Postharvest Biology and Technology 51 (2009) 414–424

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Physiological basis of UV-C induced resistance to *Botrytis cinerea* in tomato fruit. V. Constitutive defence enzymes and inducible pathogenesis-related proteins

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ARTICLE INFO

Article history:
Received 19 February 2008
Accepted 16 August 2008

Keywords:
Chitinase
Disease control
Gray mold
Glucanase
Host resistance
Hormetic
Hormic dose
Host defense mechanisms
Lycopersicon esculentum
Pathogenesis-related proteins (PR)
Polyacrylamide gel electrophoresis
Postharvest pre-storage treatment
UV-light

ABSTRACT

Changes in the protein content and profile of postharvest tomato fruit treated with the hormetic dose (3.7 kJ m^{-2}) of ultraviolet light C (UV-C) at the mature green stage was investigated. In UV-C treated fruits, the total protein content increased until 10 d after treatment and decreased thereafter during a 30 d storage period; whereas in control fruit, protein content decreased constantly throughout the storage period. Using polyacrylamide gel electrophoresis (PAGE) it was shown that UV-C treatment affected the protein profile of tomato fruit in several manners: (1) UV-C repressed the expression of some proteins presumably associated with ripening; (2) it enhanced the expression of several constitutive proteins, of which one was an acidic β-1,3-glucanase, three acidic chitinases and three basic chitinases; and (3) it induced the synthesis of at least 5 new proteins of which four were basic proteins. Among the proteins induced by UV-C, three (a basic β-1,3-glucanase and two acidic chitinases) were apparently pathogenesis-related proteins as they were also induced by inoculation with *Botrytis cinerea*. The molecular mass (MM) of five of the UV-C induced proteins was determined using SDS-PAGE. Their molecular masses were 45, 39.4, 34.6, 10 and 8.9 kDa. The UV-C induced β-1,3-glucanase had a MM of 33.1 kDa. The MM of two constitutive chitinases were 48.3 and 30.5 kDa, and those of the two UV-C and pathogenesis-induced chitinases were 37.1 and 20.6 kDa. Furthermore, the glucanohydrolase activities induced by UV-C were maintained until the end of the storage period. It is likely that the PR-proteins with glucanohydrolase activities induced by UV-C are an integral part of the long-term resistance observed in UV-C treated tomato fruit.

1. Introduction

In several plant–pathogen interactions, resistance to infection is correlated with *de novo* synthesis of pathogenesis-related (PR) proteins (Linthorst, 1991; Van Loon and Van Strien, 1999). These proteins are triggered in plant tissues in response to pathogenic attack. Non-specific stress conditions both biotic and abiotic in nature also activate the synthesis of these proteins, which are normally absent in healthy or unstressed tissue. PR proteins are classified into 17 distinct families (PR-1 to PR-17), with a putative novel PR-18 family, and have been recently revised by Van Loon et al. (2006). PR proteins within a single family have defined biochemical properties and are serologically related. Some PR proteins have catalytic activities and are characterized by their ability to degrade fungal cell wall constituents, namely β-1,3-glucan and chitin, and exhibit antifungal properties. For this reason, β-1,3-glucanases, the PR-2 family, and chitinases belonging to the PR-3, -4, -8 and -11 families (Van Loon et al., 2006) have been extensively studied in several plant–pathogen interactions. PR-1, the most abundant among the PR proteins, is induced in infected tissue (Alexander et al., 1993). A protein belonging to the PR-1 family was shown to inhibit *Phytophthora infestans* zoospore germination (Niderman et al., 1995). Some PR-4 proteins could cause lysis of germ tubes and inhibit growth of *Fusarium solani* and *Trichoderma viride* (Ponstein et al., 1994), while PR-5 proteins are thaumatin-like proteins (Van Loon and Van Strien, 1999) that could cause lysis and growth inhibition of *P. infestans* (Woloshuk et al., 1991). Members of the PR-6 family are characterized by their proteinase-inhibitor activities, while PR-7 and PR-9 proteins act as endoproteinases and peroxidases, respectively, where the latter are involved in lignin formation; and PR-10 proteins show "ribonuclease-like" activities (Van Loon and Van Strien, 1999). PR-12 proteins identified as defensins have potent antifungal activity against filamentous fungi (Van Loon and Van Strien, 1999;

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doi:10.1016/j.postharvbio.2008.08.016

Terras et al., 1992). PR-13 proteins are cysteine-rich thionins, toxic to phytopathogenic bacteria and fungi (Epple et al., 1997). PR-14 proteins are lipid transfer proteins endowed with antibacterial and antifungal properties (Garcia-Olmedo et al., 1995). PR-15 and PR-16 proteins from barley are typified by their oxalate oxidase or oxalate oxidase-like activities (Van Loon et al., 2006). PR-17 and PR-18 proteins have been detected recently and remain to be characterized (Van Loon et al., 2006).

Many studies have reported the induction of PR proteins in tomato (*Lycopersicon esculentum* Mill.) leaves in response to ultraviolet light B (UV-B, 280–320 nm) and that of UV-C (200–280 nm) radiation, ethephon, silver ion, β-aminobutyric acid, and infection by *P. infestans*, *Fulvia fulva*, and citrus exocortis viroid (Granell et al., 1987; Christ and Mösinger, 1989; Cohen et al., 1994; Green and Fluhr, 1995). In the past decade, several reports have appeared on improved resistance to pathogens in postharvest fruits and vegetables treated with biotic or abiotic elicitors of inducible defence mechanisms (Arul, 1994; Wilson et al., 1994; El-Ghaouth and Wilson, 1995; Arul et al., 2001; Terry and Joyce, 2004; Ben-Yehoshua and Mercier, 2005; Stevens et al., 2006a,b). Resistance to postharvest fungal pathogens (*Alternaria alternata*, *B. cinerea* and *Rhizopus stolonifer*) was reported to have been induced in tomato fruit by pre-storage treatment with hormetic low dose of UV-C (Liu et al., 1993; Charles et al., 1996, 2008a). Phytoalexin accumulated in UV-C treated products such as carrot root (Mercier et al., 1993a,b, 1998) and *Citrus* fruit (Kim et al., 1991; Ben-Yehoshua et al., 1992). In tomato fruit, UV-C induced resistance to *B. cinerea* was related in part to the accumulation of the sesquiterpenoid phytoalexin rishitin (Charles et al., 2008a). The resistance also involved structural reinforcement by lignin, suberin and other phenolics (Charles et al., 2008b) as well as ultrastructural modifications (Charles et al., 2008c). However, it is not known whether PR proteins play any role in the observed UV-C induced disease resistance in tomato fruit.

Thus the objective of this investigation was to detect activities of constitutive PR proteins, and determine whether PR proteins are induced in response to the abiotic elicitor, UV-C in mature tomato fruit; and relate their activities to the observed disease resistance induced by UV-C.

2. Materials and methods

2.1. Biological materials

Commercial greenhouse-grown tomato fruit (*Lycopersicon esculentum* Mill. cv. Trust) were used for this study. The fruit were manually picked from the vine at the mature green stage. Following harvest, the fruit were sorted, with only fruit free of blemishes were selected. The fruit were washed in running water, surface-sterilized with sodium hypochlorite solution ($1.0\,g\,L^{-1}$) for 5 min, rinsed in sterile distilled water and air-dried. The washed fruit were kept overnight at 13 °C until treatment with UV-C within 24 h.

The *B. cinerea* strain used in this study was isolated from diseased tomato leaves and cultured on potato dextrose agar (PDA). A pure subculture was preserved on sterile soil at 4 °C and used as starting inoculum whenever fresh culture was needed. An aliquot of soil was then plated on PDA, and as soon as mycelial growth was evident, an agar plug was once again subcultured on PDA until spore production occurred. After 10 d, the Petri dish was flooded with sterile water containing 0.02% (v/v) Tween 20. The spore suspension was filtered through sterile cheese cloth and diluted to 5×10^8 spores/L. The fruit were inoculated by placing 1 mL of the spore suspension in the stem scar of surface-sterilized tomato fruit from both the UV-C treated group and the non-treated control group, either 3 or 10 d after UV-C treatment, and the inoculated fruit were incubated at 13 °C.

2.2. Chemicals

The chemicals used for electrophoresis, the molecular mass markers, the SYPRO™ Orange protein stain and the protein assay kit were purchased from Bio-Rad Laboratories (Hercules, CA, USA). The calcofluor white M2R, silver nitrate and Triton X-100 were obtained from Sigma Chemical (St. Louis, MO, USA), and the aniline blue was obtained from BDH Laboratories (Poole, UK). Glycol chitin was prepared as described by Trudel and Asselin (1989). Yeast glucan was prepared from commercial baker's yeast according to the procedure described by Cabib and Bowers (1971). All other reagents were of analytical grade.

2.3. Low dose UV-C treatment, storage and sampling

Top and bottom parts of the fruit were treated with the hormetic dose of $3.7\,kJ\,m^{-2}$ (Maharaj et al., 1999) the day after harvest. UV-C radiation was produced by fluorescent germicidal lamps (GE 30W, General Electric, Circleville, OH) with peak emission at 254 nm. Radiation intensity was measured with a portable radiometer (UVX Digital Radiometer, UVP, Inc., San Gabriel, CA). Exposure to white light was avoided after UV-C treatement by placing the fruit in plastic containers covered with black polyvinyl chloride (PVC) sheets to prevent photoreactivation by exposure to visible light (Wade et al., 1993). A total of 192 fruit were randomly assigned to two groups (control and UV-C treated). Fruit from each group were stored in eight plastic containers containing 12 fruit each. Two containers per group were assigned for the monitoring of the protein content and profile of tomato fruit during storage. On d 3 and 10 after UV-C treatment, the fruit of two other containers per group were subdivided into lots of six fruit, placed in eight smaller containers (four containers with UV-C treated fruit and four containers with control fruit) and inoculated with *B. cinerea*. The remaining two containers per group served as non-inoculated controls and were also subdivided into lots of six fruit. Fruit were placed on a wire mesh platform in the container with a layer of water (about 1 L of sterile water) at the bottom of the container to ensure relative humidity of 90–95%, and a perforated lid was used to prevent accumulation of CO_2 in the container. The fruit were stored at 13 °C in the dark.

Protein and electrophoretic analyses were performed on the non-inoculated fruit of the control and the UV-C treated fruit on d 0 (day of harvest and treatment), 3, 10, 15, 20 and 30 after UV-C treatment. The inoculated fruit were sampled 4 d after inoculation. Tissue from four to six tomato fruit was excised from the pericarp. The excised sample was about 2 mm thick, including the cuticle and the first mesocarp cell layers. For the non-inoculated fruit, tissue was excised from the top of the fruit about 1–2 cm from the stem scar. For the inoculated fruit, the tissue was excised from the inoculation site to the edge of the expanding lesion, to a distance not exceeding 1 cm from the macerated tissue. The excised tissues were rapidly frozen in liquid nitrogen and kept stored at −80 °C.

2.4. Crude protein extract preparation

Fruit tissue frozen in liquid nitrogen was ground to a fine powder on the day of analysis using a Micro-Mill (Scienceware, Bel-Art Products, Pequannock, NJ, USA). Then, 5 g of fruit powder was homogenized with 5 mL of cold (4 °C) sodium phosphate buffer (0.1 M, pH 7) containing 1.0% (v/v) Triton X-100. The homogenate was centrifuged (10,000 × *g*, 15 min at 4 °C), and the supernatant was used for the analysis of the protein profile. The total protein content was determined according to the method of Bradford (1976) using the Bio-Rad protein assay kit with bovine serum albu-

min (BSA) as standard. Extraction of proteins was performed on the day of electrophoretic analysis.

2.5. Polyacrylamide gel electrophoresis under native conditions

Crude protein extracts were separated in one-dimensional polyacrylamide gels (15%, w/v) under native conditions using the Davis (Davis, 1964) and Reisfeld (Reisfeld et al., 1962) systems for acidic and basic proteins, respectively. The pH of the separating buffer was 8.9 for the Davis system and 4.3 for the Reisfeld system. The Bio-Rad Mini Protean II kit was used for separation with 0.75 mm thick gels. Aliquots of extracts (30 μL) that were loaded in the wells contained 15% (w/v) sucrose (Trudel and Asselin, 1989). Electrophoresis was performed at 20 mA for 1 h for Davis separation, and 35 mA for 3 h for Reisfeld separation, with both performed at 4 °C.

After separation, the Reisfeld gels were stained with SYPRO™ Orange protein stain (Bio-Rad). The gels were soaked (30 min) at room temperature in 7.5% (v/v) acetic acid containing 0.05% sodium dodecyl sulphate (SDS), and then stained (30 min) with a 1:5000 dilution of SYPRO™ Orange protein stain in 7.5% (v/v) acetic acid. The staining tray was covered with aluminum foil to protect the stain from light. After staining, the gels were rinsed rapidly (30 s) in 7.5% (v/v) acetic acid and photographed on a Chromato-Vue C-62 transilluminator (UV Products, San Gabriel, CA, USA) with Polaroid® 667 black and white film using a UV-haze filter and a yellow 15 filter (Heliopan, Einschranbgewinde, Germany).

The Davis gels were stained as described by Morrissey (1981). After electrophoresis, the gels were rinsed three times in distilled water (1 h each time) and then immersed in an aqueous solution of dithiothreitol (5 mg L^{-1}) for 30 min. The gels were then stained with 0.1% (w/v) silver nitrate and rinsed with water (30 s). The electrophoretic bands were revealed by placing the gels in 3% (w/v) sodium carbonate containing 0.5 mL L^{-1} of 37% formaldehyde. Development was arrested by quickly transferring the stained gels to 4% (v/v) acetic acid. Photographs were taken on a white fluorescent transilluminator (X-ray accessory, Picker International, Charlotte, NC, USA) with Polaroid® 667 black and white films.

2.6. Denaturing polyacrylamide gel electrophoresis

Protein extracts were also subjected to denaturing SDS-PAGE according to the procedure described by Laemmli (1970). Sodium dodecyl sulphate (SDS) was added to the separating gels and running buffer at a concentration of 0.1% (w/v). The sample extracts were denatured by boiling for 5 min under reducing conditions with 2% mercaptoethanol in a 125 mM Tris–HCl (pH 6.7) buffer containing 15% (w/v) sucrose (Trudel and Asselin, 1989). Molecular mass was determined using either broad- or low-range molecular weight markers (Bio-Rad). Staining and photography of SDS-PAGE gels were performed with SYPRO™ Orange (Bio-Rad) as described for the Reisfeld system.

2.7. Detection of β-1,3-glucanase activities

The procedure to detect β-1,3-glucanase was as described by Grenier and Asselin (1993). Alkali-soluble yeast glucan (0.1%, w/v) was used as substrate to detect β-1,3-glucanase activity under native (Davis and Reisfeld) and denaturing (SDS-PAGE) conditions. Because of the affinity of some β-1,3-glucanases for the substrate under native conditions (Davis and Reisfeld), enzymatic activities were detected with overlay gels containing the substrate. Under denaturing condition (SDS-PAGE), the substrate was directly incorporated into the separating gels before electrophoresis. Following separation, the gels were incubated for 2 h in sodium acetate buffer (pH 5, 50 mM) at 37 °C. The gels were stained for 15 min at room temperature in 0.025% (v/v) aniline blue in 150 mM K_2HPO_4 (pH 8.6). Gels were then destained in distilled water for 10 min. Lytic zones were revealed using a long-wave UV transilluminator (C-62, UV Products, San Gabriel, CA, USA). The gels were photographed (on Polaroid® 667 films) using a UV-haze filter and a yellow 15 filter (Heliopan, Einschranbgewinde, Germany).

2.8. Detection of chitinase activities

Chitinase activities were detected under both native and denaturing conditions by directly adding the substrate, 0.01% (w/v) glycol chitin, to the separating gels (Trudel and Asselin, 1989). After electrophoresis under native conditions (Davis and Reisfeld), the gels were incubated (3–4 h) in 50 mM sodium acetate buffer (pH 5) at 37 °C and stained (5 min) in 0.01% calcofluor white M2R in a 300 mM Tris–HCl buffer (pH 8.9). Excess stain was removed with distilled water under mild agitation at room temperature for 1–2 h. In the case of SDS-PAGE, the proteins were first renatured in a 100 mM Tris–HCl buffer (pH 8.0) containing 1 mM Triton X-100 and 1 mM mercaptoethanol; the latter two chemicals were also added to the incubation media. The gels were photographed on a medium-wave UV transilluminator (TM-36, UV Products, San Gabriel, CA, USA) with Polaroid® 667 black and white films using a UV-haze filter and an orange 02 filter.

3. Results

3.1. Changes in protein profile mediated by UV-C and Botrytis cinerea infection

The total protein content of the control fruit tended to decrease as ripening and maturation progressed (Fig. 1). In UV-C treated fruit,

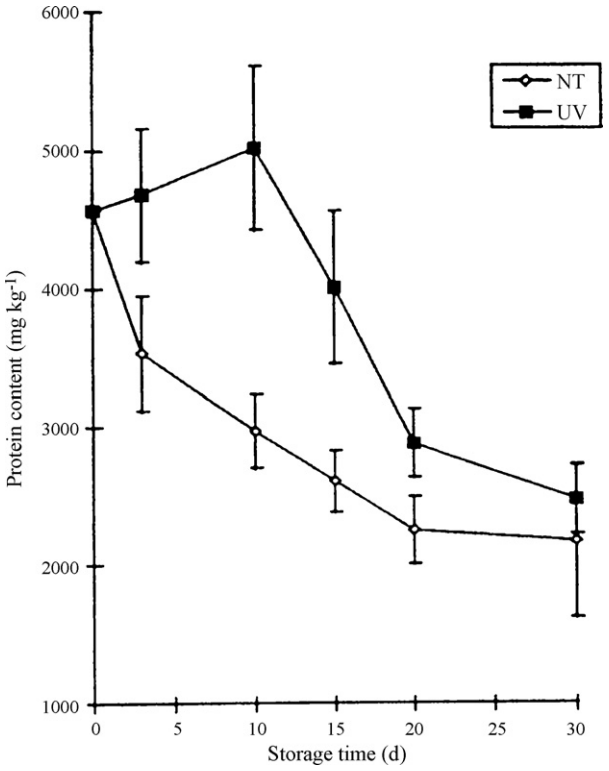

Fig. 1. Effect of UV-C treatment on protein content of fresh tissue of postharvest tomato fruit during storage at 13 °C. Data points are the means of four replicates. Vertical bars represent the SE values. NT: non-treated control; UV: UV-C treated.

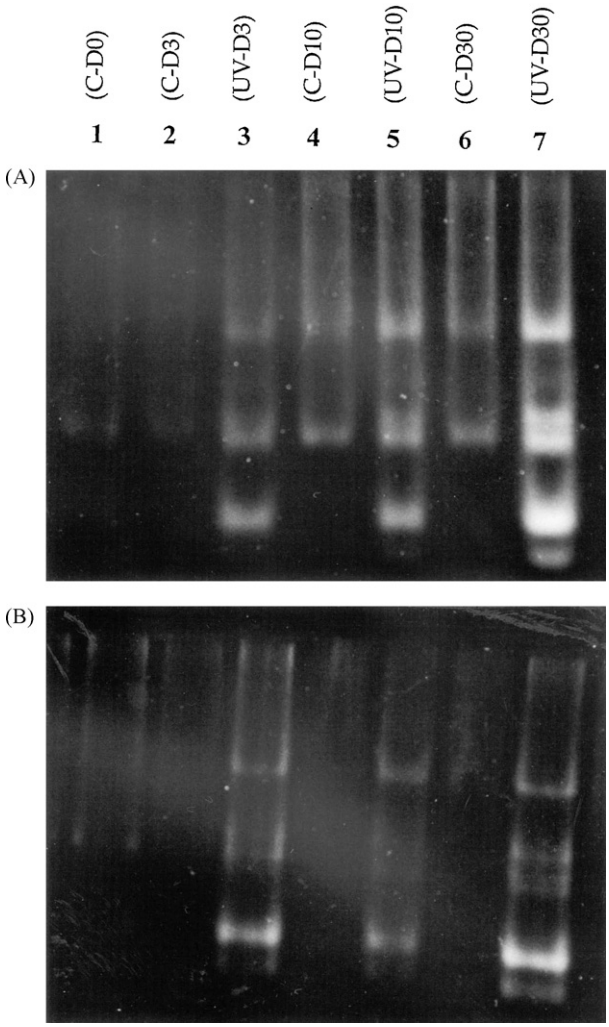

Fig. 2. Basic protein profile of non-treated (control) and UV-C treated tomato fruit after native-PAGE. Electrophoresis was carried out immediately after extraction (A) and performed with extracts stored at −80 °C for 4 d (B). Lanes: 1. control tissue at harvest and treatment day (time zero) (C-D0)); 2. control tissue 3 d after treatment (C-D3); 3. UV-C treated tissue 3 d after treatment (UV-D3); 4. control tissue 10 d after treatment (C-D10); 5. UV-C treated tissue 10 d after treatment (UV-D10); 6. control tissue 30 d after treatment (C-D30); 7. UV-C treated tissue 30 d after treatment (UV-D30).

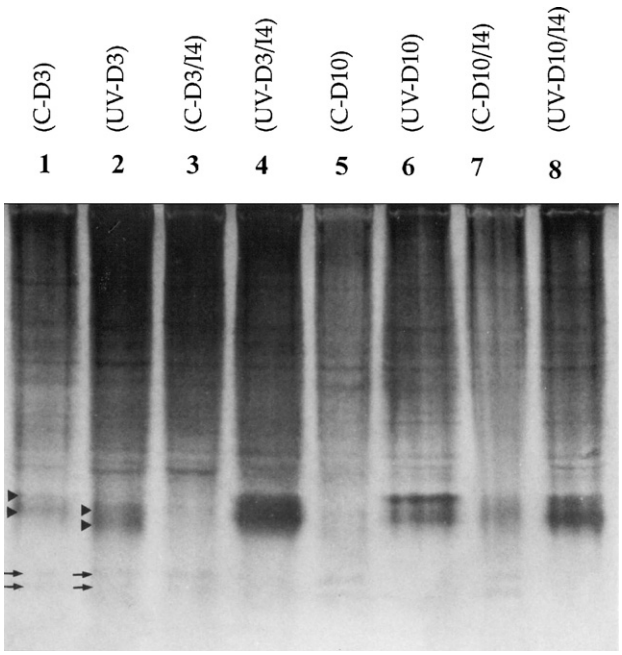

Fig. 3. Changes in the acidic protein profile of tomato fruit in response to UV-C and *Botrytis cinerea* infection. Lanes: 1. control tissue 3 d after treatment (C-D3); 2. UV-C treated tissue 3 d after treatment (UV-D3); 3. control tissue 4 d after inoculation (fruits were inoculated 3 d after treatment) (C-D3/I4); 4. UV-C treated tissue 4 d after inoculation (fruits were inoculated 3 d after treatment) (UV-D3/I4); 5. control tissue 10 d after treatment (C-D10); 6. UV-C treated tissue 10 d after treatment (UV-D10); 7. control tissue 4 d after inoculation (fruits were inoculated 10 d after treatment) (C-D10/I4); 8. UV-C treated tissue 4 d after inoculation (fruits were inoculated 10 d after treatment) (UV-D10/I4). Arrowheads highlight the proteins whose intensities increased in response to UV-C (lanes 2 and 6) and/or to infection (lanes 4, 7 and 8). Arrows highlight the protein bands whose intensities increased with advances in ripening in the control fruits (lanes 1 and 5) but decreased in UV-C treated fruits (lanes 2 and 6).

a sharp increase in protein content was observed with the highest peak (5.012 g kg^{-1}) reaching after 10 d. Thereafter, the protein content started to decrease in a pattern similar to that of the control. However, the degradation of proteins in the UV-C treated fruit was lower, and the protein levels tended to be higher than in the control fruit even on d 30.

When electrophoresis was performed on extracts from control tissue stored at −80 °C for a certain time (e.g., 4 d after extraction), protein bands faded away completely regardless of the electrophoresis conditions (Davis, Reisfeld or SDS-PAGE). All possible precautions undertaken to preserve proteins such as performing extraction protocols at 4 °C, rapid freezing with liquid nitrogen, etc. did not improve the electrophoretic profiles. But this event occurred only to a lesser extent with UV-C treated fruit extract (Fig. 2A vs. B); and in fact, protein bands could be revealed by electrophoresis even many days after storage at −80 °C. The observed low-temperature stability of proteins in UV-C treated tomato fruit can only be attributed to a stimulation of mechanisms by UV-C, which may involve cryoprotection and/or inhibition of proteases that control protein degradation. Hon et al. (1995) have shown that apoplastic proteins, which included chitinases, β-1,3-glucanases and thaumatin-like proteins (PR-5 family), exhibit anti-freeze activity in winter rye. Also UV-C was shown to induce protease inhibitors I and II in 14 d old tomato leaves (Conconi et al., 1996).

The acidic protein profile revealed by electrophoresis in the Davis system showed that there were significant changes in the protein pattern of tomato fruit pericarp in response to both UV-C treatment and infection by *B. cinerea* (Fig. 3). Two highly acidic protein bands (Fig. 3, arrowheads) were more intense in the UV-C treated fruit (lanes 2 and 6) and were further enhanced upon inoculation with *B. cinerea* (lanes 4 and 8); this was more so when inoculation was performed on d 3 (lane 4) than when it was performed on d 10 (lane 8). These two proteins that were present in the control fruit on d 3 (lane 1) faded with time and they were not detectable on d 10 (lane 1 vs. lane 5). They were not enhanced by inoculation when it was performed on d 3 (lane 3), and only slightly enhanced by *B. cinerea* with the progress of ripening (lane 7). The intensity of these bands also faded with storage time or maturity in UV-C treated fruit (lane 2 vs. lane 6). Two other acidic proteins (Fig. 3, lane 1, arrows) were observed in the control, and their intensities appeared to increase with ripening. Although these proteins were present in the UV-C treated fruit 3 d after treatment (Fig. 3, lane 2, arrows), they were not detectable by 10 d (Fig. 3, lane, 6). It is worth noting that these two acidic protein bands in the control disappeared in UV-C treated fruit; and these changes could be due to repression of the protein synthesis pathway associated with ripening. There was no significant effect of *B. cinerea* on these pro-

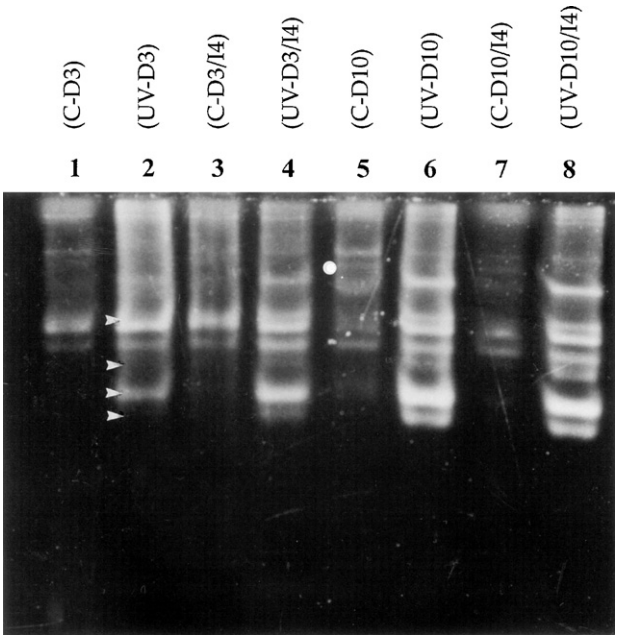

Fig. 4. Changes in the basic protein profile of tomato fruit in response to UV-C and *Botrytis cinerea* infection. Legends for gel lanes are same as for Fig. 3. Arrowheads highlight protein bands induced by UV-C treatment (lanes 2 and 6). These proteins were also detected in inoculated UV-C treated tissue (lanes 4 and 8). Circle indicates a protein band likely related to ripening in non-treated control fruits (lane 5).

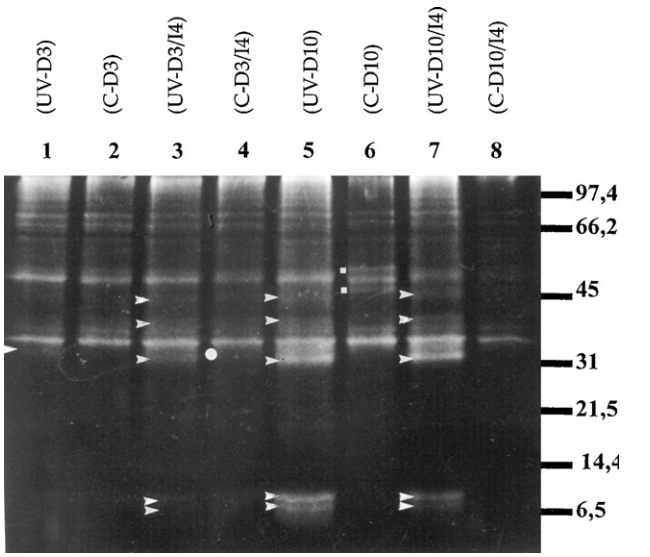

Fig. 5. Changes in the SDS-PAGE protein profile of tomato fruit in response to UV-C and *Botrytis cinerea* infection. Lanes: 1. UV-C treated tissue 3 d after treatment (UV-D3); 2. control tissue 3 d after treatment (C-D3); 3. UV-C treated tissue 4 d after inoculation (fruits were inoculated 3 d after irradiation) (UV-D3/I4); 4. control tissue 4 d after inoculation (fruits were inoculated 3 d after irradiation) (C-D3/I4); 5. UV-C treated tissue 10 d after treatment (UV-D10); 6. control tissue 10 d after treatment (C-D10); 7. UV-C treated tissue 4 d after inoculation (fruits were inoculated 10 d after irradiation) (UV-D10/I4); 8. control tissue 4 d after inoculation (fruits were inoculated 10 d after irradiation) (C-D10/I4). Molecular mass markers (kDa) are indicated at the right. Arrowheads: protein bands induced by UV treatment (lanes 1, 3, 5 and 7); circle: protein band induced in control tissue upon pathogenesis (lane 4); square dots: protein bands related to ripening (lane 6).

teins in either the control (lanes 3 and 7) or the UV-C treated fruit (lanes 4 and 8).

Electrophoresis in the Reisfeld system (basic proteins) clearly showed that UV-C induced at least four additional proteins (Fig. 4, lanes 2 and 6, arrowheads). Since these basic proteins were absent in the tissue of the control fruit (lanes 1 and 5), it is clear that the UV-C treatment induced these proteins. Furthermore, the amount of these proteins increased with storage time, as indicated by the increase in staining intensity from d 3 after irradiation (lane 2) to d 10 (lane 6). It is worth mentioning that the level of these proteins remained significantly high in the irradiated tissue until 30 d after treatment (data not shown). It was also observed that during ripening (d 10) of the control fruit tissue extracts, a new protein was detected (Fig. 4, lane 5, circle). This protein was not found in UV-C treated extracts at an equivalent time (Fig. 4, lane 6). Inoculation of the control tissue by *B. cinerea* did not induce the accumulation of the UV-C induced proteins in the control fruit (Fig. 4, lane 1 vs. lane 3 and lane 5 vs. lane 7). Furthermore, *B. cinerea* infection did not appear to enhance these proteins in the UV-C treated fruit as well (lane 2 vs. lane 4 and lane 6 vs. lane 8).

SDS-PAGE revealed at least five UV-C induced new proteins (Fig. 5, lanes 1 and 5, arrowheads) with estimated molecular masses of 45, 39.4, 34.6, 10 and 8.9 kDa, respectively. The intensities of these proteins increased with storage time. In the UV-C treated fruit, the intensities of the protein bands were not apparently affected by *B. cinerea* infection (lanes 3 and 7). These bands were not present in the healthy control fruit (lanes 2 and 6). However, in the control fruit inoculated on d 3, a faint band was detectable at 34.6 kDa (lane 4, circle). Older tissue apparently lost the ability to express this protein in response to infection, since no protein band was detected when the control was inoculated on d 10 (lane 8).

In control fruit, two proteins with molecular masses of 54.6 and 48.4 kDa (Fig. 5, lane 6, square dots) appeared to increase in intensity as ripening progressed. These proteins may correspond to the acidic proteins seen in Fig. 3 (arrows) and may be related to the ripening process. These proteins were not detectable in the UV-C treated extracts (Fig. 5). It is also possible that four of the five proteins whose molecular masses were estimated by SDS-PAGE (Fig. 5) correspond to the basic proteins induced by UV-C (Fig. 4, arrowheads). However, these observations (acidic proteins in the control fruit and basic proteins induced by UV-C) should be confirmed by further analysis of these proteins by two-dimensional SDS-PAGE and sequencing of purified proteins.

3.2. β-1,3-glucanase (PR-2) activities induced by UV-C and *Botrytis cinerea* inoculation

In the Davis system (acidic proteins), two significant protein bands showing β-1,3-glucanase activity (Fig. 6) were detected. One of these activities was constitutive of tomato fruit, since that activity was also detected in the non-inoculated control (Fig. 6, lanes 1 and 5, solid triangle). This constitutive activity was enhanced by UV-C (lanes 2 and 6, empty triangle), and its intensity was also enhanced in response to inoculation in both the control fruit (lanes 3 and 7) and the UV-C treated fruit (lanes 4 and 8). In addition, a protein with glucanase activity was induced by UV-C (lanes 2 and 6, solid square), which increased in the fruit tissue with storage time (lane 2 vs. lane 6) as well as in response to infection (lanes 4 and 8, empty square). This protein exhibited a weak electrophoretic mobility and hence was retained at the top of the separating gel, characteristic of low-charge-density proteins. Furthermore, this glucanase was not induced by pathogenesis in the control fruit (lanes 3 and 7).

In the Reisfeld system (basic proteins), one protein band with a strong β-1,3-glucanase activity was elicited in UV-C treated fruit (Fig. 7, lanes 2, 6 and 10). The hydrolytic activity of this induced protein increased with storage time and also in response to infection (lane 2 vs. lane 6, lane 2 vs. lane 4, and lane 6 vs. lane 8). While it was absent in the non-inoculated control (lanes 1, 5 and 9), mod-

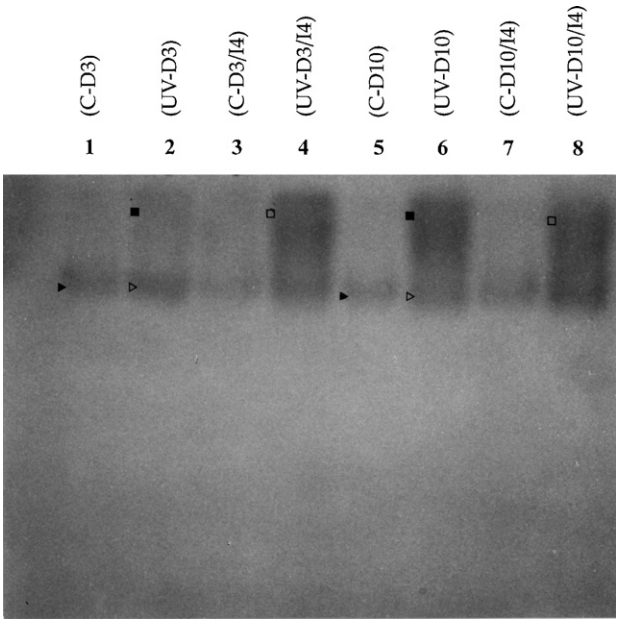

Fig. 6. Detection of acidic β-1,3-glucanase activities in postharvest tomato fruit in response to UV-C and *Botrytis cinerea* infection. Legends for gel lanes are same as for Fig. 3. Solid triangle: constitutive β-1,3-glucanase activity; solid square: UV-C induced β-1,3-glucanase activity; empty triangle: constitutive β-1,3-glucanase whose activity increased with storage in UV-C treated fruit; empty square: UV-C induced β-1,3-glucanase whose activity were increased by pathogenesis.

erate expression was detectable in response to *B. cinerea* infection (lanes 3 and 7). The high glucanase activity induced by UV-C was maintained until 30 d after treatment (lane 10).

SDS-PAGE revealed only one band with glucanase activity. This band, with an estimated molecular mass of 33.1 kDa, was detected only in the UV-C treated tissue (Fig. 8, lanes 2, 4, 6 and 8, solid triangle). The absence of glucanase activity in both the non-inoculated controls (lanes 1 and 5) and the inoculated controls (lanes 3 and 7) can be attributed to irreversible denaturation of the constitutive acidic glucanase and/or to lower concentration of the pathogenesis-induced basic glucanase. Since this activity is not detectable in the control tissue, it might be either the low-charge-density protein (Fig. 6), which was induced only by UV-C, or the basic glucanase that was induced by both UV-C and *B. cinerea* (Fig. 7). However, further characterization of this protein is necessary for precise identification.

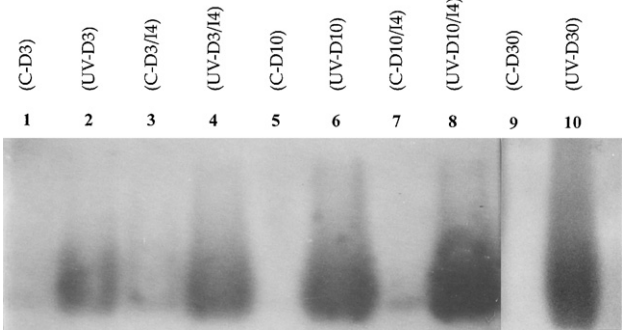

Fig. 7. Detection of basic β-1,3-glucanase activities in postharvest tomato fruit in response to UV-C and *Botrytis cinerea* infection. Legends for gel lanes 1–8 are same as for Fig. 3. Lanes: 9. control tissue 30 d after treatment (C-D30); 10. UV-C treated tissue 30 d after treatment (UV-D30).

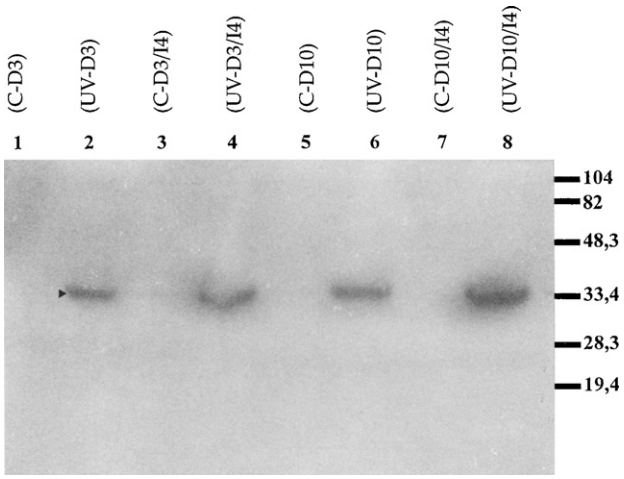

Fig. 8. Detection of β-1,3-glucanase activities after SDS-PAGE in postharvest tomato fruit in response to UV-C and *Botrytis cinerea* infection. Legends for gel lanes are same as for Fig. 3. Molecular mass markers (kDa) are indicated at the right.

3.3. Chitinase (PR-3) activities induced by UV-C and Botrytos cinerea inoculation

Electrophoretic separation in the Davis system revealed that at least four acidic chitinases were constitutive in tomato fruit (Fig. 9, lanes 1 and 5, empty triangle and empty circle). These activities were not affected by ripening and senescence, as judged by the fact that the intensities of the bands on d 10 (lane 5) remained comparable to the ones observed on d 3 (lane 1). The enzyme activity of these bands was enhanced by UV-C (lane 1 vs. lane 2 and lane 5 vs. lane 6). In both the UV-C treated fruit and the control fruit, these chitinase activities were enhanced by *B. cinerea* infection (lanes 3, 4, 7 and 8). Furthermore, two additional bands were induced by both UV-C and infection (lanes 3, 4, 6, 7 and 8, solid triangle). However,

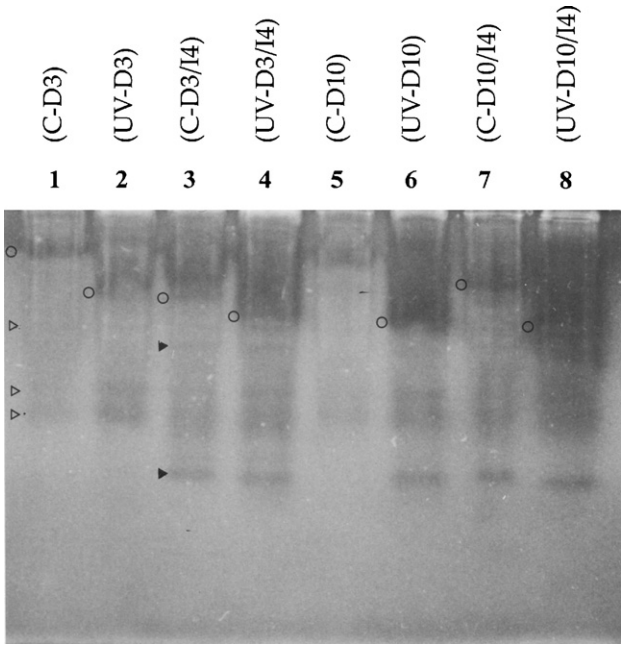

Fig. 9. Detection of acidic chitinase activities in postharvest tomato fruit in response to UV-C and *Botrytis cinerea* infection. Legends for gel lanes are same as for Fig. 3. Empty triangles: constitutive chitinases; empty circle: constitutive chitinase modified by UV-C and pathogenesis; solid triangles: UV-C and pathogenesis-induced chitinases.

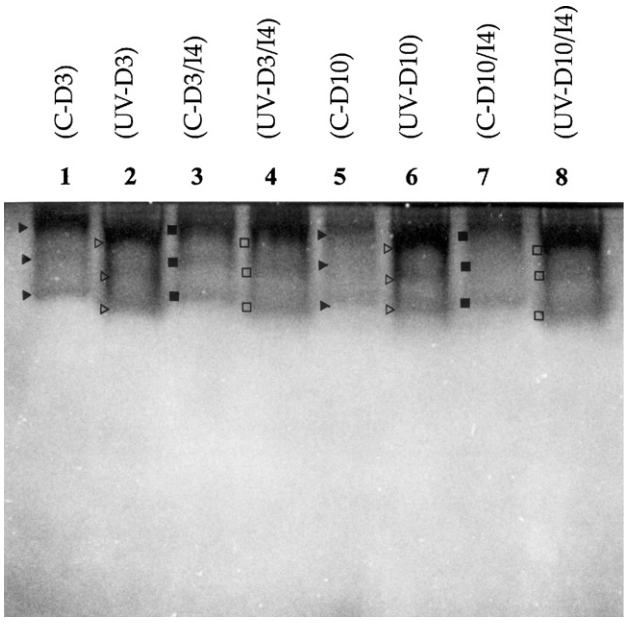

Fig. 10. Detection of basic chitinase activities in postharvest tomato fruit in response to UV-C and *Botrytis cinerea* infection. Legends for gel lanes are same as for Fig. 3. Solid and empty triangles highlight the position of the chitinase activities detected in the non-treated control and UV-C treated tissues, respectively. Solid and empty squares indicate chitinase activities in the inoculated control and UV-C treated tissues, respectively.

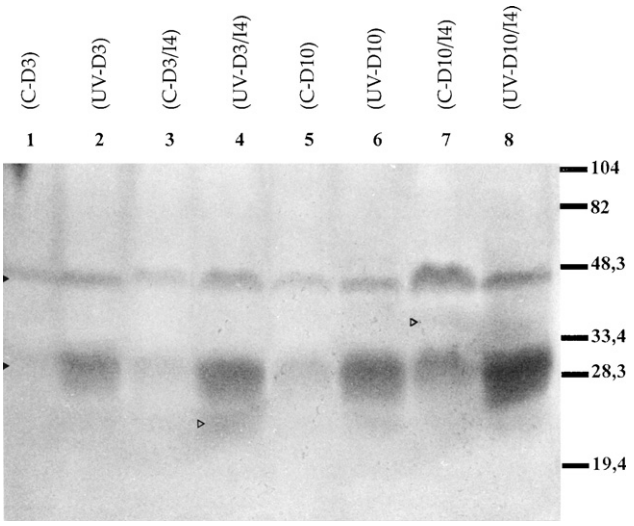

Fig. 11. Detection of chitinase activity after SDS-PAGE in postharvest tomato fruit in response to UV-C and *Botrytis cinerea* infection. Legends for gel lanes are same as for Fig. 3. Molecular mass markers (kDa) are indicated at the right. Solid triangles indicate the position of constitutive chitinases; empty triangles indicate the position of UV-C and pathogenesis-induced chitinases.

UV-C induced accumulation of these PR proteins was not rapid, since the presence of these activities was not very evident 3 d after treatment (lane 2); but it was intense 10 d after treatment (lane 6). The intensified acidic chitinase activities induced by UV-C were also maintained in the tissue until 30 d after irradiation (data not shown).

One of the constitutive proteins was retained at the top of the gel and tended to trail, rendering its characterization difficult. Its migration pattern was modified by UV-C treatment (lanes 2 and 6) as well as by pathogenesis (lanes 3, 4, 7 and 8), suggestive of a lower negative charge density. It is possible either that this constitutive protein is modified by UV-C and biotic stresses or that it is a different protein. Both UV-C treatment (lanes 2 and 6) and *B. cinerea* increased the activity of this chitinase (lanes 3, 4, 7 and 8).

Three constitutive basic chitinases were detected in tomato fruit (Fig. 10, solid triangle). The activities of these hydrolases appeared to decrease with ripening, as indicated by the diminishing intensity of the bands from d 3 (lane 1, solid triangle) to d 10 (lane 5, solid triangle). Infection of the control fruit did not induce *de novo* synthesis of any basic protein with chitinase activity. However, a small increase in the activity of the constitutive chitinases in response to infection was observed (lanes 3 and 7, solid square). UV-C alone did not induce any chitinase, although it did enhance the levels of the three constitutive basic chitinases (lanes 2 and 6, empty triangle). Infection by *B. cinerea* neither induced any chitinase nor increased the activities expressed in the UV-C treated fruit (lanes 4 and 8, empty square). The activity of these chitinases remained intense in the UV-C treated tissue throughout the storage period.

Even though the migration pattern of proteins with basic chitinase activities in the UV-C treated fruit (lanes 2, 4, 6 and 8) was quite similar to the pattern in the control fruit (lanes 1, 3, 5 and 7), the electrophoretic mobility of the former was greater than the mobility of the latter. This seems to imply either that these proteins are different or that UV-C brought about certain modification of the constitutive proteins, resulting in alteration of their charges.

The estimated molecular mass of the proteins exhibiting chitinase activity was determined using one-dimensional SDS-PAGE. Two constitutive chitinase activities at 48.3 and 30.5 kDa (Fig. 11, solid triangle) were detected. The activity of these chitinases was enhanced in response to UV-C treatment (lanes 2 and 6). The constitutive chitinases increased with the onset of ripening in both the control fruit and the UV-C treated fruit but was more pronounced in the UV-C treated fruit (lanes 1 and 5 vs. lanes 2 and 6). Infection also enhanced the expression of these two proteins (lanes 3, 4, 7 and 8), and they can be any one of the constitutive basic or acidic chitinases that were detected under native conditions (Figs. 9 and 10).

In addition, two faint bands with chitinase activity were induced in response to infection, with respective estimated molecular masses of 37.1 and 20.6 kDa. However, only the 20.6 kDa chitinase was expressed in the UV-C treated fruit when inoculation was performed 3 d after irradiation (Fig. 11, lane 4, empty triangle), whereas both hydrolases were induced in both the control tissue (Fig. 11, lane 7, empty triangle) and the UV-C treated tissue in response to infection by *B. cinerea* when inoculation was performed 10 d after treatment. It should be noted that the expression of these defence enzymes was always more intense in the UV-C treated tissue. It is possible that the 20.6 kDa chitinase is one of the acidic chitinases (Fig. 9) induced by UV-C and pathogenesis.

4. Discussion

This study showed that ripening, UV-C and *B. cinerea* affected the protein profile of tomato fruits in different manners as summarized in Table 1. UV-C also slowed down the overall degradation of proteins in tomato fruit, which is generally associated with ripening and senescence. The higher protein content observed in UV-C treated tomato fruit is likely related to the effect of UV-C treatment on senescence (Liu et al., 1993; Maharaj et al., 1993, 1999; Ait-Barka et al., 2000a; Stevens et al., 1998, 2004) and the activation of defense related genes (Logemann et al., 1995). This study showed that UV-C repressed the synthesis of two proteins detected in senescing control fruit. Furthermore, UV-C enhanced the synthesis of several constitutive proteins and induced *de novo* synthesis of stress- and/or pathogenesis-related proteins, presumably contributing to the higher protein content in UV-C treated fruit during

Table 1
Overall effects of ripening, UV-C treatment and inoculation with *Botrytis cinerea* on the protein profile of tomato fruit pericarp.

Protein	Ripening (NT)[a]			UV-C			NT + *B. cinerea*			UV + *B. cinerea*		
	Ind.[b]	Enh.[c]	Dec.[d]	Ind.	Enh.	Mod.[e]	Ind.	Enh.	Mod.	Ind.	Enh.	Mod.
Native-PAGE Acidic	–	2	2	–	2	2	–	2	–	–	2	–
Native-PAGE Basic	1	–	–	4	–	–	–	–	–	–	4	–
SDS-PAGE[f]	45, 39.4, 34.6, 10 and 8.9 (54.6 and 48.4)[g]											
Acidic β-1,3-glucanase	–	–	–	1	1	–	–	1	–	–	2	–
Basic β-1,3-glucanase	–	–	–	1	–	–	1	–	–	–	1	–
β-1,3-glucanase SDS[f]	33.1											
Acidic Chitinase	–	–	–	2	3	1	2	4	1	–	–	–
Basic Chitinase	–	–	–	–	3	3	–	3	–	–	3	–
Chitinase SDS[f]	48.3 and 30.5 (37.1 and 20.6)[h]											

[a] NT: Non-treated control.
[b] Ind.: Induced.
[c] Enh: Constitutive and enhanced.
[d] Dec: Constitutive and decreased.
[e] Mod: modification in protein mobility.
[f] Molecular mass, kDa.
[g] Molecular mass in parenthesis was expressed only in ripe NT.
[h] Molecular mass in parenthesis was expressed in response UV-C and infection.

the storage period. The possible induction of protease inhibitors by UV-C (Conconi et al., 1996) may also have contributed to the slower rate of protein decline.

Treatment of tomato fruit with the hormetic UV-C dose enhanced the expression of several constitutive proteins and induced *de novo* expression of several proteins, of which four were basic in nature (Table 1). Of the UV-C enhanced constitutive proteins, one was characterized as an acidic β-1,3-glucanase, three exhibited acidic chitinase activities, while three others displayed basic chitinase activities. Among the three induced proteins, two acidic ones showed chitinase activities and the third one a basic β-1,3-glucanase activity. These three proteins are likely true PR proteins, since they are also induced in control fruit in response to *B. cinerea*. The molecular mass of the five UV-C induced proteins was determined using SDS-PAGE. Their respective molecular masses were 45, 39.4, 34.6, 10 and 8.9 kDa. The 34.6 kDa protein was also induced by pathogenesis but the response was weak and observed only in younger fruit. The molecular masses of one β-1,3-glucanase (33.1 kDa) induced by UV-C, two constitutive chitinases (48.3 and 30.5 kDa) and two UV-C and pathogenesis-induced chitinases (37.1 and 20.6 kDa) were also determined.

It is interesting to note that UV-C treatment induced two low-molecular-mass proteins (10 and 8.9 kDa). Low molecular mass of these proteins suggests that they may be thionins, which are a family of peptides of less than 100 amino acid residues, containing six or eight cysteine residues and three or four disulphide bridges (Broekaert et al., 1997). Thionins (PR-13) are antimicrobial peptides that have been isolated from several plant species and are expressed constitutively or are induced in plant tissue in response to infection by microbial pathogens (Broekaert et al., 1997; Van Loon and Van Strien, 1999). In immature pistils and stamens of tomato, the constitutive expression of γ-thionin has been reported (Milligan and Gasser, 1995). The potential of UV-C to induce thionin-like proteins in postharvest tomato fruit and the putative role of those proteins in the observed disease resistance deserve further attention.

The data presented here indicate constitutive expression of glucanohydrolases in tomato fruit, which may have a potential role in defence against pathogen attack. The ability of the stored fruit to accumulate PR proteins in response to pre-storage irradiation with UV-C and infection by *B. cinerea* was also demonstrated. To the best of our knowledge, this is the first report on the constitutive expression of glucanohydrolases (chitinases and β-1,3-glucanases) with a potential defensive function in postharvest tomato fruit, although the possible involvement of β-1,3-glucanase in tissue softening of ripening tomato fruit has been shown by Hinton and Pressey (1980). In the vegetative bodies of tomato plants, however, several studies have reported on the constitutive or induced expression of hydrolases with defence roles (Harikrishna et al., 1996; Lawrence et al., 1996; Pozo et al., 1996; Enkerli et al., 1993).

In several plant–pathogen interactions, the putative role of both chitinases and glucanases in disease resistance is related to their capacity to degrade fungal cell wall, the mainly composed of chitin and β-1,3-glucan (Joosten et al., 1995). *B. cinerea* is an ascomycete, and as such its cell wall contains chitin microfibrils embedded in a glucan matrix (Epton and Richmond, 1980). The potential of enhanced or induced glucanohydrolase activities to contribute to effective defence of UV-C treated tomato fruit against *B. cinerea* is worthy of consideration. Moreover, this hypothesis is strengthened by the fact that some biological agents, such as the yeasts *Cryptococcus laurentii* and *Rhodotorula glutinis*, which have shown potential for controlling postharvest decay of fruit, are able to produce significant levels of extracellular β-1,3-glucanase activity in the presence of *B. cinerea* hyphal cell walls (Castoria et al., 1997).

Basic isoforms with chitinase and β-1,3-glucanase activities are generally reported to act synergistically (Mauch et al., 1988; Joosten et al., 1995) and to be more inhibitory than acidic isoforms (Joosten et al., 1995; Sela-Buurlage et al., 1993). The difference in the inhibitory power of the basic (class I) and the acidic (class II) isoforms have been related to their cellular localization, the former being intracellular and the latter extracellular. The results of this study show that only a basic isoform with glucanase activity is elicited by either UV-C or infection by *B. cinerea* and no basic chitinase counterpart is induced, but two acidic chitinases are induced. However, it was found that the activity of constitutive basic chitinases was significantly enhanced by both UV-C and infection. It is probable that the enhancement of constitutive hydrolases intensifies the natural defence of the tissue.

We observed the induction by UV-C of one β-1,3-glucanase with a molecular mass of 33.1 kDa, in tomato fruit. Lawrence et al. (1996) reported the detection of two constitutive β-1,3-glucanases with estimated molecular masses of 33 and 35 kDa in tomato leaves. They also found two constitutive chitinases at 30 and 32 kDa and two pathogen-induced chitinases at 26 and 27 kDa. The molecular masses reported by Lawrence et al. (1996) for constitutive and induced chitinases are in agreement with the masses reported by Joosten and De Wit (1989) and Joosten et al. (1995) in tomato

leaves, but are different from the molecular mass profile detected in tomato fruit that we have presented here. Furthermore, we found two constitutive chitinases with molecular masses of 48.3 and 30.5 kDa and two induced chitinases at 20.6 and 37.1 kDa. The difference in the molecular masses of the proteins expressed in the leaves (Lawrence et al., 1996) and in the fruit may possibly illustrate that expression of the isoforms is regulated by different genes in the two organs. Differential regulation of chitinases has been reported in barley grain and leaves inoculated with *Erysiphe graminis* (Collinge et al., 1993).

Short periods of irradiation with UV-C induced changes in the protein pattern of tomato leaves (Christ and Mösinger, 1989) and tobacco leaves (Yalpani et al., 1994; Brederode et al., 1991). In tomato leaves, the same eleven PR proteins induced by infection by *Phytophthora infestans* and *Fulvia fulva* were also induced by UV-C (Christ and Mösinger, 1989). Brederode et al. (1991) have shown that in tobacco leaves the genes encoding the full spectrum of known acidic and basic PR proteins responded similarly to UV-C and infection by tobacco mosaic virus. We found a more modest induction of new proteins in response to UV-C. The level of induction was even lower in response to infection, suggesting that activation of stress-related proteins by an abiotic factor such as UV-C may proceed through a different pathway than that of the induction of PR proteins by biotic elicitors. UV-C alone induced the synthesis of four basic proteins, of which only one, a β-1,3-glucanase, was also induced by pathogenesis. Analyses of the acidic protein profile indicate that UV-C elicited two chitinases that were also induced by infection by *B. cinerea*. The reduced capacity of mature tomato fruit (compared to leaves) to accumulate PR proteins may be related to a reduced ability or necessity of seed-bearing fruits to activate this defence mechanism.

Ozone, another oxidative stressor that resembles fungal elicitors, is known to induce PR proteins including β-1,3-glucanases and chitinases (Kangasjärvi et al., 1994; Sanderman et al., 1998). Is is increasingly apparent that reactive oxygen species (ROS), ethylene, salicylic acid and jasmonic acid are involved in the signal transduction pathways in the outcome of response of plants to oxidative stress such as ozone and UV-B and in incompatible plant–pathogen interactions (Enyedi et al., 1992; Kangasjärvi et al., 1994, 2005; Brosché and Strid, 2003; Baier et al., 2005). A similar hormonal signalling may be at play in response to increased oxidative load in UV-C exposed tomato fruit. A biphasic H_2O_2 accumulation and significant lipid peroxidation (liposuchsin-like compounds, malondialdehyde, aldehydes, pentane and ethane) was observed within 5 d following exposure of tomato fruit to hormic dose of UV-C (Ait-Barka et al., 2000b). An early increase in stress ethylene and significant increase salicylic acid were also observed in tomato fruit treated with UV-C (Maharaj, 1995; Maharaj et al., 1999).

Resistance of UV-C treated commodities to postharvest pathogens has generally been correlated with phytoalexin accumulation (Ben-Yehoshua et al., 1992; Droby et al., 1993; Mercier et al., 1993a,b, 1998). In a previous report (Charles et al., 2008a), we pointed out that a significant level of resistance was observed between 3 and 7 d when the rishitin level was low in UV-C treated fruit and it persisted even after the protection offered by rishitin began to ward off. This indicates that other defence mechanisms, such as PR proteins, may have been playing a role. This assumption is validated by the fact that the activity of the UV-C induced basic β-1,3-glucanase and acidic chitinases remained high even 30 d after treatment. The maintenance of the activities of these enzymes is possibly related either to a continuous synthesis of these proteins or that these proteins are protected against degradation. The first assumption is unlikely, because *de novo* synthesis of defence molecules cannot indefinitely be sustained by primary metabolites that are not replenished in the detached plant organ. Therefore, it is possible that the turnover of these defence molecules is reduced by the presence of factors such as proteinase inhibitors in the treated tissues. The latter possibility is very likely, given that UV-C locally induced class I and II proteinase inhibitors in tobacco leaves (Balandin et al., 1995; Linthorst et al., 1993) and tomato leaves (Conconi et al., 1996).

The expression of PR proteins in plant tissue is often associated with hypersensitive response (HR) and systemically acquired resistance (SAR), and has long been recognized as an effective part of the defence response to pathogen attack. The efficiency of activated defence mechanisms is dependent on the timing (rapidity), the site of accumulation and the intensity of the response. We showed that UV-C treatment activated proteins with glucanohydrolase activities (acidic chitinase and basic glucanase) before inoculation with *B. cinerea*, allowing the plant tissue to gain an advantage in fighting the infection. It is likely that PR proteins are an integral part of the long-term resistance induced in UV-C treated tomato fruit, as seen by the persistence of the activity of the defence hydrolases.

Acknowledgements

This work was supported by grants from the Natural Sciences and Engineering Research Council (NSERC) and the Conseil des Recherches en Pêche et en Agro-alimentaire du Québec (CORPAQ). The authors are grateful to Mr. Jean Grenier, Mr. Ronan Corcuff and Ms. Dominique Roussel for their technical assistance.

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