effects of CHX on the general plant protein synthesis, 4CL protein accumulation and β -glucosidase activity were analyzed within total protein extracts. For western blot analysis of recombinant and extracted 4CL, bacterial lysates containing recombinant 4CL were loaded on a Ni NTA Spin Column (Qiagen) and eluted with an imidazole buffer (250 mM) according to the manufacturer's recommendation. Approximately 1 μ g of purified 4CL recombinant proteins and 20 μ g of total plant proteins from I+M+ and I+M+(CHX) cucumber plants were electrophoresed in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis denaturing gel. As controls, total protein extracts prepared from *A. thaliana* and poplar plants were electrophoresed (20 μ g) using the same conditions. Electrophoresed proteins were then blotted on a nitrocellulose membrane and probed by reaction with a polyclonal antiserum raised against parsley 4CL, according to Ragg et al. (35). For analysis of β -glucosidase activity, total protein extracts were first prepared from the tissues of I+M-, I+M+, I-M-, and I+M+(CHX) cucumber plants, as well as from *A. thaliana*. The level of β -glucosidase activity was quantified within these extracts according to the protocol developed by Politycka (34), and expressed as millimole of para-nitrophenyl pyranoside per microgram of protein. The activity was measured from leaves harvested in three separate experiments.

Fig. 2. A, A leaf harvested from an inoculated, nonelicited (I+M-) cucumber plant, and B, a leaf harvested from an inoculated and elicited (I+M+) cucumber plant. C and D, Cucumber leaves harvested from inoculated and elicited cucumber plants infiltrated with cycloheximide I+M+(CHX) and 3,4-methylenedioxy cinnamic acid I+M+(MDCA), respectively. All leaves were harvested 2 weeks after infiltration with inhibitors.

CHS activity was determined within total protein extracts prepared from the tissues of I+M-, I+M+(CHX), and I+M+ cucumber plants using the methodology developed by O'Neill et al. (32), with minor modifications. Briefly, in a total volume of 110 μ l, 13.5 μ l of coumaroyl-CoA (205 μ M), 5 μ l of (14 C) malonyl-CoA (2.2 Gbq mmol $^{-1}$, 60 mCi mmol $^{-1}$), and 20 μ l of crude CHS enzyme extract were incubated in 71.5 μ l of reaction buffer (700 mM K-Pi, pH 8.0; 2% bovine serum albumin) at 37°C for 1 h. Following two extractions of the reaction product with 200 μ l of ethyl acetate (2 × 200 μ l), the amount of radioactivity within a 250- μ l sample of the combined ethyl acetate extracted phase was estimated by scintillation counting. The identity of the flavonoid reaction product was confirmed using thin layer co-chromatography with toluene/chloroform/ethyl acetate (40:25:35) as the developing solvent system and a standard for naringenin purchased from Aldrich Chemical Company. The activity was measured from leaves harvested in three separate experiments.

Light and transmission electron microscopy. Seven days after infiltration with inhibitors, tissue samples were harvested from the third leaf of all plants. Leaf samples were dissected into 1-mm 2 sections, fixed overnight at 4°C in 3% (vol/vol) glutaraldehyde per 0.1 M sodium cacodylate buffer (pH 7.2), postfixed for 1 h at ambient temperature using 1% (wt/vol) osmium tetroxide, dehydrated by soaking in a series of aqueous ethanol solutions (30 to 100%), and embedded in Jemdeb 812 Resin (Canemco Inc., St. Laurent, Quebec). For light microscopy observations, thick sections (1 to 2 μ m) were cut with a glass knife and stained for 45 s with 0.2% (wt/vol) toluidine blue O in 2% (wt/vol) sodium borate.

For transmission electron microscopy, thin sections (0.1 μ m) were cut with a diamond knife and samples were examined with a transmission microscope (JEOL, Tokyo) operating at 80 kV. Cytochemical labeling of chitin within tissue samples was performed according to Benhamou et al. (3). Briefly, gold particles (12 nm in diameter) were first prepared according to the method reported by Frens (18). Wheat germ agglutinin (WGA), a lectin with *N*-acetylglucosamine binding specificity, was used as the second-step reagent. A highly specific cytochemical ovomucoid-gold conjugate probe (pH 6.0) was added to tissue samples as the third and final reagent.

RESULTS

Disease incidence following elicitor and inhibitor treatments. In this study, Milsana-treated plants inoculated with *P. xanthii* (I+M+) had a low level of infection that never exceeded 5% of leaf surfaces for the duration of the experiment (4 weeks). In contrast, infection for nonelicited plants (I+M-) progressed rapidly and covered approximately 80% of leaf surfaces (Fig. 2A and B). These results were consistent with previous studies examining the prophylactic effects of Milsana in powdery mildew-infected cucumber plants (9,10,17,41).

Infiltration with a blue-green CuSO $_4$ solution demonstrated extensive translocation of fluids within cucumber leaf tissues at 24 h after injection; the margins and venation of infiltrated leaves were typically blue-green in color (data not shown). Despite receiving an eliciting treatment, the level of infection for CHX-infiltrated I+M+(CHX) plants was significantly higher (46% coverage of leaf surface) compared with that of I+M+ plants (Fig. 2C). Infiltration with MDCA in I+M+(MDCA) yielded an intermediate level of infection (27%) between that of I+M+(CHX) plants and I+M+ plants (Fig. 2D). Disease assessment for I+M+(PA) plants was difficult because infiltration with PA caused cytotoxic reactions at both 0.5 and 1 mM, as evidenced by the chlorotic appearance of leaf tissue. Injection with water alone in I+M+(H $_2$ O) plants caused no substantial differences compared with that of noninfiltrated I+M+ plants (data not shown).

Analysis of protein synthesis and CHS enzymatic activity.

Western blot analysis of purified recombinant bacterial lysates confirmed the affinity of the polyclonal antiserum raised against parsley 4CL for 4CL3 and 4CL2 (Fig. 3A). Similarly, total protein extracts prepared from the tissues of all plants investigated reacted with the 4CL antibodies (Fig. 3A). Of particular importance, no differences were observed for the presence of 4CL within I+M+(CHX) plants compared with I+M+ cucumber plants, *A. thaliana*, or poplar, indicating that CHX had no effect on 4CL protein synthesis (Fig. 3A). When β -glucosidase activity was assayed, no significant differences were observed for I+M+(CHX) plants compared with uninhibited I+M-, I+M+, I-M- cucumber plants, or *A. thaliana* (Fig. 3B), suggesting that CHX did not have a general damaging effect on synthesis of proteins such as β -glucosidase. In contrast, analysis of CHS revealed greater enzymatic activity for I+M+ plants compared with that of I+M- or I+M+(CHX) cucumber plants (Fig. 3C). In particular, the level of CHS activity for I+M+(CHX) plants was 1.6 times lower than that of susceptible I+M- plants and 4.4 times lower than that of disease-resistant I+M+ cucumber plants (Fig. 3C).

Light microscope observations. Light microscope observations paralleled the disease incidence observed at the whole plant level (Fig. 4). On inoculated nonelicited I+M- cucumber plants, fungal cells formed germ tubes at the cell surface and penetrated the epidermis to form well-developed multilobed haustoria (Fig. 4A). In inoculated Milsana-elicited (I+M+) plants, papilla formation was observed (Fig. 4B) and haustoria were often encased in an amorphous material that formed at the point of fungal penetration (Fig. 4C to E). Papilla development at the site of appressorium formation was also observed as a primary plant defense response (Fig. 4E).

Treatment with PA resulted in pronounced plant tissue alterations as judged by the extent of collapsed and disorganized leaf cells (Fig. 4F), corroborating the phytotoxic reactions observed for the leaves of PA-infiltrated plants. For elicited MDCA-infiltrated I+M+(MDCA) cucumber leaf tissue, fungal haustoria were sometimes collapsed and degraded (Fig. 4G and H). Although aggregated haustoria were often encased in a material that stained densely with toluidine blue, the presence of amorphous material was not as extensive and regular as that observed in I+M+ plants (Fig. 4B). In contrast, multilobed haustoria were abundant in epidermal cells of CHX-infiltrated I+M+(CHX) plants, and mycelial structures at the plant leaf surface appeared healthy and turgid (Fig. 4I and J).

Transmission electron microscope observations. Electron microscope observations of I+M- plants revealed that infection with *P. xanthii* resulted in the formation of multilobed haustoria characterized by ovoid central bodies surrounded by an extra-haustorial membrane (Fig. 5A). Following cytochemical labeling, it was shown that the haustorial wall was labeled by the WGA/ovomucoid-gold complex, whereas the extra-haustorial matrix and extra-haustorial membrane were free of labeling (Fig. 5B).

Within the epidermal cells of inoculated elicited I+M+ cucumber plants, haustoria were visibly degraded and collapsed (Fig. 6A to C). In most cases, haustorial lobes were retracted and compressed against the haustorial body, and organelles within haustoria of I+M+ plants were no longer discernible (Fig. 6A to C). Fungal hyphae at the plant cell surface frequently contained aggregated cytoplasm (Fig. 6A), and collapsed haustoria were surrounded by an amorphous material that did not react with the WGA/ovomucoid-gold complex that labels chitin (Fig. 6B and C). In contrast, the walls of fungal haustoria were frequently heavily labeled (Fig. 6C).

For I+M+(MDCA) epidermal cells, irregular defense reaction was associated with elongated vesicular and collapsed haustoria encased in a granular matrix resembling callose (Fig. 7A). For I+M+(CHX) plants, the morphology and ultrastructure of haustoria were very similar to those observed for I+M- plants (Fig. 7B

to D). Of particular importance, healthy multilobed haustoria were consistently observed within the epidermis of I+M+(CHX) cucumber plants.

DISCUSSION

During the last 4 decades, the cucumber-powdery mildew pathosystem has been investigated relative to mechanisms under-

Fig. 3. A, Western blot analysis of recombinant (r), and extracted 4-coumarate:CoA ligase (4CL) enzymes prepared from elicited cucumber plants inoculated with *Podosphaera xanthii* (I+M+), *Arabidopsis thaliana*, poplar, and inoculated elicited cucumber plants infiltrated with cycloheximide I+M+(CHX). Immunodetection was performed using a polyclonal antiserum raised against parsley 4CL. Protein loading was equal in all treatments. B, β -Glucosidase enzyme activity in nonelicited cucumber plants inoculated with *P. xanthii* (I+M-), elicited inoculated cucumber (I+M+) plants, nonelicited uninoculated (I-M-) cucumber plants, elicited inoculated cucumber plants infiltrated with cycloheximide I+M+(CHX), and *A. thaliana* plants. C, Chalcone synthase (CHS) enzyme activity in nonelicited cucumber plants inoculated with *P. xanthii* (I+M-), elicited inoculated cucumber plants infiltrated with cycloheximide I+M+(CHX), and elicited inoculated (I+M+) cucumber plants. Vertical bars in B and C represent the standard error from the means of three separate measurements.

Fig. 4. Light micrographs of cucumber leaves infected with *Podosphaera xanthii*. Tissue samples were harvested 7 days after treatment with inhibitors, and resin-embedded sections were stained with 0.2% toluidine blue O in 2% sodium borate. **A**, An inoculated nonelicited (I+M–) epidermal cell (E) containing a healthy multilobed fungal haustorium (H) (×400). **B to E**, Inoculated elicited (I+M+) leaves showing infected epidermal cells and the accumulation of amorphous material (AM) surrounding collapsed haustoria. Wall appositions were at times observed at fungal penetration sites (**B**, ×1,000; **C to E**, ×400). **F**, Leaf tissue sample from an piperonyl acid-infiltrated I+M+(PA) plant showing signs of phytotoxicity (×400). **G and H**, Fungal haustoria (H) within the epidermal cells of 3,4-methylenedioxy-infiltrated I+M+(MDCA) leaves (×400). **I and J**, Ovoid, multilobed healthy haustoria within the epidermal cells (E) of cycloheximide-infiltrated I+M+(CHX) cucumber leaf samples (×400). FM, fungal mycelium; and P, papilla.

lying induced resistance. The first defense reactions reported were associated with penetration resistance and were lignification of cell walls or formation of cell wall papilla (8,20,21). Later, putative roles were demonstrated for PR proteins like chitinases, peroxidases, and β -1,3-glucanases (11,22,29,36). Recently it was shown that powdery mildew-resistant cucumber plants also produced phytoalexins as a site-specific response to fungal penetration. Using fluorescence microscopy coupled with phytochemical analyses, McNally et al. (26) showed that rapidly synthesized C-glycosyl flavonoid phytoalexins accumulated exclusively within infected epidermal cells of disease-resistant plants.

Thus, plant pathologists are now faced with the challenge of evaluating the role of each response. Complicating this research are the obvious difficulties in untangling the contributions of defense responses, which naturally occur in synchrony within the tissues of diseased plants (12,16,21). In this study, elicited cucumber plants expressing induced resistance against *P. xanthii* were infiltrated with potential inhibitors of C4H, 4CL, and CHS to evaluate the role of flavonoid phytoalexin production. Microscope and biochemical investigations supported the emerging hypothesis that induced resistance in cucumber is correlated with rapid, de novo biosynthesis of flavonoid phytoalexin compounds.

The level of induced resistance observed for elicited cucumber plants inoculated with *P. xanthii* confirmed previous findings related to the prophylactic effects of Milsana (9,10,26). Light and electron microscope investigations of these plants corroborated the results obtained by Wurms et al. (41). For instance, collapsed, electron-dense fungal haustoria surrounded by an amorphous material were typical defense reactions observed for elicited plants during this study. A recent study examining phytoalexin production in cucumber demonstrated that an amorphous material sur-

rounding collapsed haustoria contained high levels of the C-glycosyl flavonoid phytoalexins cucumerin A and B, along with minor C-glycosyl flavonoids such as vitexin, isovitexin, orientin, and isoorientin (26). These results obtained for elicited plants differed markedly from nonelicited plants, whose leaves supported dense fungal colonies and epidermis contained healthy multilobed fungal haustoria. It is worth noting that papillae were at times observed as an initial response to fungal penetration. Generally, papillae relate to powdery mildew fungal penetration in two ways: in some instances, penetration fails when papillae are present and alternatively, penetration may succeed and the papilla becomes a collar for the haustorial neck (21,41). Using transmission electron microscopy, both outcomes were observed for elicited and non-elicited cucumber plants, strongly suggesting that papilla formation alone may not be sufficient to explain the level of induced resistance observed for elicited plants. Moreover, chitin labeling revealed that the walls and lobes of fungal haustoria within both treatments were undisturbed, suggesting that PR proteins, such as chitinases and β -1,3-glucanases, may not play a major role in the early events of induced resistance for cucumber.

Treatment of cucumber plants with PA, an inhibitor of C4H, was phytotoxic as evidenced by highly necrotic leaf tissue and disorganization at the cellular level. Therefore, in this study it was not possible to form accurate conclusions regarding the role of C4H in disease resistance for this pathosystem. Alternatively, infiltration with MDCA, an inhibitor of 4CL, had no toxic effect on leaves of I+M+(MDCA) plants. In this case, a moderate level of infection coincided with the expression of induced resistance, albeit in a somewhat altered state. Light microscope observations confirmed that fungal haustoria within the epidermis of I+M+(MDCA) plants were often collapsed and resembled those

Fig. 5. Electron micrographs of ultrathin sections of nonelicited, *Podosphaera xanthii*-inoculated (I+M-) cucumber leaves. **A**, A well-developed healthy fungal haustorium (H) ($\times 6,000$). **B**, Chitin localization showing specific deposition of gold particle labeling along the walls of the haustorial lobes (L) and the haustorial main body (HB) ($\times 30,000$). **E**, epidermal cell; **EHMa**, extra-haustorial matrix; **EHMe**, extra-haustorial membrane; **FM**, fungal mycelium; and **HW**, haustorial wall.

observed within I+M+ plants, but the surrounding material was different (Figs. 6B and 7A). Since MDCA interferes primarily with the biosynthesis of lignin (19), these results suggest a minor role for lignification as a defense response in induced resistance in cucumber. Examination of I+M+(MDCA) plants with electron microscopy showed that defense reactions often differed from

those observed in I+M+ plants and from other studies on Milsana-elicited defense reactions in cucumber (41). For instance, collapsed fungal haustoria within MDCA-infiltrated plants frequently were elongated and surrounded by granular material resembling callose. A possible explanation is offered by the fact that some 4CL-derived phenylpropanoids are used in the biosyn-

Fig. 6. Electron micrographs of ultrathin sections of elicited cucumber leaves inoculated with *Podosphaera xanthii* (I+M+). **A**, An infected epidermal cell (E) showing the site of fungal penetration through an existing papilla (P). The haustorium is showing signs of collapse and is connected to the fungal mycelium (FM) which contains dense, aggregated cytoplasm ($\times 8,000$). **B**, A collapsed haustorium (H) surrounded by amorphous material (AM) ($\times 10,000$). **C**, Chitin labeling of a collapsed haustorium (H) within an infected epidermal cell (E) ($\times 16,000$). HB, haustorial main body; and L, haustorial lobe.

thesis of flavonoid compounds (Fig. 1). For example, the B-rings of most flavonoids, including those of the flavonoid phytoalexins in cucumber, are synthesized directly from the C6-C3 phenylpropanoid metabolites in plants (12,27). It is conceivable that disruption of 4CL in I+M+(MDCA) plants reduced the concentration of the phenylpropanoid metabolite pool and therefore caused a reduction in flavonoid phytoalexin biosynthesis. This may explain the difference in defense reactions often observed for I+M+(MDCA) plants and the higher level of infection on the leaves of these plants.

Infiltration with CHX resulted in a nearly complete loss of induced resistance in elicited plants. The leaves of I+M+(CHX) plants supported dense fungal colonies paralleling disease severity observed for I+M- plants, and light microscope observations confirmed the development of healthy, multilobed fungal haustoria

within the epidermal cells of these plants. A closer examination with transmission electron microscopy showed that the internal structures of fungal haustoria within I+M+(CHX) plants were clearly discernible and were free of the amorphous material observed in I+M+ plants. Biochemical analyses of I+M+(CHX) plants revealed a clear correlation between CHS enzyme activity and induced resistance in cucumber. For instance, CHS activity was significantly lower in susceptible CHX-infiltrated plants compared with that in disease-resistant I+M+ plants. In fact, similar levels of CHS activity were observed for I+M+(CHX) and nonelicited plants, reasonably suggesting that a comparable level of flavonoids may be produced in these two treatments. These results support the findings of a recent study that showed the leaves of cucumber plants elicited with Milsana containing 29 times more flavonoid phytoalexins compared with that of nonelicited

Fig. 7. Electron micrographs of ultrathin sections of elicited cucumber leaves inoculated with *Podosphaera xanthii* and infiltrated with 3,4-methylenedioxy cinnamic acid I+M+(MDCA) or cycloheximide I+M+(CHX). **A**, I+M+(MDCA) leaf with a poorly developed and collapsed haustorium (H) encased in a granular matrix (GM) ($\times 8,000$). **B**, An I+M+(CHX) leaf showing a healthy multilobed fungal haustorium (H) ($\times 10,000$). **C and D**, Chitin localization within I+M+(CHX) leaves showing specific deposition of gold particle labeling within the walls of the haustorial lobes (L) and main haustorial bodies (HB) (**C**, $\times 10,000$; **D**, $\times 24,000$).

plants (26). Of particular importance, phytotoxic reactions were never observed for CHX-infiltrated leaves and levels of β -glucosidase activity and 4CL synthesis resembled uninhibited plants, suggesting that CHX-induced alteration of nontargeted function may be minimal. Nevertheless, there is a possibility that CHX, as an inhibitor of translation, can interfere with the expression of other unmeasured proteins involved in resistance or of transcription factors that regulate CHS protein synthesis and, hence, down regulate this gene product. In conclusion, although direct causality cannot be established in absence of a specific inhibitor, this study presents for the first time strong evidence that induced resistance in cucumber can be suppressed through down regulation of CHS and disruption of the flavonoid pathway. This finding strongly suggests that flavonoid phytoalexins play a key role in determining the outcome of plant-powdery mildew interactions for this species. In contrast, other defense reactions reported to occur in cucumber such as lignification, papilla formation, and certain PR proteins may be relatively minor components contributing to induced disease resistance. Overall, the results of this study support the hypothesis that induced resistance in cucumber is largely correlated to de novo synthesis of flavonoid phytoalexin compounds.

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