Novel Extracellular Chitinases Rapidly and Specifically Induced by General Bacterial Elicitors and Suppressed by Virulent Bacteria as a Marker of Early Basal Resistance in Tobacco

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Early basal resistance (EBR, formerly known as early induced resistance) is triggered by general bacterial elicitors. EBR has been suggested to inhibit or retard expression of the type III secretion system of pathogenic bacteria and may also prevent nonpathogenic bacteria from colonizing the plant tissue. The quickness of EBR here plays a crucial role, compensating for a low bactericidal efficacy. This inhibitory activity should take place in the cell wall, as bacteria do not enter living plant cells. We found several soluble proteins in the intercellular fluid of tobacco leaf parenchyma that coincided with EBR under different environmental (light and temperature) conditions known to affect EBR. The two most prominent proteins proved to be novel chitinases (EC 3.2.1.14) that were transcriptionally induced before and during EBR development. Their expression in the apoplast was fast and not stress-regulated as opposed to many pathogenesis-related proteins. Nonpathogenic, saprophytic, and avirulent bacteria all induced EBR and the chitinases. Studies using these chitinases as EBR markers revealed that the virulent *Pseudomonas syringae* pv. *tabaci*, being sensitive to EBR, must suppress it while suppressing the chitinases. EBR, the chitinases, as well as their suppression are quantitatively related, implying a delicate balance determining the outcome of an infection.

Additional keywords: avoidance, innate immunity, PAMP, tolerance, virulence.

Recognition of the broadest possible range of foreign invading organisms and setting up protection measures to anticipate their potentially harmful activity is a prerequisite for successful survival of plants and animals in natural habitats. These basic protective functions are performed by innate immunity mechanisms that share conserved molecular structures in both kingdoms. Accordingly, there are conserved, recognizable molecular fingerprints of pathogens (pathogen associated molecu-

lar patterns (PAMPs) or general elicitors) that serve as its elicitors (Nurnberger and Brunner 2002; Schulze-Lefert 2004). Nonpathogens also bear PAMPs, as most known PAMPs are essential components of a bacterial or fungal cell. Thus, probably every microbe bears several PAMPs (Zipfel et al. 2004) whose molecular characterization is crucial to dissect the complex and diverse processes of disease and resistance. A few earlier studies of induced resistance in plants used whole bacterial cells devoid of pathogenic activity (e.g., saprophytes, killed cells, or pathogenicity mutants of pathogens, i.e., collections of PAMPs) or purified cell components as elicitors. There was no pathogenesis or hypersensitive reaction (HR) that would complicate plant responses. Thus, these phenomena represent instances of an ancient form of plant innate immunity, basal resistance, which also has molecular similarities to plant host-specific pathogen recognition systems (Jones and Takemoto 2004). For example, there were bacterial treatments that interfered with the HR or the normosensitive reaction induced by a subsequent challenge bacterium. These results (Burgýán and Klement 1979; Lovrekovich and Farkas 1965; Lozano and Sequeira 1970) indicated broad induction specificity with an indication of conserved elicitors. Comparable results were obtained with putative bacterial glycoproteins (Sequeira et al. 1972), the lipid A part (Graham et al. 1977), and other domains of lipopolysaccharides (Kecskés et al. 1999; Newman et al. 1997), lipopolysaccharide-protein complexes (Mazzucchi et al. 1979), and bacterial oligosaccharides (Stefani et al. 1994). These and possibly other surface-associated PAMPs like flagellin (Asai et al. 2002) may provide the first opportunity for the plant to mount a sufficiently quick and broadly effective defense against any intruder, not only within a pre-treatment/challenge artificial inoculation system but also where the naturally infecting bacterial cell itself spurs protective response against itself. We are aware of only one such rapid response described, the early basal resistance (EBR), formerly known as early induced resistance. In tobacco, EBR and late basal resistance (LBR; previous name, late induced resistance) constitute a system that is part of the plant's innate immunity. Several types of bacteria induce protection against the HR-associated death by a subsequent avirulent pathogen or against the normosensitive (susceptible) reaction by a virulent pathogen. The inducer bacteria include live and killed cells of saprophytes and avirulent pathogens, *hrp* (hypersensitive reaction and patho-

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genicity) mutants of avirulent pathogens and killed cells of the virulent *Pseudomonas syringae* pv. *tabaci*. The protection develops within 2 to 6 hpi (hours postinoculation) against HR (Burguán and Klement 1979) and after about two days against the normosensitive reaction (Lovrekovich and Farkas 1965). The rapid and slower responses are manifestations of EBR and LBR, respectively. Probably because of the presence of one or more conserved and constant bacterial elicitors, EBR is triggered even during an incompatible interaction independently of pretreatment (Burguán and Klement 1979; Klement et al. 1999, 2003b). The protection against hypersensitive death (by EBR) occurs at a very early stage of the incompatible interaction, i.e., during the 0.5- to 3-h span of the HR induction period, which is the time needed for bacteria to express and operate their type III secretion system. EBR can be so rapid that the relatively 'slow' *P. syringae* pv. *phaseolicola* (induction period of HR about 3 h) often fails to induce the HR in the absence of pretreatment (Klement et al. 1999). This implies that the bacterium becomes unable to translocate its plant-activated effector proteins into the plant cytoplasm. In agreement with this, blocking EBR with hot-water heat treatment superinduced the expression of most *hrp* genes of *P. syringae* pv. *syringae* in planta (Bozsó et al. 1999). Although EBR was found not to be effective against the virulent pathogen *P. syringae* pv. *tabaci* (Burguán and Klement 1979), in leaves where EBR is inhibited by heat treatment or by pretreatment with *P. syringae* pv. *tabaci*, *hrp* mutant cells of *P. syringae* pv. *syringae* do not suffer the otherwise normal 90% drop in their number (Bozsó et al. 1999; Klement et al. 2003b). The mechanism behind the EBR effect is largely unknown. Direct inhibition of challenge bacteria by the inducer bacteria can be excluded in cases where killed bacteria—often of the same strain as the challenge—are used as EBR elicitors. Also, several types of live bacteria, irrespective of their known antibiotic behavior, are successful EBR elicitors. Given the huge surface area of plant cells, spatial exclusion is also improbable. EBR does not prevent cell death provoked by harpin (Klement et al. 1999) or *P. syringae* pv. *tabaci* (Burguán and Klement 1979). EBR develops sooner at a higher temperature (e.g., 30 vs. 20°C) and in younger leaves. This observation along with the time requirement and sensitivity of EBR to cycloheximide and heat treatment suggests that induced active plant participation is necessary for EBR (Klement et al. 1999, 2003b). All these comply with criteria of induced resistance proposed by Steiner and Schönbeck (1995). EBR may thus affect a broad range of pathogens and nonpathogens, presumably employing extracellular factors such as proteins with rapid expression kinetics, in order to provide an expeditious basic protection mechanism. Our objective was to identify tobacco extracellular proteins that could participate in EBR. A few such proteins were identified as novel chitinases. Their correlation with EBR highlights the specificity and complexity of this immune response.

RESULTS

EBR induced by one or more general bacterial elicitors correlates with two intercellular washing fluid proteins.

For preparing the standard bacterial inocula for tobacco (crude preparation of PAMPs), cells of nonhost pathogens *P. syringae* pv. *phaseolicola* or *P. syringae* pv. *syringae* were heat-killed (70°C, 12 min), in order to preserve the potential bacterial elicitors while excluding plant-activated bacterial effector activities. In addition, we wanted to ensure that only the plant will be able to produce new proteins. A simplified small-scale method to quickly obtain intercellular washing fluid (IWF) yielded good quality samples; the centrifugal force applied was strong enough to hold the leaf strips in place while

mild enough not to cause mechanical damage to the tissue. Using a native 1-D electrophoresis technique with silver staining, it was found that only proteins below approximately 30 kDa distinguished EBR-inducing treatments from the uninoculated (0 hpi) or water-treated control (Fig. 1C). Protein bands of more than 30 kDa were not clearly distinguishable by silver staining because of a high background. Attempts to reduce this background by binding phenolics with PVPP (polyvinyl polypyrrolidone) were not fully satisfactory. Moreover, PVPP partially removed some proteins, which resulted in weaker bands. Six to eight protein bands from the region of tobacco leaf mesophyll cell walls could be reproducibly detected 2 to 12 h after heat-killed *P. syringae* pv. *phaseolicola* treatment. Two of these proteins, designated as '215' and '250,' appeared induced. This period comprises the development (2 to 5 hpi, depending on the ambient temperature) and maximal expression (approximately 12 to 20 hpi) of EBR, with heat-killed *P. syringae* pv. *phaseolicola* as an inducer (Fig. 1A; Klement et al. 2003b). In particular, at 20°C, the two new IWF proteins appeared later (between 2.5 and 6 hpi) but persisted longer than at 30°C. Accordingly, at 30°C EBR levels had a shorter but more intense course than at 20°C. Darkness and old age attenuated the level of the two (and other) proteins. These two effects were additive (Fig. 1A). It has been shown that old leaves develop only weak EBR and that light has an important EBR-maintaining role, especially in older leaves (Klement et al. 2003b). We observed that EBR persists for about one week in the dark, albeit its strength diminished after 1 to 2 days (Fig. 1A). The two bands disappeared from leaf samples during this week whether in light or not (Fig. 1A). Several additional controls were included to warrant proper selection of EBR-related IWF proteins. No more additional protein bands were detected when IWF was concentrated about 10 times with ultrafiltration (not shown). Thus, silver-staining was sensitive enough to detect all proteins in IWF that is dilute with respect to proteins (approximately 5 to 20 µg ml⁻¹), while other staining methods (Coomassie brilliant blue, fluorescent staining) usually fell short of detecting the less abundant proteins, e.g., 215 and 250. There was a negligible change in the protein pattern upon using protease inhibitor cocktails. The quantity of two very small proteins decreased, indicating activity of extracellular proteases. No changes were observed for any of the induced proteins (not shown). There were no proteins in the bacterial inocula with a migration distance similar to the two induced bands (Fig. 1C). When IWF from noninoculated leaves was mixed with heat-killed *P. syringae* pv. *phaseolicola* inoculum in a test tube or when IWF was taken out of heat-killed *P. syringae* pv. *phaseolicola*-inoculated leaves before expression of the induced proteins and was incubated in vitro at 24°C, none of these protein bands were detected as compared with the starting material (Fig. 1C). This indicates that induction of the new proteins by heat-killed *P. syringae* pv. *phaseolicola* requires an active plant response. Appearance of the two new bands was not due to the cutting of the leaves (not shown). Because of general differences between individual gels, the absolute band densities varied considerably. The percentages of the individual proteins within total band densities in gels were much more consistent. Figure 1B shows the ratio of the two proteins in the protein mixture after combinations of bacterial and heat treatments. A short but intense heat treatment (51°C, 15 s) just before inoculation with heat-killed *P. syringae* pv. *phaseolicola* delayed the EBR effect by 12 to 16 h, so EBR recovered by 24 hpi (not shown). Also, the two bands did not appear or appeared only very weakly during this time. However, the proteins appeared by 24 hpi (Fig. 1B). This reinforces that EBR inhibition by heat treatment is the result of blocking either the protein synthesis, transport, or both, as such processes were reported to be

Fig. 1. Correlation of two IWF (intercellular washing fluid) proteins (asterisks) and early basal resistance (EBR), induced by heat-killed bacteria. Experiments were carried out at least three times with similar results. **A**, Timecourse of EBR and IWF protein patterns in leaves treated with heat-killed *Pseudomonas syringae* pv. *syringae*. The bar chart shows intensity of EBR in midposition leaves. A standardized scale from 1 (approximately 50% hypersensitive reaction [HR]) to 3 (0% or HR fully inhibited) was used. Average values of nine experiments are shown. The gel below the chart shows early timepoints with lanes corresponding to the chart columns. The bottom gel shows the influence of leaf age and illumination on the protein patterns on a longer time scale. Numbers on the left of the gels are proportional to the relative mobility of the proteins and are used as reference for the respective bands. Only the lower half of the gels are shown, with proteins below approximately 30 kDa. **B**, Band densities of two candidate EBR-related proteins. Two proteins ('215' and '250') are shown that coincided with EBR (A and C) and proved consistent through several plant sets. HT = heat-treatment of leaves; K-Pph = infiltrated with heat-killed *P. syringae* pv. *phaseolicola*; + = treatments together; numbers after treatments indicate hours after inoculation (hpi); * = except basic proteins. Error bars = standard error of the mean, $n=3$. **C**, Protein pattern of control treatments. Uninoculated = variations of IWF samples from untreated leaves; K-Pph = heat-killed *P. syringae* pv. *phaseolicola* inoculum; *in vitro* = not in planta study; numbers after treatments indicate hpi; K-Pph + IWF = bacterial cells were mixed with IWF from untreated leaves in a test tube and were incubated at 22°C for the time indicated. Only bands smaller than 30 kDa are shown. The superscript a indicates two times more sample was loaded. Below the lanes, (+) or (−) signs indicate whether EBR was operative or not, respectively, as evidenced by the HR test; −(+) indicates weak and less durable EBR in a minority of cases, and n.d. indicates not determined.

blocked by severe heat shock (Welch and Suhan 1985; Yost and Lindquist 1986). The identical delay in the EBR effect and in the proteins' appearance further strengthens their relationship.

Fig. 2. IWF (intercellular washing fluid) protein patterns from leaves inoculated with live bacteria. Only bands smaller than 30 kDa are shown from the gels. Experiments were carried out at least three times with similar results. Below the lanes, (+) or (-) signs indicate whether EBR (early basal resistance) was operative or not, respectively, as evidenced by the hypersensitive reaction test. K- = heat-killed (included for reference); numbers after treatments indicate hours after inoculation. **A**, IWF protein pattern of leaves treated with 5×10^8 CFU of nonvirulent bacteria per milliliter. Pph = *Pseudomonas syringae* pv. *phaseolicola*; E. coli = *Escherichia coli*; P. flu = *P. fluorescens*; Pss hrpJ = hrpJ mutant of *P. syringae* pv. *syringae*; Pcor = *P. syringae* pv. *coriandricola*. **B**, Growth of *P. syringae* pv. *tabaci* in tobacco leaves, starting from an inoculum density of 10^5 CFU ml⁻¹. Left chart, effect of heat-treatment (HT); right chart, effect of 6 h pretreatment with 5×10^8 cells ml⁻¹ heat-killed *P. syringae* pv. *phaseolicola* (EBR). Error bars indicate 2 $\times$ standard error of the mean. In the left chart, only the lower error bars are shown for the HT and the upper ones for the control. **C**, IWF protein patterns of tobacco leaves of different ages after infiltration with *P. syringae* pv. *tabaci* (Pta, 2×10^5 CFU per milliliter unless stated otherwise); old = older leaf; young = younger leaf; 10⁹ = high-density (10^9 CFU ml⁻¹) inoculum; Pta+K-Pph = *P. syringae* pv. *tabaci* infection for 24 h, challenged with heat-killed *P. syringae* pv. *phaseolicola* (5×10^8 cells per milliliter) and sampled at 6 h thereafter.

Pathogenic bacteria are sensitive to EBR, but only the virulent *P. syringae* pv. *tabaci* suppresses it and the expression of the two proteins in the cell wall.

The next question was how specific was the induction of the two proteins by living bacteria that represent different types of interactions with tobacco. Non-plant pathogenic bacteria (*Escherichia coli*), the saprophytic or hrp mutants (hrpJ is shown here) of *P. syringae* pv. *syringae*, triggered the appearance of the 215 and 250 bands within 6 hpi. Treatment with live avirulent bacteria (*P. syringae* pv. *phaseolicola*, *P. syringae* pv. *syringae*, *P. syringae* pv. *coriandricola*) also resulted in the appearance of the representative bands, and the HR also occurred here later.

Apart from some small (<5 kDa; not shown) polypeptides, the polyacrylamide gel electrophoresis (PAGE) separation pattern was similar to that induced by heat-killed *P. syringae* pv. *phaseolicola* (Fig. 2A). The above experiments with nonvirulent bacteria indicate that two relatively small proteins could be part of the EBR.

As for the compatible interaction, the most important type, we found that leaf tissue hosting a rapidly multiplying high *P. syringae* pv. *tabaci* population did not express or only weakly expressed the two proteins (Fig. 2C). In the late samples (from 30 hpi on), bacterial number reached or exceeded the amount used for routine EBR induction (5×10^5 to 5×10^7 cells cm⁻² of leaf area) (Fig. 2B, control growth curves). On the other hand, heat-killed *P. syringae* pv. *tabaci* induced the 215 and 250 proteins (Fig. 2C). To deactivate *P. syringae* pv. *tabaci* cells and otherwise preserve their elicitor quality, we tried gamma radiation on cells frozen in liquid nitrogen. A 3.2-kGy dose sufficiently killed the bacteria but did not produce any differences between elicitor activities of the heat-killed and gamma ray-killed cells. Live *P. syringae* pv. *tabaci* moderately induced the proteins if inoculated at an unnaturally high (10^9 CFU ml⁻¹) cell density at 6 hpi (Fig. 2B).

The above results with *P. syringae* pv. *tabaci* show that, in spite of bearing the elicitors of the proteins 215 and '250,' the virulent bacterium has the ability to keep their expression at a low level. This suggested that EBR may have significance during infection that has been underestimated so far. Therefore, we examined possible pathogenic strategies of *P. syringae* pv. *tabaci*, such as avoidance, tolerance, and suppression, with respect to the EBR, to assess its significance in the pathogenesis. The fact that this bacterium multiplies rapidly and causes necrotic symptoms fits to all or any one of the three strategies. Killed *P. syringae* pv. *tabaci* induced EBR, such as cells of several other bacteria (Burguán and Klement 1979; Table 1, sequence C). EBR (induced by heat-killed *P. syringae* pv. *pisi*) did not inhibit the normosensitive necrosis by a high dose of *P. syringae* pv. *tabaci* (above 10^6 CFU ml⁻¹; Table 1, sequence D) but greatly retarded infection by a lower-dose inoculum (10^5 CFU ml⁻¹; Table 1, sequence E). This delay lasted up to 2 weeks, whereas plants treated with only *P. syringae* pv. *tabaci* developed necrotic symptoms after 4 to 5 days. The delay was also reflected by a lower multiplication rate (Fig. 2B, growth curve EBR). When a tissue pretreated with rifampin-sensitive *P. syringae* pv. *tabaci* is challenged with a rifampin-resistant avirulent bacterium, e.g., *P. syringae* pv. *pisi* along with rifampin, HR appears in the outer zone, which has been interpreted as lack of EBR in the tissue infected with *P. syringae* pv. *tabaci* (Klement et al. 2003b, Table 1, sequence F). When this series of treatments was done after an EBR-inducing treatment, there was no HR (Table 1, sequence I). When heat-killed *P. syringae* pv. *tabaci* (5×10^8 cells ml⁻¹) was mixed with a lower number of live *P. syringae* pv. *tabaci* cells (2×10^5 cells ml⁻¹ or less), it also greatly delayed, if not prevented, the compatible symptoms (Table 1, sequence A). Lastly, pretreatment

with only active *P. syringae* pv. *tabaci* inhibited EBR (Table 1, sequences G and H) as assessed by the HR test (Table 1, sequence B), and again, this activity of the virulent bacterium could be disturbed by pretreatment with heat-killed bacterial cells (Table 1, sequence J).

Taken together, elicitor activity of a cell constituent can delay or impair disease by overwhelming another activity of live *P. syringae* pv. *tabaci* cells that, under normal progression of disease, actually suppresses EBR and the expression and secretion of 215 and 250 proteins.

Abiotic stresses or defense signaling compounds (salicylic acid, jasmonic acid, and ethylene) are poor inducers of proteins 215 and 250^a.

Salt stress (0.2 M NaCl), osmotic stress (0.4 M mannitol) induced to a low level (Fig. 3A), 300 mM H₂O₂, and 20 μM paraquat, a generator of intracellular superoxide, did not induce appearance of the two proteins in the IWF (Fig. 3B). Notably, in some instances, even water proved a weak elicitor (Fig. 1C), approximately at the level of NaCl or mannitol (not shown). In these cases, water also resulted in an EBR-like effect, but this kind of protection against HR induction lasted only for a few (below 20) hours. At 0.5 M, NaCl caused necrotic symptoms to a greater extent in older leaves than in younger ones. Mannitol treatment was symptomless. Leaf infiltration with 300 mM H₂O₂ did not cause necrotic symptoms. Younger leaves tolerated even 1 M H₂O₂. At higher concentrations, yellowish discoloration and spotty death occurred, especially close to the injection point. In older leaves, 40 to 50 μM paraquat caused necrotic spots at the injection point that could spread beyond the initial infiltration area, mainly along major leaf veins.

Salicylic acid (SA), jasmonic acid (JA), and ethylene pathways are often involved in stress- and defense responses to pathogens, often along with the accumulation of these compounds. Ethylene (1-aminocyclopropane-1-carboxylic acid at 1 mM), JA (100 μM), or SA (0.6 mM) did not induce or only poorly induced the expression of the two proteins or EBR at 6 hpi or later (Fig. 3C). However, we observed protection from

HR by JA between 24 and 96 hpi that was pronounced only in older leaves. Here, JA treatment acted on the death process by slowing it considerably. SA treatment resulted in only a short period (approximately 4 to 10 hpi) of an EBR-like effect. Moreover, EBR developed in NahG plants that do not accumulate SA (data not shown).

In summary, based on their coincidence with EBR under a variety of conditions, at least two proteins of less than 30 kDa proved to be good candidates for EBR-related proteins. The corresponding proteins, detectable only with silver-staining in the unconcentrated IWF, were shown to correlate with EBR development qualitatively and semiquantitatively.

The two proteins are novel chitinases (EC 3.2.1.14) with early transcriptional control.

Tryptic digests of the two PAGE-purified proteins were analyzed unfractionated by matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS). Each digest yielded more than 20 peptides, approximately 80% of which were present in both samples (data not shown). Thus, we concluded that the proteins were related. Database search with the singly charged protonated peptide masses (MH⁺) detected in the tryptic digest did not identify any protein. Then PSD (post source decay) experiments were performed on the six most abundant components present: MH⁺ at m/z 779.43, 1,588.76, 1,748.84, 1,899.96, 2,464.2, and 2,649.2 (data not shown). Two PSD spectra, those of m/z 779.43 and 1,588.76 were manually interpreted and yielded amino acid sequences DAVI/LSFK and PDI/LVAR, respectively. (Isomeric ile and leu residues cannot be differentiated by PSD analysis.) These sequences showed similarity (MS pattern search with Protein Prospector software; Clauser et al. 1999) to a 26-kDa chitinase isolated from Jack bean (National Center for Biotechnology Information [NCBI] number 10120702), corresponding to positions 144 to 150 and 138 to 143, respectively. Interestingly, a MS-TAG database search (Protein Prospector software; Clauser et al. 1999) with the PSD data of m/z 1,748.84 'identified' a tryptic peptide, GPLQISWNFNYGPAGK, from a 25-kDa rice chitinase (NCBI number 7435372). The identity of the under-

Table 1. Phenotypic outcomes of bacterial treatments with mixed or sequential inocula

Sequence	First inoculation ^a	Time gap ^b	Challenge inoculation(s) ^c	HR (NN) ^d	Notes ^e
A	Heat-killed cells (5 × 10 ⁸) mixed with live <i>P. syringae</i> pv. <i>tabaci</i> (2 × 10 ⁵ or less)			(-); (±)	Tissue death is delayed or even prevented
B	Heat-killed avirulent 5 × 10 ⁷	7 to 18	Avirulent	-	Establishment of EBR, test for EBR
C	Heat-killed <i>P. syringae</i> pv. <i>tabaci</i> 2 × 10 ⁸	6	Avirulent	-	Cellular constituent(s) of <i>P. syringae</i> pv. <i>tabaci</i> induce the EBR
D	Heat-killed avirulent 5 × 10 ⁸	7	<i>P. syringae</i> pv. <i>tabaci</i> 10 ⁷ to 10 ⁹	(+)	EBR does not inhibit NN
E	Heat-killed avirulent 5 × 10 ⁸	7	<i>P. syringae</i> pv. <i>tabaci</i> 5 × 10 ⁵ and below	(-)	EBR delays pathogenesis by <i>P. syringae</i> pv. <i>tabaci</i>
F	<i>P. syringae</i> pv. <i>tabaci</i> 5 × 10 ⁷	7	Avirulent	+	Live <i>P. syringae</i> pv. <i>tabaci</i> pretreatment does not result in EBR
G	<i>P. syringae</i> pv. <i>tabaci</i> 10 ⁶	24	'B'	+	<i>P. syringae</i> pv. <i>tabaci</i> can inhibit development of the EBR
H	Heat-killed <i>P. syringae</i> pv. <i>tabaci</i> 10 ⁶	24	'B'	-	Result of 'G' is dependent on metabolic activity of <i>P. syringae</i> pv. <i>tabaci</i>
I	Heat-killed avirulent 5 × 10 ⁸	16	'F'	-	<i>P. syringae</i> pv. <i>tabaci</i> infection does not abolish established EBR
J	Heat-killed avirulent 5 × 10 ⁸	6	'G'	-	EBR inhibits EBR-inhibitory activity of <i>P. syringae</i> pv. <i>tabaci</i>

^a Heat-killed bacteria, avirulent (*Pseudomonas syringae* pv. *pisi*, *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *syringae*), and virulent (*P. syringae* pv. *tabaci*) bacteria themselves cause symptomless responses, hypersensitive reaction (HR), and normosensitive necrosis (NN), respectively. Numbers indicate cell density, cells ml⁻¹.

^b Hours between the first and the following inoculation (or inoculation series).

^c Single capital letters in quotes indicate inoculation sequences identified in the first column.

^d + = tissue death; - = no tissue death; ± = variable with high challenge inoculum. NN is shown in parentheses. Distinction between HR and NN was possible in 'F' and 'G' using parallel controls, checking times for death development and symptoms.

^e EBR = early basal resistance.

lined amino acids was confirmed by N-terminal and C-terminal fragment ions (data not shown). These data suggest the presence of a novel chitinase in tobacco plants.

Using in-gel chitinase activity analysis, the earliest and the most consistent activities could be coupled to the two above-characterized proteins, 215 and 250 (Fig. 4). Importantly, the electrophoretic band densities of the silver-stained chitinases were proportional to their in-gel enzymatic activities. An additional chitinase activity was also obvious for a larger protein (Fig. 4, at or close to band 128) that is also inducible by heat-killed bacteria. This chitinase activity was also suppressed by *P. syringae* pv. *tabaci* infection (Fig. 4). There is a constitutive chitinase activity as well, provided by an even larger protein. This activity was less subject to changes in the course of treatments and was the only one found in uninoculated leaves. The

size of chitinases 215 and 250 is estimated at approximately 15 kDa, as judged from ultrafiltration studies and running size standards (not shown).

To determine whether the chitinases are subject to transcriptional control related to EBR, cDNA samples were prepared from tobacco leaves treated with a *P. syringae* pv. *syringae* hrp mutant (*hrcC* mutant, a known inducer of EBR) in a time-course of 1 to 48 hpi. Redundant gene-specific PCR primer was designed on the conserved region of the chitinase peptides, with the help of known tobacco and *Arabidopsis* chitinase sequences. Polymerase chain reaction (PCR) yielded four visible products, three of which were cloned. Nucleotide sequences were obtained and interpreted using the BLAST program (Altschul et. al. 1990).

Two of the obtained sequences were novel, with relatively low similarities to any known gene. Highest identities were to CHTO3D9, a *Sambucus nigra* chitinase class II (PR-3), GenBank accession number Z46950, 73%, and CHTO3E10, *Oryza sativa*, GenBank accession number XM_483800, 75%. The third sequence, CHTO1A2, corresponded (99% identity) to a known gene, the *N. tabacum* endochitinase, GenBank accession number X16939.

MS and PSD analysis of the tryptic digests of gel bands 215 and 250 suggest that both may be the products of gene CHTO3D9 (Fig. 5A). The two protein digests were almost identical (80%), and the amino acid sequence deduced from PCR product CHTO3D9 matched components identified from both digests. Since the sequences underlined seem to be identical in both proteins, we assume that the missing N-terminal region must be different, possibly due to heterogeneity in the still unknown N-terminal part, posttranslational modifications, or both. Inverse PCR deciphering the N-terminal sequence is underway.

Expression levels of the corresponding genes were assessed by real time reverse transcription (RT)-PCR in a timecourse, using specific primers for the above three sequences. Expression patterns obtained this way corresponded well to the original 3'Race PCR (not shown) and the electrophoretic data as well. CHTO3D9 was strongly and selectively induced in bacterially treated cDNA samples, such as chitinases 215 and 250. Gene activity was already high at 1 hpi and declined after 12 hpi (Fig. 5B). CHTO1A2 was constitutive. CHTO3E10 was strongly induced in water- and bacteria-treated samples at 1 hpi and was already low at 3 hpi (data not shown).

Thus, CHTO3D9 corresponds to either chitinase '215,' '250,' or both, the extracellular accumulation of which may be transcriptionally regulated.

DISCUSSION

Our objective was to find EBR-related proteins in the IWF, in which plant effector proteins are likely to be found with roles in a putative initial recognizing function, the final bacteriostatic function, or both. Selection criteria were early induction and a good correlation between proteins and EBR under experimental conditions known to arouse, stimulate, or inhibit this form of innate immunity. The two most relevant proteins proved to be novel soluble extracellular chitinases, a fact that implies several possibilities for their function in a broadly effective resistance response. EBR, possibly with the related chitinases, is a factor to combat with for any bacterium that contacts the leaf parenchyma, including the most specialized virulent pathogen.

Specificity of EBR-related chitinase induction.

Having used a variety of bacterial elicitors, controls, environmental conditions and stresses, as well as leaves of different ages, we conclude that these chitinases are specific for the

Fig. 3. Stress-related expression of IWF (intercellular washing fluid) proteins. Only bands smaller than 30 kDa are shown. Below the lanes, (+) or (-) signs indicate whether EBR (early basal resistance) was operative or not, respectively, as evidenced by the hypersensitive reaction test. Numbers after treatments indicate hours after inoculation (hpi). **A.** Salt and osmotic stresses. NaCl and mannitol (man.) were used at 0.1 M. **B.** Oxidative stresses. H_2O_2 was used at 0.3 M, paraquat (Pq) at 20 μ M. **C.** Stress- and defense-related plant compounds. 1-Aminocyclopropane-1-carboxylic acid (ACC) and jasmonic acid (JA) were used at 1 mM and salicylic acid (SA) at 0.6 mM in older and younger leaves at 19 hpi. Experiments were carried out at least three times with similar results.

EBR machinery. First, several bacterial cell types not having a functional type III secretion system (saprophytic bacteria, heat-killed avirulent, virulent, or live *hrp* mutant bacteria) known to trigger EBR also induced the chitinases before or within the EBR time frame. There is probably a common, general elicitor of EBR as well as these proteins, most likely on the bacterial cell surface, as the surface is exposed towards the plant cell and killed cells also are active elicitors. Experiments are now conducted to test known PAMPs such as flagellin (Felix et al. 1999), cold shock protein (Felix and Boller 2003), and lipopolysaccharide preparations (Kecskes et al. 1999). The broad induction specificity suggests effectivity against a broad range of bacteria. EBR might lead to an answer to the old and neglected question why saprophytes cannot grow in living tissue in spite of having all the necessary nutrients (Klement et al. 1964). Second, conditions and treatments manipulating EBR similarly affected the presence of the chitinases. These conditions include inhibition by (Visnyovszky et al. 1983) and recovery from heat treatment, acceleration at higher (30°C) as compared with lower (20°C) temperatures, and persistence and reinforcement by light and juvenility (Klement et al. 1999, 2003b). Moreover, quantity of the chitinases seemed to be proportionally related to the strength of EBR, when the latter was not artificially blocked by heat treatment or provoked by challenge. Nevertheless, as EBR was never observed in the absence of these proteins, they might be components of the EBR machinery. Third, abiotic stresses such as salt and osmotic stresses did not trigger, or only very poorly triggered, appearance of neither the chitinases nor the EBR itself. Chitinases and EBR coincided also at these low levels, as exemplified in some water-treated control samples (Fig. 1C). The broad-range bacterial induction suggested a possible incorporation of abiotic stress responses into the EBR signal pathway. This possibility now seems unlikely. Many pathogenesis-related proteins are induced by abiotic stresses and rather late (Ohashi and Ohshima 1992). In contrast, the EBR-related chitinases seem to discriminate between abiotic and biotic stress stimuli. Biotic and abiotic stresses are generally associated with elevated production of reactive oxygen species extracellularly and intracellularly, respectively (Mittler 2002). We found relatively low and transient H₂O₂ accumulation during the development of EBR as compared with the HR (Klement et al. 2003b). However, neither H₂O₂ nor superoxide induced the chitinases or EBR, indicating that reactive oxygen species elevation is rather a

consequence or, at least, not the single cause of EBR development. Fourth, application of defense-related plant compounds, such as ethylene or SA, did not result in an EBR-like effect or resulted in a very short-living one. The chitinases were expressed accordingly. Independence of EBR from SA is apparent from the fact that EBR was observed in NahG plants. Longer SA pretreatment seemed even to potentiate the HR in the leaves, a fact that was previously known (Alvarez 2000). JA treatment, such as heat-killed bacterium pretreatment, delayed HR by a challenge bacterium, but JA is an unlikely substitute for heat-killed bacteria because EBR (induced by heat-killed bacteria) inhibits rather than slows down the HR and takes effect significantly earlier than the protection caused by JA. This indicates that JA acts on the plant-dictated part of the HR, i.e., the latent or death period. JA, antagonizing SA, was shown to abolish cell death induced by O₃ (Rao et al. 2000) or by harpin (Andi et al. 2001). Also, EBR is more functional in younger leaves than in older ones, as opposed to JA. Further studies are necessary to decide whether the ethylene or JA pathways, which often interact with each other and with the SA pathway (Kunkel and Brooks 2002), are required for EBR.

EBR-related chitinases and EBR after infection with plant pathogenic avirulent and virulent bacteria.

Recognition of PAMPs is considered as the evolutionary predecessor of avirulence factor recognition (Klement et al. 2003a; Navarro et al. 2004; Nurnberger and Brunner 2002). This reflects a temporally defined relationship of EBR and HR during defense. The PAMPs are available for perception by the plant from the very beginning of the bacterium-plant interaction, well before the bacteria are supposed to translocate their effector proteins into the plant cell. Thus, EBR signaling commences whether or not the bacteria perform translocation, in which case, EBR buildup runs parallel with that of the translocation machinery. When translocation succeeds, EBR may have failed to inhibit bacteria during their HR-induction period (Klement et al. 1999). Such stepwise progress of inducible defense was put forward in the *P. syringae* pv. *phaseolicola*–*Arabidopsis* nonhost interaction (Tao et al. 2003), which does not result in HR (Lu et al. 2001) and thus appears similar to the *P. syringae* pv. *phaseolicola*–tobacco interaction at 30°C.

The EBR-related chitinases are expressed during the HR, so it is plausible to assume that the bacteriostatic effect of EBR constitutes part of the strong successive resistance provided by

Fig. 4. In-gel chitinase activity of IWF (intercellular washing fluid). K-Pph = heat-killed *P. syringae* pv. *phaseolicola*; Pta = *P. syringae* pv. *tabaci*; Pta+K-Pph = *P. syringae* pv. *tabaci* (2×10^5 CFU ml⁻¹) infection for 24 h, challenged with 5×10^8 cells of heat-killed *P. syringae* pv. *phaseolicola* per milliliter and sampled at 6 h thereafter; numbers after treatments indicate hours after inoculation; the superscript a indicates an aligned piece of a silver-stained gel; the lane indicated by the superscript b was included to show pattern variation; below the lanes, (+) or (–) signs indicate whether EBR (early basal resistance) was operative or not, respectively, as evidenced by the hypersensitive reaction test; –(+) indicates weak and less durable EBR in a minority of cases. Experiments were carried out at least three times with similar results.

the HR. In accordance, when a HR response was stripped of cell death and oxidative burst by La³⁺ treatment, there was a drop in the resistance level as well, but the extent of the residual resistance was similar to that of EBR (Ott et al. 2002). Common biochemical reactions thought to be involved in defense, like deposition of callose and phenolics in papillae, were shown to occur in response to *hrp* mutants and their HR-inducing parent strains (Keshavarzi et al. 2004). There is a high degree of similarity between gene expression profiles induced by a HR-less nonhost pathogen (Tao et al. 2003; Z. Bozsó, unpublished data) or a PAMP (Navarro et al. 2004) and those induced early by race-specific elicitor-induced genes, whereby the possibility of stimulation of PAMP-mediated innate immune response during the HR also emerged (Navarro et al. 2004).

Fig. 5. Transcriptional regulation of early basal resistance (EBR)-related chitinases. **A**, Amino acid sequence alignment between three translated chitinase-specific polymerase chain reaction (PCR) product sequences and masses of tryptic fragments of both chitinases ('215' and '250') observed in the mass spectra. Underlined sequences in CHO3D9 were confirmed by post source decay; pointed line, identified only by mass; bars mark border amino acids of peptide fragment sequences; areas with black background show partial identities between peptides and PCR products. **B**, Early chitinase gene induction during EBR. Transcripts corresponding to sequence CHO3D9 were quantified with real-time PCR in leaf tissue infiltrated with the *P. syringae* pv. *syringae* *hrcC* mutant (EBR induced) or water (control). Ordinates show the ratio of CHO3D9 and constitutive actin transcripts. The asterisk (*) indicates that first samples were actually taken immediately before inoculation. Error bars = standard error of the means, $n = 3$.

Virulent pathogens are normally able to create a favorable environment in their host by reprogramming its metabolism. Adaptation of *P. syringae* pv. *tabaci* to the host also means coping with plant defense by strategies possibly involving suppression, tolerance, and avoidance of recognition. Importantly, heat- (Burguán and Klement 1979) or gamma ray-killed *P. syringae* pv. *tabaci* had no special characteristics as a PAMP. It did elicit EBR along with the expression of the two proteins. At unnaturally high inoculum density, even live bacteria triggered the appearance of the proteins. This observation indicates that *P. syringae* pv. *tabaci* does not avoid its initial recognition. Later, however, when bacteria multiply, either avoidance, tolerance, or both cannot be excluded as pathogenic strategies. For example, *P. syringae* pv. *tabaci* produces exopolysaccharides (Leigh and Coplin 1992) that may mask its presence, further backing the act of suppressors.

Besides, *P. syringae* pv. *tabaci* showed susceptibility to EBR in tandem, and even mixed, inoculation experiments. This says that EBR is a plant protection factor during pathogenesis. PAMP (flagellin) recognition has also been implicated in the basal resistance of *Arabidopsis* against the compatible pathogen *P. syringae* pv. *tomato* (Zipfel et al. 2004). It follows that *P. syringae* pv. *tabaci*, facing EBR, must possess a certain level of tolerance or suppressive activity. Indeed, its tolerance to EBR was found to be higher than that of the avirulent pathogens, when it was used as a challenge bacterium after an EBR-inducing pretreatment (Klement et al. 2003b). Increased tolerance of a virulent bacterium to defense responses induced by its *hrp* mutant is well documented by the fact that the wild type as a challenge could still promote growth of the mutant used as pretreatment (Keshavarzi et al. 2004). The exopolysaccharides may contribute to tolerance to, e.g., host-derived reactive oxygen species (Király et al. 1997).

EBR and EBR-related chitinase suppression by *P. syringae* pv. *tabaci* was evident from the absence or very low expression of the proteins during disease and from the lack of recognition avoidance discussed above. Heat treatment failed to promote the growth of the virulent bacterium (Fig. 2B), while it greatly assisted the survival of *hrp* mutants and late growth of the incompatible *P. syringae* pv. *syringae* in tobacco (Bozsó et al. 1999). Heat treatment provides here a fenocopy of the *Arabidopsis* *nho1* mutation. NHO1-mediated basal resistance (Lu et al. 2001), HR-mediated resistance, and the NHO1 glycerol kinase itself (Kang et al. 2003) were shown to be suppressed by means of an Hrp-dependent virulence mechanism of virulent bacteria. Also, *P. syringae* pv. *tabaci* as a pretreatment enabled *hrp* mutants of *P. syringae* pv. *syringae* to retain their numbers and greatly stimulated *hrp* gene expression in tobacco (Klement et al. 2003b), indirectly demonstrating suppression of EBR by *P. syringae* pv. *tabaci*. Several PAMP-induced genes were put forward as subjects to suppression by bacterial effector proteins (Navarro et al. 2004), as were genes involved in cell-wall-based, SA-independent responses (Hauck et al. 2003) and biochemical reactions in papillae (Keshavarzi et al. 2004). The necessity and the bacterial effectors (delivered by the type III secretion system) of basal defense suppression by virulent and avirulent bacteria has recently been postulated (Alfano and Collmer 2004).

When leaves infected with *P. syringae* pv. *tabaci* were challenged by heat-killed *P. syringae* pv. *phaseolicola*, the chitinase bands appeared (Figs. 2B and 4). In similar conditions, EBR itself is suppressed (Table 1, sequence G), which means that EBR has other substantial mechanisms besides the chitinases. It also suggests that at least parts of the EBR recognition are functional during disease. Thus, a reason behind this result can be an upset balance between elicitation and suppression of the EBR-related chitinases. Accordingly, the low and

varying amounts of the chitinases during *P. syringae* pv. *tabaci* infections (Figs. 2B and 4) suggest a dynamic equilibrium between the efforts of the pathogen and the plant to control the interaction. This calls for considering quantitative relationships. Indeed, EBR seems to be a quantitative (elicitor dose dependent) rather than a qualitative response. Varying levels of EBR can be measured in timecourse studies using different challenge-inoculum densities (Fig. 1A). Results with disturbed *P. syringae* pv. *tabaci* inocula (mixture of killed and live cells, challenge by heat-killed *P. syringae* pv. *phaseolicola* cells) also show that elicitation can outweigh suppression and hinder the progress of disease. Reviving early concepts (Klement 1982; Klement et al. 1978), now based on gene array data, a quantitative difference between incompatible (a high level of gene expression, high 'output intensity') and compatible (low output intensity) interactions was established. The compatible interaction was considered not robust because it was sensitive to changes of input signal intensity, which was itself intrinsically low (Tao et al. 2003). We also observed high variability—low robustness—in the expression of the EBR-related chitinases during the compatible interaction. In this context, bacterial suppressors and avoidance may decrease input signal intensity.

Our current view is that, for successful pathogenesis, suppression of or tolerance to EBR (and the related chitinases) should anticipate the inevitable EBR-elicitor potential of PAMPs that accumulate as virulent bacteria multiply.

Possible functions of the EBR-related extracellular chitinases.

The EBR has rather limited bacterium-killing effect as compared with the HR (Ott et al. 2002), but its quick development seems to make amends. EBR might be sufficient against the less specialized nonpathogenic or avirulent pathogenic bacteria. Analogously to that in plant-fungal interactions (Mauch and Staehelin 1989), the possible role of chitinases in plant-bacterial interactions may be multiple. Chitinases hydrolyze beta-1,4-linked sugar units on *N*-acetylglucosamines, and some chitinases exhibit lysozyme (EC 3.2.1.17) activity working on a related substrate, peptidoglycan, a general component of the bacterial cell wall (Collinge et al. 1993). Plant chitinases comprise a very diverse group with respect to their physical properties (Collinge et al. 1993) and the range of potential substrates, e.g., for lysozyme activity (Brunner et al. 1998). Besides, some chitinases possess an antibacterial effect that lies in their structural properties rather than lysozyme activity (Düring et al. 1999). Extracellular chitinases may thus directly affect either metabolism, viability, or both of bacteria, microorganisms known to stay outside the cell walls of living plant cells. So the EBR-related chitinases may be plant effectors

against bacterial effectors. Few indications point to this possibility in the literature. Recently *Arabidopsis* leaf peroxidase and exochitinase activity were shown to negatively correlate with growth of the virulent *P. syringae* pv. *tomato* (Traw et al. 2003). Participation in the input signal generation and amplification is again a function that is compatible with the earliness of EBR-related chitinases. Early induced chitinases with proposed similar function are known (de A Gerhardt et al. 1997). The constitutive extracellular chitinase activity seen in the present study is a particularly 'early' candidate to release elicitor-active microbial fragments from just-arrived invaders. Among plant-bacterium interactions, chitinases have a firmly established role only in nodulation, where they cleave bacterial Nod factors to control the plant response (Schultze et al. 1998). During EBR, activity of the chitinases may release further intracellular elicitors from the bacteria, leading to an enrichment of the resistance response, which might be, e.g., cold shock proteins (Felix and Boller 2003).

The EBR phenomenon is also compatible with potentiation of a response to a challenge infection by faster, improved signal transduction pathways. These activities fit within the scope of the so-called type I nonhost resistance (Mysore and Ryu 2004) that is induced by general elicitors and provides the first line of inducible, basal defense from nonself organisms.

MATERIALS AND METHODS

Plant material and treatments.

Tobacco plants (*Nicotiana tabacum* L. cv. Samsun) were grown in a greenhouse on commercial garden soil. For experiments two- to three-month-old plants with five to eight fully expanded leaves were used. One to two days before inoculation, the plants were placed in a growth chamber at 20 to 22°C with 16 h of light (12,000 lux intensity at plant height) and an 8-h dark period, unless indicated otherwise. Leaf interveins were inoculated with bacterial inocula using a hypodermic syringe fitted with a 26-gauge needle (Klement 1963).

For routine detection of EBR (the HR test), bacterial at 5×10^8 cells ml⁻¹ were used as elicitors, and this pretreated area (approximately 2 cm²) was challenged later with avirulent bacteria that have the potential to induce the HR in intact plants at a rate of 10^8 CFU ml⁻¹. Absence or partial absence of the HR indicated that EBR has been developed. EBR was scored using a standardized scale from 1 (approximately 50% HR) to 3 (0% or fully inhibited HR). For more precise, semiquantitative assessment of EBR, three different challenge cell densities (10^7 , 10^8 , and 10^9 CFU ml⁻¹) were used, and their scores were averaged. For heat treatment of leaves, whole or half-leaves of plants kept at 20°C were immersed into a 51°C water bath for 15 s (Visnyovszky et al. 1983). Depending on a given plant

Table 2. Bacterial strains used in this work

Bacterium	Designation	Relevant characteristics	Phenotype ^a	Source ^b
<i>Escherichia coli</i>	DH5α	Not pathogenic in plants	HR –	Life Technologies, Inc.
<i>Pseudomonas syringae</i> pv. <i>coriandricola</i>		Pathogenic in coriander, avirulent in tobacco	HR +	GSPB 2784
<i>P. fluorescens</i>		Saprophyte	HR –	ATCC No. 13525
<i>P. syringae</i> pv. <i>phaseolicola</i> (Burkholder)	Race 6	Pathogenic in bean, avirulent in tobacco	HR +	Mansfield et al. 1994
<i>P. syringae</i> pv. <i>pisi</i> (Sackett) Young et al.		Pathogenic in pea, avirulent in tobacco	HR +	ATCC No. 11055
<i>P. syringae</i> pv. <i>syringae</i>	61	Weakly pathogenic in bean, avirulent in tobacco	HR +	Xiao et al. 1992
	<i>hrpJ</i> mutant	Hypersensitivity and pathogenicity mutant	HR –	Xiao et al. 1992
	<i>hrcC</i> mutant	Hypersensitivity and pathogenicity mutant	HR –	A. Collmer, Cornell University, Ithaca, NY, U.S.A.
<i>P. syringae</i> pv. <i>tabaci</i>	H10	Pathogenic, virulent in tobacco	NN +	NCAIM B.01601

^a On tobacco. HR = hypersensitive response and NN = normosensitive necrosis.

^b GSPB, Göttinger Sammlung Phytopathogener Bakterien (Göttingen Collection of Phytopathogenic Bacteria); ATCC, American Type Culture Collection, Manassas, Virginia, U.S.A.; NCAIM, National Collection of Agricultural & Industrial Microorganisms, Budapest, Hungary.

material, minor adjustments of these parameters ($\pm 1^{\circ}\text{C}$, ± 3 s) were made, e.g., younger plants, which were less tolerant.

Bacterial material and treatments.

Bacterial strains used in this study are shown in Table 2. During the experiments *Pseudomonas* strains and *Escherichia coli* were grown on King's B media (King et al. 1954) at 26 to 28°C. When required, media were supplemented with antibiotics kanamycin and rifampin at concentrations of 50 and 20 $\mu\text{g ml}^{-1}$, respectively. To prepare inocula, bacterial cells were washed and resuspended in distilled water at the required density, using a spectrophotometer (CO8000 cell density meter; WPA, Cambridge, U.K.). For EBR induction, late-exponential phase cells ($A_{600} = 1.6$), derived from liquid culture to prepare the challenge inocula, 1-day-old cells from agar plates were used. When needed, the cells were heat-killed by putting cell suspensions into a 70°C water bath for 13 min. The cell suspension was then transferred to ice and was used within an hour. Survival rate was approximately 10^{-9} . *P. syringae* pv. *tabaci* frozen in liquid nitrogen was also killed by gamma irradiation (2 to 3 kGy) from a type RH- γ self-shielded laboratory Co₆₀ source (Central Food Institute, Budapest). The number of viable bacteria in leaves was monitored as follows: eight leaf disks (0.9-cm diameter, excised with a cork borer) were ground with a mortar and pestle in 10 mM potassium phosphate buffer (pH 7.0). Serial dilutions of the extracts were plated on King's B agar medium with or without antibiotics. Agar plates were inoculated according to an economical method (Bertoni and Mills 1987).

Chemicals.

1-Aminocyclopropane-1-carboxylic acid, SA, glycol chitin and fluorescent brightener 28 were from Sigma (St. Louis), JA from Apex Organics Ltd. (Devon, U.K.), paraquat from Imperial Chemical Industries PLC (Bracknell, England), protease inhibitor cocktails (Complete Mini) from Roche (Mannheim, Germany). Electrophoresis grade (ammonium persulfate, Bromophenol Blue, sodium acetate, glycerol, glycine) chemicals were from Sigma and others from Reanal (Budapest, Hungary).

Sampling for electrophoresis.

For critical comparisons, opposite halves of the same leaves or leaves of the same developmental stage with a similar intercellular space/cell surface ratio were used. Preparation of IWF was based on the method of Klement (1965) and its modification (Rohringer et al. 1983). Tobacco leaf panels were cut out, were washed briefly under tap water and then distilled water, and were vacuum-infiltrated with distilled water. IWF was obtained by centrifuging plastic strip-supported leaf rolls with 500 $\times g$ force parallel to the leaf plane in a swinging rotor for 15 min at 4°C. IWF was centrifuged (22,000 $\times g$, 15 min at 4°C to remove insoluble material and bacterial cells) and was stored at -70°C . Smaller samples of 50 to 150 μl could be obtained with a novel method that was developed for a fixed angle rotor. Here, one or two interveinal areas of vacuum-infiltrated leaves were placed in a standard 25-mm diameter plastic centrifuge tube onto the side distant from the rotor axis. Protease inhibitors were used only during preparation of proteins for mass spectrometry but not for routine detection.

Electrophoresis.

Discontinuous acrylamide gradient (10 to 20% for small gels, Bio-Rad mini protean 3 system [Bio-Rad, Hercules, CA, U.S.A.] and 3 to 20% for large gels (Hoefer SE600 system Hoefer, San Francisco) native gels were prepared according to the Ornstein-Davis buffer system (pH 6.8 for stacking gels, pH 8.8 for resolving gels, and pH 8.3 for running buffer).

A 25- μl aliquot of IWF was used with bromophenol blue indicator. Its protein level (1 to 2 μg) was not adjusted to allow comparisons between individual bands. Large gels were run at constant 15 mA current at 4°C for 6 to 8 h, small gels at constant voltages, first at 110 V for 20 min and then at 160 V for 100 min. The gels were stained with silver (Tschernuschko 1996), were photographed, and were subjected to densitometry using an Alphainnotech TM-26 imager.

In-gel chitinase activity assay of IWF was conducted by analyzing band intensity using ultraviolet transillumination in an overlay gel containing 0.04% glycol chitin and a fluorescence enhancer, fluorescent brightener 28 (Trudel and Asselin 1989).

Mass spectrometry.

IWF from plants treated with heat-killed *P. syringae* pv. *phaseolicola* cells was collected at 12 to 19 hpi and concentrated about 40-fold by ultrafiltration with a 5-kDa cut-off regenerated cellulose filter (Millipore, Bedford, MA, U.S.A.). Protease inhibitors were used for concentration of the two proteins, but the number of filtration steps should be kept to a minimum, as the proteins tended to bind to the filter membranes. Concentrated (about 40-fold) samples were subjected to electrophoresis on large gels. Gels were stained with Coomassie brilliant blue, and gel pieces containing distinct protein bands were cut out. Coomassie brilliant blue and salts were removed using a 1:1 50-mM NH_4HCO_3 /acetonitrile solution. The disulfide-bridges were reduced with dithiothreitol, alkylated with iodoacetamide. The protein was in-gel digested with Promega (Madison, WI, U.S.A.) side chain-protected porcine trypsin. The resulting peptides were extracted with a 1:1 H_2O /acetonitrile mixture containing 5% trifluoroacetic acid. Mass spectrometry analyses of peptides were performed on a Bruker Reflex III matrix-assisted laser desorption-ionization time of flight (MALDI-TOF) mass spectrometer (Bruker Daltonic, Leipzig, Germany). The tryptic digest was purified on a C18 Zip-tip (Millipore) and was analyzed unfractionated by MALDI-TOF MS using 2,5-dihydroxy-benzoic acid as the matrix. Trypsin autolysis products m/z 842.51 and 2211.1 served as standards for internal calibration (Protein Prospector software, Clauser et al. 1999). PSD analysis was performed on selected components in 10 to 12 steps, lowering the reflectron voltage by 25% at each step, and the data was eventually stitched together. While the MS conditions permitted monoisotopic mass measurements, in PSD mode, average masses were determined.

RNA extraction and cDNA construction.

Total RNA was extracted using the plant total RNA extraction miniprep system (Viogene Biotek, Taipei, Taiwan) as suggested by the manufacturer. Approximately 0.1 g of leaf tissue was ground in liquid nitrogen 1 to 48 hpi. RNA content was assayed by UV spectrophotometry at 260 nm.

Reverse transcription of 2.5 μg of total RNA was carried out using RevertAid H minus first strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania), using the TP primer (TP: 5' GAC TCG AGT CGA CAT CGA(T)₁₇; Frohman 1990) in the case of 3'Race PCR or the oligo(dT) primer from the kit in the case of real-time PCR according to the manufacturer's instructions.

3'Race cloning of homologous sequences.

A cDNA pool containing fragments of EBR-related genes was constructed from tobacco leaves treated with the *P. syringae* pv. *syringae* *hrcC* mutant. Redundant gene-specific PCR primer was designed on the conserved region of the chitinase peptides. Conserved regions (HNFNYG) were sought using known tobacco and *Arabidopsis* chitinase sequences. The re-

sulting primer was CHTO, 5' CAC AAC T(A/T)(C/T) AAC TA(C/T) GG(A/G) C 3'. The 3' end of the cDNA fragment was obtained by the EP primer (5' GAC TCG AGT CGA CAT CG3') on the TP adaptor region (Frohman 1990). cDNA from an EBR time series was PCR-amplified using the above pair of primers at 94°C for 2 min, 94°C for 45 s, 55°C for 1 min, 72°C for 1 min 35× to step 2, and finally 72°C for 10 min. Total volume was 25 µl, comprising 2.5 µl of cDNA sample, 100 pmol of each primer, 200 µM dNTPs (Fermentas), and 2.5 units of *Taq* DNA polymerase LC (recombinant) in the provided buffer (Fermentas). Samples were run on a 2% agarose gel, were visualized by ethidium bromide. Bands were excised by a scalpel and gel-eluted by gel-extraction miniprep kit (Viogene). Fragments were cloned by Promega's pGEM-T Vector System-I.

Sequencing of clones was carried out using plasmid DNA at the sequencing facility of MWG-Biotech AG (Ebersberg, Germany). Vector and adaptor sequences were removed manually. Clone identification was performed using BLAST search (Altschul et al. 1990).

Quantitative RT-PCR analysis of gene expression.

Upper and lower primer sequences for the three clones described in the text were as follows:

CHO1A2: 5' TGG ATT TGG CGT CAT CA 3'; 5' TCC CTT CAG GTC ACA ATC A 3';
 CHO3D9: 5' CAA CCA TTC GAG CCA 3'; 5' TAA GCC TCA CAA CCG TG 3';
 CHO3E10: 5' GCT ATC TGG TGG GTA CGG TT 3'; 5' GGA GCC AAA AGC AAC TTA GG 3'.

We used 2.5 µl of a 10-fold dilution of the cDNA stock (prepared as described above) in 15-µl reactions. Primer concentrations were 3 µM. PCR was carried out using the iQ SYBR green 2× supermix (Bio-Rad) on the DNA Engine Opticon 2 (MJ Research [Bio-Rad Laboratories], Waltham, MA, U.S.A.). Cycling parameters were as follows: initial 95°C for 6 min, followed by 40 cycles of 95°C for 30 s, annealing for 1 min, and 72°C 40 s, plate-read step. Annealing temperatures for CHO1A2, CHO3D9, and CHO3E10 were 55, 52, and 60°C, respectively. Measured cycle threshold values were always normalized against actin (internal control) values. Every sample was measured at least twice and standard deviations were calculated. Values of the noninoculated controls were given the arbitrary value of 1; other sample values are relative to the controls.

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