

NUCLEOLYTIC ACTIVITIES IN PEPPER LEAVES REACTING HYPERSENSITIVELY TO *XANTHOMONAS CAMPESTRIS* PV. *VESICATORIA*

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SUMMARY

A substrate-based gel assay was used to determine RNase and DNase activities in the incompatible interaction between pepper leaves (cv. 'Early Calwonder 10R') and the avirulent race 2 of *Xanthomonas campestris* pv. *vesicatoria* from 0 to 24 hours after inoculation, and for comparison in leaves inoculated with the virulent race 1 of the same bacterium (compatible interaction). Two major bands of RNase activity, with apparent molecular mass of 18 and 27 kDa were detected in pepper leaf extracts. Both activities were markedly inhibited by Mg^{2+} and Zn^{2+} ; activity of the 18 kDa RNase was stimulated by Ca^{2+} . Activity banding at 25 kDa activity, degrading both RNA and DNA as substrate (nuclease activity) and inhibited by EDTA and Zn^{2+} , was detected in bacterial cell extracts and in culture fluids of both races 1 and 2 of *X. campestris* pv. *vesicatoria*. No significant changes in plant nucleolytic activity were detected in hypersensitively reacting leaves, whereas an increase in the bacterial 25 kDa activity band was observed in both compatible and incompatible interactions.

Key words: *Capsicum annuum*, DNase, RNase.

INTRODUCTION

The hypersensitive reaction (HR), associated with resistance to plant disease, is induced by several plant pathogens and is characterized by rapid cell death at the infection site. Several lines of evidence suggest that death of host cells during HR is not caused directly by toxic substances produced by the pathogen but rather results from the activation of suicide processes encoded by the plant genome. In fact, HR is thought to be a form of programmed cell death (PCD) (Greenberg, 1997; Gilchrist, 1998; Heath,

1998; Pontier *et al.*, 1998), a process well known in animals, characterized by a range of morphological features and in which a number of regulatory genes, stimulatory events and signalling pathways are involved (Gilchrist, 1998).

A widely studied form of PCD in mammalian cells is apoptosis, characterized by distinct morphological features such as chromatin condensation, nuclear blebbing, cytoplasm condensation and vacuolisation, and fragmentation of the cell into apoptotic bodies (Wyllie *et al.*, 1984; Schwartzman and Cidlowski, 1993; Chinnaiyan and Dixit, 1996). Associated with these features are usually several biochemical markers such as DNA fragmentation into 300 and/or 50 kbp pieces and/or internucleosomal-sized fragments (multimers of about 180 bp) with 3'OH ends indicative of endonucleolytic cleavage, degradation of the DNA repair enzyme poly(ADP-ribose) polymerase, and activation of a specific family of cysteine proteases (caspases) related to interleukin-1 β -converting enzyme (Oberhammer *et al.*, 1993a; Chinnaiyan and Dixit, 1996; Greenberg, 1997; Heath, 1998).

Similarities between HR and mammalian apoptosis have been reported in a number of studies. Chromatin condensation at the nuclear periphery (Polverari *et al.*, 2000), and condensation and vacuolization of cytoplasm (Levine *et al.*, 1996; Mittler *et al.*, 1997) have been documented in some incompatible plant-pathogen interactions characterized by HR. On the contrary, there are numerous light and electron microscope studies on HR, where apoptotic morphological features were not observed (Goodman and Novacky, 1994; Bestwick *et al.*, 1995). Plant DNA cleavage has been reported in examples of HR triggered by a virus (Mittler and Lam, 1997), bacteria (Levine *et al.*, 1996; Mittler and Lam, 1997) and a fungus (Ryerson and Heath, 1996). Stimulation of caspase-like activity in tobacco plants reacting hypersensitively to TMV has recently been described (del Pozo and Lam, 1998).

DNA degradation during apoptosis in mammals can be accompanied by induction of specific endonucleases that are thought to mediate chromatin cleavage (Schwartzman and Cidlowski, 1993). Mittler and Lam

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(1995, 1997) studied the relation between DNA degradation and nuclease activity. They found that at least four different endonucleases were induced in the HR of tobacco to TMV infection, this activation being correlated with degradation of DNA into approximately 50 kbp fragments. However, they also reported that the increase in nuclease activity did not always correlate with DNA fragmentation. In fact, an *Hrp*- strain of *Pseudomonas syringae* pv. *phaseolicola* (Burkholder) Young, Dye and Wilkie, incapable of inducing HR and DNA degradation in tobacco leaves, provoked stimulation of nuclease activity when infiltrated in the leaves (Mittler and Lam, 1997).

Polverari *et al.* (2000) reported that ultrastructural alterations, which recall certain aspects of apoptosis, occurred in the HR induced in pepper leaves by an avirulent race of *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye and that these alterations were not associated with DNA fragmentation. On the basis of these results, we set out to ascertain whether the absence of DNA fragmentation is associated with the lack of nuclease changes in this incompatible plant-bacterium interaction.

MATERIALS AND METHODS

Plants, bacteria, inoculation and sampling. Pepper (*Capsicum annuum* L.) plants, cv. 'Early Calwonder-10R' ('ECW-10R'), with the *Bs1* resistance gene to *X. campestris* pv. *vesicatoria*, were grown in sterilized compost-enriched soil in a greenhouse at 22-28°C, under natural lighting. About 40 days after sowing, plants were potted and transferred to a growth chamber at 28±2°C, 60-70% RH, 65 µE m⁻² s⁻¹ illumination and 14 h light period.

Race 1 (strain Xcv 82-8) and race 2 (strain Xcv E3) of *X. campestris* pv. *vesicatoria*, respectively virulent and avirulent to pepper cv. 'ECW-10R', were used in all experiments. Bacterial cultures were stored as suspensions in 15% glycerol at -80°C.

To prepare inocula, bacteria were grown on nutrient agar at 27±1°C for 48 h, suspended in deionized water and spectrophotometrically adjusted to 10⁸ cfu ml⁻¹ ($A_{600} = 0.3$).

The 3rd-6th leaves of pepper plants at the 8th true leaf stage were completely infiltrated with the bacterial suspensions using a syringe with a 30 G needle. Corresponding leaves infiltrated with water served as controls. Leaf samples collected 0 to 24 h after inoculation were snap-frozen in liquid nitrogen and stored at -80°C before use.

Nuclease assays. Plant nuclease activity was determined in compatible and incompatible interactions in two independent experiments. Leaf samples (about 0.5 g fresh wt) were disrupted with liquid nitrogen in a mortar and the powder ground with 50 mM Tris-HCl buffer, pH 7.0 (2.5 ml g⁻¹ fresh wt) and sand (0.2 g g⁻¹ fresh wt). The slurry was filtered through 4 layers of nylon cloth and the fluid centrifuged at 15,000 *g* for 20 min at 4°C. Nuclease activity was determined in the supernatants after SDS-PAGE according to the procedure of Young *et al.* (1997), with some modifications. Protein content of the supernatant was determined according to Bradford (1976). Extracts were diluted to a final concentration of 0.5 mg protein ml⁻¹ in 6x SDS sample buffer (0.3 M Tris-HCl, pH 6.8, 10% SDS, 0.01% bromophenol blue, 30% glycerol) and kept in a boiling water bath for 3 min. Twenty µg of each protein sample were loaded onto a 12% SDS-PAGE gel containing 30 µg ml⁻¹ sheared, denatured salmon sperm DNA to detect DNase activity, or 300 µg ml⁻¹ of *Torulopsis utilis* RNA to detect RNase activity. After electrophoresis, the gels were washed three times to remove the SDS (10 min/wash) with 10 mM Tris-HCl, pH 7.5 containing 25% (v/v) isopropanol (preincubation buffer). The gels were then washed three times (10 min/wash) with 10 mM Tris-HCl, pH 7.5, to remove isopropanol and incubated at 50°C in 50 mM Tris-HCl, pH 7.5 (incubation buffer) for 1 h. DNase activity was revealed by staining the gels in aqueous ethidium bromide (1 µg ml⁻¹) for 10 min and destaining for 1 h with several changes of water. Gels were photographed under UV. To detect RNase activity, the gels were stained with aqueous 0.2% toluidine blue, destained with several changes of water and photographed on a light box.

Nuclease activity was also determined in bacterial cells and in the bacterial culture fluids in two independent experiments. Race 1 and race 2 strains of *X. campestris* pv. *vesicatoria* were grown in flasks of nutrient broth on an orbital shaker (100 rpm) at 28°C for 24 h. Bacterial cells were separated from culture fluid by centrifugation at 12,000 *g* for 15 min and washed three times with 50 mM Tris-HCl buffer, pH 7.0; cultural fluid was passed through a 0.45 µm membrane. Bacterial cells were disrupted with liquid nitrogen in a mortar and the powder ground with 50 mM Tris-HCl buffer, pH 7.0 and sand. The slurry was filtered through 4 layers of nylon cloth and the fluid centrifuged at 15,000 *g* for 20 min at 4°C. Nuclease activities were determined in supernatant and culture fluid with the same procedure used for plant nucleases. The apparent molecular masses were estimated using prestained markers (GibcoBRL). The effect of pH on

nuclease activities was evaluated by preincubation and incubating gels in 100 mM Tris-acetate buffer, at pH 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. To estimate the effect of divalent cations and EDTA on nucleolytic activities, these were added to the preincubation and incubation buffer. Calcium and magnesium chloride at 0.5, 1 and 2 mM, $ZnCl_2$ at 2, 20 and 200 μM , EDTA at 0.1, 1 and 10 mM were used.

Evaluation of bacterial growth *in planta*. Bacterial growth *in planta*, was determined at 0, 4, 8 and 12 hours after inoculation in two independent experiments. For each experiment, 2 plants per collection time were used, inoculating 4 leaves (3rd-6th) per plant. Inoculated leaves were longitudinally cut in two halves; one half was used to determine bacterial multiplication *in planta* while the other half served for nuclease analysis. Leaf samples (about 0.5 g fresh wt) were ground in a mortar with sterile 0.01 M potassium phosphate buffer, pH 7.0 (2.5 ml g^{-1} fresh wt). Leaf homogenates were diluted tenfold and several 10 μl drops of each dilution were placed on nutrient agar. After 24-48 h incubation at $27 \pm 1^\circ C$, bacterial colonies were counted using a stereomicroscope.

RESULTS

Symptoms and bacterial growth *in planta*. When pepper leaves were inoculated with the avirulent race 2 (incompatible interaction), they first showed macroscopic tissue collapse after 7-8 h. At this time, a more intense green colour with respect to the water-infiltrated control leaves was seen on the lower leaf surface. Subsequently, large areas of affected leaves turned greenish brown and then gradually withered and coalesced until almost the entire infected leaf blade wilted and shrivelled (12 h after inoculation). Symptoms were not detected in pepper leaves inoculated with the virulent bacterial race 1 (compatible interaction), during the experimental period (0-12 h after inoculation); symptoms (water soaking of leaf tissue) first appeared after 36 h.

No significant differences were observed between growth of race 1 and race 2 in pepper leaves from 0 to 12 h after inoculation (Fig. 1).

Characteristics of the nucleolytic activities. Healthy pepper leaf extracts, fractionated by SDS-PAGE, revealed two major bands of RNase activity, one with an apparent mass of 18 kDa and the other 27 kDa (Fig. 2a). Sometimes a faint 25 kDa RNase band was

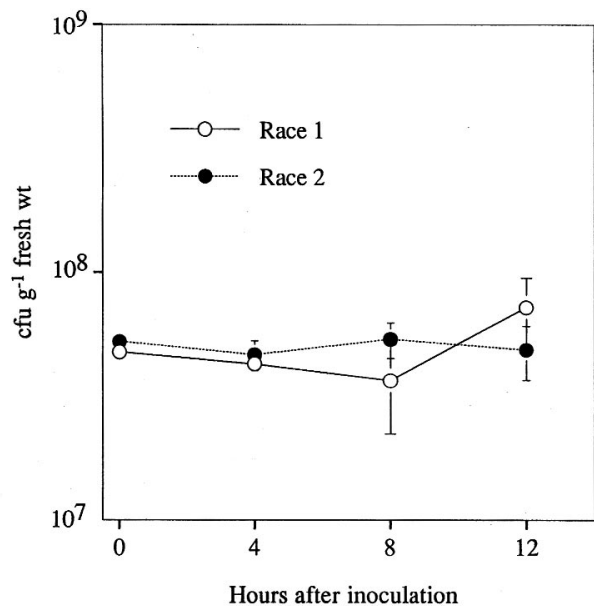


Fig. 1. Growth of the virulent race 1 or the avirulent race 2 of *X. campestris* pv. *vesicatoria* in leaves of pepper (cv. 'Early Calwonder 10-R') plants. Each value is the mean of two independent experiments. Vertical bars represent SE of each mean ($n = 2$).

resolved (see control in Fig. 3). No significant DNase activity bands were detected in pepper leaf extracts (data not shown). Optimum pH activity ranged from 5.5 to 6.0 and from 6.0 to 7.0 for the 18 and 27 kDa bands, respectively (Table 1). Mg^{2+} and Zn^{2+} strongly inhibited the activity of both 18 and 27 kDa bands, while Ca^{2+} stimulated only the 18 kDa activity (Table 1).

A 25 kDa band with both RNase and DNase activity, therefore a nuclease, was intracellularly and extracellularly produced by race 1 (Fig. 2b). This activity had a pH optimum of 6.5-7.5 and was strongly inhibited by EDTA and Zn^{2+} (Table 1). Similar results were obtained with race 2 (data not shown).

Effect of bacterial infection on nucleolytic activities. Marked increases in DNase activity (25 kDa band) were detected in bacterially infected pepper leaves in both compatible and incompatible combinations. High intensity bands were detected at 4 and 8-12 hours after inoculation in incompatible and compatible combinations, respectively (Fig. 3).

No significant changes in RNase activity were detected in infected plants except for higher intensity of the 25 kDa band in both plant-bacterium combinations than in the control, where this band was barely visible (Fig. 3).

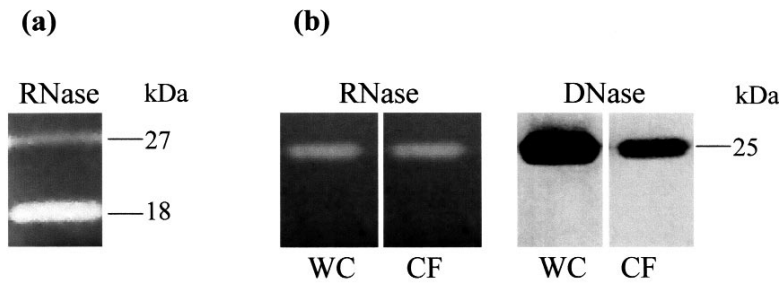


Fig. 2. (a) SDS-PAGE of pepper leaf extracts and gel assay for RNase activity. (b) SDS-PAGE of extracts from whole cells (WC) and culture fluid (CF) of *X. campestris* pv. *vesicatoria* (race 1) and gel assay for RNase and DNase activities. The apparent molecular mass of each activity band is indicated on the right. Since similar data were obtained in several experiments, only results of a representative experiment are shown.

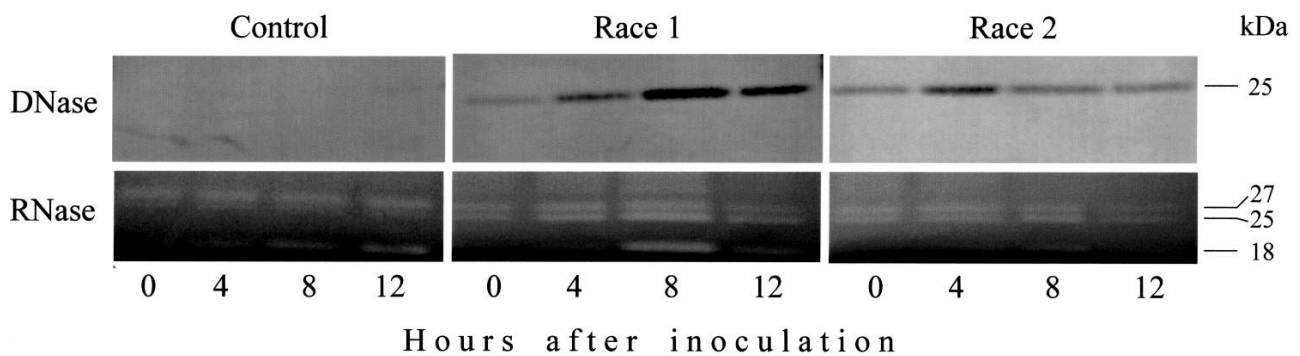


Fig. 3. SDS-PAGE of DNase and RNase activities in leaves of pepper plants (cv. 'Early Calwonder 10-R') inoculated with the virulent race 1 or the avirulent race 2 of *X. campestris* pv. *vesicatoria*. The apparent molecular mass of each activity band is indicated on the right. Since similar data were obtained in several experiments, only results of a representative experiment are shown.

Table 1. Properties of the major pepper leaf and *X. campestris* pv. *vesicatoria* nucleases.

Source	Molecular mass (kDa)	Enzyme type	pH optimum	Response ^a			
				Ca ²⁺	Zn ²⁺	Mg ²⁺	EDTA
Plant	18	RNase	5.5-6.0	+	-	-	0
	27	RNase	6.0-7.0	0/-	-	-	0
Bacterium	25	nuclease	6.5-7.5	0	-	0/-	-

^a See Materials and Methods for further details on the effect of divalent cations and EDTA.
 -: reduced activity.
 0: no change in activity.
 +: increased activity.

DISCUSSION

It has recently been shown that pepper leaves undergoing a HR to *X. campestris* pv. *vesicatoria* had nuclear and cytoplasmic ultrastructural alterations recalling certain aspects of apoptosis (e.g. chromatin condensation), which were not associated with DNA fragmentation (Polverari *et al.*, 2000). The fact that we did not find

plant nuclease stimulation in this context could explain the lack of DNA degradation. PCD occurring in the HR model studied, apart from the apoptosis-like morphological alterations, was different from that induced by other pathogens (Mittler and Lam, 1995, 1997) or that occurring in plant senescence (Xu and Hanson, 2000); this was also true for D-mannose treated plant cells (Stein and Hansen, 1999) and in maize endosperm

development (Young *et al.*, 1997), where nuclease activation together with DNA degradation has been reported. Our findings together with those of Polverari *et al.* (2000) are consistent with the results of Oberhammer *et al.* (1993b), where chromatin condensation in apoptotic cultured hepatocytes is not associated with nuclease activation and DNA fragmentation.

We also found that both virulent race 1 and avirulent race 2 of *X. campestris* pv. *vesicatoria* intracellularly and extracellularly produced *in vitro* a 25 kDa nuclease activity. Besides being secreted *in vitro*, this activity is also produced in bacteria infected pepper plants. In fact, a nuclease activity with the same apparent mass (25 kDa) of those produced *in vitro* by the bacterium was stimulated in infected leaves, both in compatible and incompatible combinations. This stimulation was particularly evident in gels stained for DNase activity, because of the absence of plant DNase interfering with the analysis (see Fig. 3). Since 25 kDa activity increased with time, especially in the compatible interaction, we suggest that the stimulation is not related to the growth of the bacterium *in planta* (since bacterial growth remained essentially constant from 0 to 12 h after the inoculation, see Fig. 1), but to compounds present in the apoplast which stimulate nuclease activity.

Although many bacteria secrete extracellular proteins, few extracellular nucleases have been reported (Benedik and Strych, 1998). They are limited to a small number of bacterial species, *e.g.* *Staphylococcus aureus* (Cuatrecasas *et al.*, 1967) and *Serratia marcescens* (Nestle and Roberts, 1969). It will be interesting to establish what role *X. campestris* pv. *vesicatoria* nuclease might play in the interaction with pepper plants. Although nucleic acids are not a particularly suitable carbon source, it is possible that bacterial nucleases together with other bacterial extracellular degradation enzymes could better exploit the host plant nutrients, probably after lysis of the plant cell, when plant nucleic acids are released. In addition, nucleotides derived from nucleolysis could be used by the bacterium for nucleic acid synthesis.

Further studies are necessary to characterize *X. campestris* pv. *vesicatoria* nucleases at biochemical and molecular levels in order to establish the role, if any, played by this enzyme in the pathological process.

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