BIOLOGIA PLANTARUM 38 (3): 437-443, 1996

β-1,3-glucanase and chitinase as pathogenesis-related proteins in the defense reaction of two *Capsicum annuum* cultivars infected with cucumber mosaic virus

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Abstract

Pathogenesis-related (PR) proteins from pepper (*Capsicum annuum* L.) cv. Americano (tolerant) and cv. Smith-5 (sensitive), both elicited by infection with cucumber mosaic virus (CMV), were assayed for chitinase and glucanase activities. Two basic PR-proteins, M_r 49.0 and 28.0 kD, were elicicited from the intracellular fraction (INTRA-F) of both cvs. by CMV infection, while four acidic M_r 15, 19, 36 and 40 kD and two basic M_r 21.2 and 24 kD PR-proteins were elicited from the intercellular fluid (IF) of cv. Americano leaves. Five acidic M_r 21.5, 23.2, 24.4, 25.2 and 36 kD and five basic M_r 23.3, 26, 28.8, 30 and 32.3 kD PR proteins were elicited from the IF of cv. Smith-5. Isoelectric focusing (IEF) of the IF and the INTRA-F proteins revealed the occurrence, in both pepper cultivars, of one acidic M_r 36 kD and one basic M_r 25 kD PR-protein with glucanase activity. After native-PAGE for acidic proteins, the acidic PR-protein of Rf 0.7 and M_r 36 kD present in the IF of both pepper cvs. showed glucanase activity. Native-PAGE for basic proteins of the INTRA-F showed the presence of one band (Rf 0.61, M_r 25 kD) common to both cvs. and two additional bands (Rf 0.49, M_r 26 kD and Rf 0.79, M_r 33 kD) in the cv. Americano with glucanase activity. The specificity shown by the basic PR-proteins suggests glucanase activity is involved in the mechanisms of resistance to CMV in the cv. Americano. There was no difference in chitinase isoform patterns between the two pepper cultivars analyzed. After IEF of the IF proteins, one acidic chitinase isoform was detected. Native-PAGE separation of the IF showed one band

Received 25 October 1995, *accepted* 2 February 1996.

Abbreviations: CMV - cucumber mosaic virus; IEF - isoelectric focusing; IF - intercellular fluid; INTRA-F - intracellular fraction; PAGE - polyacrylamide gel electrophoresis; SDS - sodium dodecyl sulfate.

Acknowledgements: The authors thank Prof. R. Muñoz (University of Murcia) for his help in revising the manuscript. This work has been partly supported by Project from the Consejería de Agricultura de la Comunidad Autónoma de la Región de Murcia, and by the Instituto de Fomento de la Comunidad Autónoma de la Región de Murcia.

(M_r 30 kD) with chitinase activity. Chitinase activity was not detected in the INTRA-F of either cultivar.

Additional key words: isoelectric focusing, intercellular fluid, intracellular fraction, PAGE, pepper, resistance.

Introduction

Pathogenesis-related (PR) proteins are host proteins induced by various pathogen and stress-related factors (Stinzi *et al.* 1993). Many PR-proteins still have an unknown biological function, while others exhibit glycanhydrolase activity which can be related to an antimicrobial defense response (Bowles 1990). PR-proteins were identified as β-1,3-glucanases and chitinases in many plant species. β-1,3-glucanases (E.C.3.2.1.39) are reported to hydrolyse the β-1,3-glucan component in the cell walls of some pathogenic fungi (Pegg 1977) and the enzyme chitinase (E.C.3.2.1.14), is involved in the degradation of chitin, a homopolymer consisting of β-1,4-N-acetylglucosamine units. Chitinase has been induced in response to viral, bacterial or fungal infection (Metraux and Boller 1986). *In vitro*, the growth of a number of fungi is inhibited by combinations of chitinase and glucanase (Arlorio *et al.* 1992). This suggests a direct antimicrobial role for these enzymes in plant defense although they also act indirectly by their anti-viral activity (Kopp *et al.* 1989).

In this paper, we have investigated the induction of PR-proteins with β-1,3-glucanase and chitinase activities, in the leaves of two cultivars (Americano and Smith-5) of *Capsicum annuum*, clearly differing in their sensivity to cucumber mosaic virus (CMV), during virus provoked systemic infection. The involvement of these enzymes in the defense response of pepper plants against the virus is discussed. As far as we are aware, this is the first report on glucanase and chitinase elicitation by a virus as CMV. The paper offers a method for quantifying glucanase activity directly on electrophoresis and isoelectrofocus gels.

Materials and methods

Plant, virus and inoculation procedure: Pepper plants (*Capsicum annuum* L.), cv. Smith-5 (sensitive) and cv. Americano (tolerant) were grown from seed as previously described (Espín *et al.* 1994). 10 - 12 leaves from plants of each cultivar were inoculated with CMV type I, under the conditions previously described (Candela *et al.* 1993).

Pathogenesis-related protein analysis: Thirty days after infection, leaves from the top of the plants, with clear systemic symptoms of CMV virosis, were collected for PR-protein and enzymatic activity analysis. Ten plants per treatment were used and each experiment was repeated twice. Assays were performed in the intercellular fluid (IF) and in the intracellular fraction (INTRA-F) of the leaves from both cultivars.

IF extraction: Leaves (3 g) were cut into small pieces and infiltrated under vacuum (Pan *et al.* 1989). The solution recovered from the bottom of the tubes was considered as IF (Rathmell and Sequeira 1974) and frozen at -20 °C until the protein studies. Cytoplasmic contamination, estimated by the activity of the cytoplasmic enzyme glucose-6-phosphate dehydrogenase (EC 1.1.1.49, D-glucose-6-phosphate: $NADP^+$ oxidoreductase) as described by (Löhr and Waller 1974), was less than 1.2 % in the IF.

INTRA-F extraction: Sections of IF-free leaves were ground with a mortar and pestle with liquid nitrogen and the fine powder extracted with 5 cm^3 of 0.05 M acetate buffer, pH 5.0. The solution was vigorously stirred in a tube mixer at maximum velocity for 2.5 min at 4 °C. The resultant homogenate was filtered through two layers of cheesecloth and centrifuged at 26 000 g_{max} for 30 min at 4 °C. The supernatants were stored at -20 °C and used for INTRA-F protein analysis (Ye *et al.* 1990).

Protein determination: Total protein was measured according to the method of Lowry *et al.* (1951). Samples of IF were concentrated by centrifugation in *Centripep 10 AMICON* (*Amicon*, USA) before analysis.

IEF and electrophoresis: The proteins from the IF and INTRA-F were analyzed by IEF and native-PAGE by using *PhastSystem* separation equipment (*Pharmacia,* Upsala, Sweden). IEF was carried out as described by Candela *et al.* (1994). Bidimensional electrophoresis of both acidic and basic proteins were carried out as previously reported (Candela *et al.* 1993).

Staining of proteins, β–1,3-glucanase and chitinase activities: Proteins were stained with *Coomasie Blue* or $AgNO_3$ (Candela *et al.* 1993). Staining of β–1,3 glucanase activity was performed according Pan *et al.* (1989). Staining of chitinase activity in the gels was carried out following the method of McBride *et al.* (1993).

Densitometry and quantification of β–1,3-glucanase isoenzymes: To quantify the β-1,3-glucanase activity of the protein bands, the stained gels were scanned by transmittance at 540 nm using a *PhastImage* densitometer (*Pharmacia*, Upsala, Sweden). Signal areas were used to express the β-1,3-glucanase activities as the percentage of each band area with respect to the total peak area of the highest enzymatic activity obtained along each experiment. β-1,3-glucanase activity was ascertained at 4, 8, 12, 16, 20, 24, 28 and 32 d after CMV infection.

The M_r of proteins was calculated by bidimensional electrophoresis (SDS-PAGE in the 2nd dimension).

Results

The different parameters of the PR-proteins elicited in *Capsicum annuum* leaves, cvs. Smith-5 and Americano, after infection with CMV are summarized in Table 1.

Table 1. PR-proteins of *Capsicum annuum* leaves, cvs. Smith-5 and Americano, at 30 d after infection with CMV, separated by non-dissociated electrophoresis (native-PAGE). IF - intercellular fluid; INTRA-F - intracellular fraction; Rf - reference ; M_r - relative molecular mass [kD].

Cultivar	IF acidic		IF basic		INTRA-F basic	
	Rf	M_r	Rf	M_r	Rf	M_r
Smith-5	0.52	24.4	0.40	30.0	0.47	49.0
	0.70	36.0	0.48	32.3		
	0.76	25.2	0.53	26.0		
	0.80	23.2	0.53	28.8		
	0.87	21.5	0.74	23.3	0.77	28.0
Americano	0.28	40.0	0.58	21.2	0.47	49.0
	0.62	19.0				
	0.70	36.0				
	0.81	15.0	0.81	24.0	0.77	28.0

Staining for 1,3-β-glucanase activity of the proteins from leaves of CMV-infected plants, showed glucanase activity in the IF and in the INTRA-F of the two cultivars assayed (Table 2).

Table 2.- PR-protein with glucanase activity, of *Capsicum annuum* leaves, elicited 30 d after inoculation with CMV. IEF - isoelectric focusing, PAGE - polyacrylamide electrophoresis, Ip - isoelectric point, Rf - reference. M_r - relative molecular mass [kD].

Cultivar	IF			INTRA-F		
	IEF	Native-PAGE	SDS-PAGE	IEF	Native-PAGE	SDS-PAGE
	Ip	Rf	M_r	Ip	Rf	M_r
Smith-5	4.8	0.70	36.0	8.9	0.61	25.0
Americano	4.8	0.70	36.0	8.9	0.49	33.0
				8.9	0.61	25.0
				8.9	0.79	26.0

The time course of leaf glucanase activity in both cvs. after CMV infection (Fig. 1) showed that the level of glucanase in both the IF and INTRA-F increased progressively until day 28 post inoculation and, then started to fall. The greatest increase was observed at day 20 in both cultivars. Glucanase activity was elicited by CMV in both cvs. The induction of two intracellular isoenzymes which were expressed belatedly and in very low concentrations in the tolerant cv. Americano (Fig. 1C) is of note. In healthy leaves (control), no glucanase activity was detected in either cultivar.

IEF of the IF proteins recovered from the leaves of both pepper cvs. after CMV-infection, revealed one protein (pI 4.6) with chitinase activity. The elicitation of this PR-protein was confirmed by native-PAGE (Rf 0.40) and SDS-PAGE with M_r 30 kD, (results not shown). This protein was present from the 5th to 30th day

following infection, increasing continuously during this time. No basic chitinases were found in the IF from leaves of either cultivar. No protein with chitinase activity was elicited from the INTRA-F.

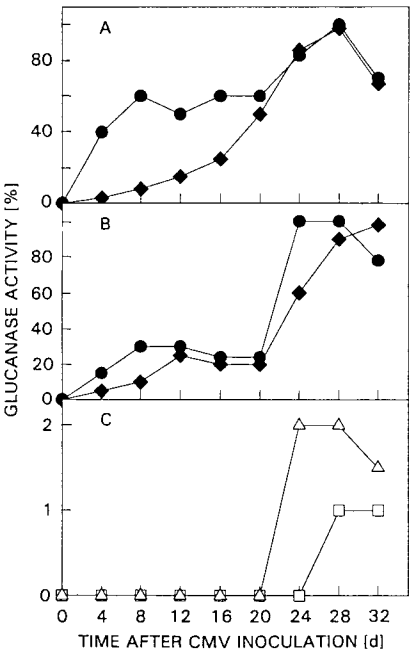

Fig 1 Time-course of glucanase induction in leaves of pepper cv. Smith-5 (*A*) and cv. Americano (*B* and *C*), inoculated with CMV and analyzed 4, 8, 12, 16, 20, 24, 28 and 32 d after incubation. *Closed circles* - IF Rf 0 70, glucanase isoform of Rf 0.70 after native-PAGE for acidic proteins, extracted from intercellular fluid. *Closed rhombs* - INTRA-F Rf 0.61, *open triangles* - INTRA-F Rf 0.49 and, *open squares* - INTRA-F Rf 0 79, glucanase isoforms of Rf 0.49, Rf 0.61 and Rf 0.79, respectively, after native-PAGE for basic proteins, extracted from intracellular fraction. IF - intercellular fluid. INTRA-F - intracellular fraction. Standard deviation was less than 5%.

Discussion

CMV infection on *Capsicum annuum* leaves resulted in the synthesis of new proteins, some of which showed glucanase and chitinase activity. The different electrophoretic patterns of both cvs. reflect the certain degree of tolerance against the virus shown by cv. Americano. In this cultivar the virotic symptoms produced by CMV were less extensive and the number of PR-proteins was also lower. Our results concerning the glucanases isoenzymes lead to three main conclusions. First, in untreated leaves, no isoenzyme was detected with 1,3-β-glucanase activity. Second, virus infection induced the secretion of one acid glucanase (Rf 0.70) into the extracellular compartment of both Smith-5 and Americano pepper leaves and an increase in basic glucanases, although it did not induce their secretion. Third, CMV

infection induced two new basic glucanases in cv. Smith-5 which were not detected in cv. Americano and which must be vacuolar enzymes. Since it has been proposed that the intracellular enzymes function late in the infection process, when cell breakage releases the vacuolar contents into the extracellular compartments (Steicher *et al.* 1992), our results indicate that the basic vacuolar glucanases (Rf 0.49 and 0.79) may be part of the cv. Smith-5 chemical defense system against a CMV infection.

As regards chitinases, no isoenzyme was detected with chitinase activity in untreated leaves. After virus infection, only one chitinase was detected by EF and this was acidic in nature. The fact that no chitinases were detected in the intracellular fraction is in accordance with reports on the basic nature and vacuolar localization of the chitinase activity elicited by ethylene (Mauch *et al.* 1992) and auxins (Grosset *et al.* 1990). Acidic chitinases seem to be released to their apoplast upon cell death during the hypersensitive response. However, Kurosaki *et al.* (1987) have demonstrated that chitinase in infected carrot cells is transported to the apoplastic space by an active transport system and not as a result of the destruction of the cellular structure following hypersensitive cell death.

Our results suggest that the protein is unspecifically synthetized and secreted to the intercellular fluid of both pepper cvs. as a consequence of CMV infection, possibily coded by a defense gene, and is not related with the tolerance that the cv. Americano has developed against the virus. This statement agrees with Vogelsang and Barz (1993), who did not obtain cultivar-specific differences in the elicitation of chitinase isoforms after the inoculation of chickpea with *Ascochyta rabiei*. This result led us to the fourth conclusion, that the induction of chitinase in pepper is a general defense mechanism, which does not explain the tolerance of cv. Americano towards CMV. In summary, the present study demonstrates the induction of PR-proteins, some of them with 1,3-β–glucanase or chitinase activity, in pepper in response to CMV infection. The new glucanase isoforms induced in the susceptible cultivar may indicate that this enzyme plays an important function in the defense mechanism, although this is not true for chitinase, which did not appear to be related with the resistance or susceptibility, but which constitutes part of the general and inspecific defense mechanism which *Capsicum annuum* has built up against CMV invasion.

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