INHIBITION OF APOPTOTIC CELL DEATH INDUCED BY *PSEUDOMONAS SYRINGAE* PV. *TABACI* AND MYCOTOXIN FUMONIZIN B1

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ABSTRACT

The impact of programmed cell death (PCD) inhibitors on lesion formation and biochemical events in transgenic (ttr line) and non-transgenic (Nevrokop 1164) tobacco infected with Pseudomonas syringae pv. tabaci was tested. Programmed cell death in tomato cell culture was induced by Fumonisin B1 (FUM) and effectively abolished by the administration of protease inhibitors and lanthanum. Caspase inhibitors Ac-YVAD-CMK and Z-asp-CH_2-DCB, serine protease inhibitor TLCK and $LaCl_3$ were inoculated together with P. syringae pv tabaci in detached tobacco leaves or applied simultaneously with fungal toxin FUM in the tomato cell suspension. The results illustrate that cell death in normal Nevrokop 1164 and ttr transgenic tobacco at sites infected with P. syringae pv tabaci is apoptotic-like and caspase-like proteases and serine proteases are involved in lesion formation. The cell death in the lesions was accompanied with enhanced H_2O_2 accumulation and MDA production in the wild type and with reduced levels of both metabolites in the transgenic line. Lanthanum, caspase inhibitors and TLCK reduced the amount of H_2O_2 and MDA in the affected lesions. FUM-induced cell death in tomato suspension cells was greatly suppressed by the application of protease inhibitors and lanthanum. The results indicated that apoptotic cell death occurs at bacteria inoculation of tobacco leaves, and in fumonisin treated tomato cells.

Introduction

Programmed cell death (PCD) is found throughout the animal and plant kingdoms and is an active process in which a cell suicide machinery is activated resulting in controlled disassembly of the cell. PCD described in animal systems is known as *apoptosis*, a cell death process characterised by specific features such as cell shrinkage, blebbing of the plasma membrane, condensation and fragmentation of the nucleus and internucleosomal cleavage of DNA. The final stage of apoptosis is a fragmentation of the cell into cellular debris-containing vesicles called "apoptotic bodies" (32). Inappropriate apoptosis has been implicated in many human diseases, including birth defects, ischemic vascular diseases, neurodegenerative diseases (e.g. Alzheimer's and Parkinson's diseases), autoimmune diseases, AIDS and *Diabetes mellitus* type I.

Examples of cell death during plant development that conform to the general definition of PCD are cell death during xylogenesis, aerenchyma formation, plant reproductive processes, leaf and petal senescence, and endosperm development. Furthermore, cell death in response to pathogen attack, and in response to a variety of abiotic factors such as ozone, UV radiation, heavy metals also fall within the definition of PCD.

Plants have elaborated sophisticated and efficient system to counteract the spread of pathogen invasion. The hypersensitive response (HR) is a form of cell death associated with plant resistance to pathogen infection and occurs at incompatible plant-pathogen interactions (53). HR is characterized by rapid, localized death of tissues at the site of infection limiting further pathogen multiplication and spread (21, 29, 63). HR also involves activation of host defense-related genes (65) and various defense responses (23, 44). PCD-inducers in plants are toxins from a number of pathogens – harpins from *Pseudomonas syringae* and *Erwinia amylovora*, the fungal toxin victorin, xylanase from *Trichoderma viridae* (22, 43), *Alternaria alternata* AAL toxin, Fumonizin B1 (FUM) from *Fusarium moniliforme* (69). Also, plant viruses such as *tobacco mosaic virus* (TMV) have been reported to elicit PCD (51). There is not always a requirement for a living pathogen to trigger the HR. Purified elicitors, such as fungal toxin fumonizin B1 or harpins can induce physiological changes associated with disease resistance (55). It has been demonstrated that spraying plants with harpin coordinately induces systematic resistance to pathogens and micro-HR and that both *NDR*1 and *EDS*1 genes are required for the development of resistance (59).

Morphological similarities have been found between animal cells undergoing apoptosis and dying plant cells, including condensation and shrinkage of the cytoplasm and nucleus, DNA and nuclear fragmentation and formation of DNA-containing (apoptotic-like) bodies (13,69). Biochemical changes involving formation of reactive oxygen species (ROS) (20, 44, 35), Ca release (6, 46), proteinases (8, 72), and ethylene (27, 14, 33, 64) are found to actively participate in the signal transduction at PCD performance in plants.

Generally, apoptotic cell death involves a sequence of cysteinyl aspartate specific proteases (caspases) activation events in which initiator caspases activate downstream executioner caspases that process a variety of target proteins eventually leading to the apoptotic phenotype (11). Caspases belong to a class of specific cystein proteases that show a high degree of specificity with an absolute requirement for cleavage adjacent an aspartic acid residue (71).

Some of the compounds implicated in the triggering of PCD signaling cascades have been identified in cell culture with addition of elicitors and specific inhibitors. Caspases can selectively be inhibited by small peptides, mimicking the substrate recognition site, carrying electrophiles such as aldehydes, nitriles or ketones at their C terminal that react with the active site cystein. There is already evidence that caspases-like proteases, as in animal systems, participate in the programmed cell death in plants. Macromolecular proteins such as the cowpox serine proteases inhibitor crmA, members of the inhibitor of apoptosis protein (IAP) family and the

broad spectrum caspase inhibitors such as baculovirus Op-IAP and p35 has been reported to inhibit pathogen-induced cell death (15b, 17, 19, 25).

Cell death in tomato suspension cells was induced by anticancer drug camptothecin (CPT) – topoisomerase-1 inhibitor and was markedly inhibited by inhibitors of human caspase-1 (Ac-YVAD-CHO, Ac-YVAD-CMK) and caspase-3 (Ac-DEVD -CHO) as well as by the broad-range caspase inhibitor Z-asp-CH_2- DCB (13). These studies suggest that cell death-associated caspase-like activity is present in plant cells.

The production of reactive oxygen spices (ROS) is a key event in plant and animal apoptosis and a sustained oxidative burst is required for induction of plant hypersensitive cell death (35, 44, 47). ROS are components of the hormonally regulated cell death pathway in barley aleurone cells (9) and an enhanced production of ROS is involved at different cellular stresses (chilling, ozone, toxic chemicals). When attacked by incompatible pathogens plants respond by activating a variety of defence responses, including ROS-generating enzyme complex (10). The overall response comprises formation of small necrotic lesions, resulting from HR. Upon pathogen recognition, one of the earlier cell reactions are opening of specific ion channels, and the formation of superoxide and H_2O_2 (38). Is has been shown that H_2O_2 drives the cross-linking of cell wall structural proteins and functions as a local trigger of PCD in pathogen challenged cells. In addition, H_2O_2 acts as a diffusible signal inducing genes encoding cellular protectants in adjacent cells (47). CPT-induced PCD in tomato cell culture is accompanied by a release of ROS into the culture medium. Cell death and accumulation of H_2O_2 were effectively suppressed by addition of lanthanum, catalase, NADPH oxidase inhibitor diphenileneiodonium and caspase inhibitors (14, 70). ROS enhance the lipid catabolism resulting in lipid peroxidation of polyunsaturated fatty acids in the cell membranes that in turn leads to structural decomposition and change in permeability. The augmented level of malondialdehyde (MDA) is an indication of initiated destructive processes and oxidative stress (26).

HR is also characterized by other metabolic disturbances, such as ion influx (Ca^{2+} and K^+) and ion efflux (Cl^-) across the plasma membrane as well as changes in pH and membrane depolarization (29,47). An increase of Ca^{2+} has been detected in association with xylogenesis, aleurone layer cell death, leaf senescence (24, 45, 47, 73). Calcium is an important element in elicitor-mediated cell suicide signaling. La^{3+} exerts inhibitory effect on Ca channels and has been reported to completely block the cell condensation and shrinkage of cultured carrot cells triggered by ethanol, heat shock and H_2O_2 (50). In tomato suspension cells La^{3+} treatment prevented CPT-stimulated cell death thus indicating that Ca^{2+} is implicated in CPT-triggered deadly cascade (14).

In the present study we have tested the impact of apoptosis inhibitors on lesion formation and biochemical events in transgenic and non-transgenic tobacco infected with *Pseudomonas syringae* pv. *tabaci*. The amount of malondialdehyde, hydrogen peroxide and total protease activity were measured. Programmed cell death in tomato cell culture was induced by Fumonisin B1 (FUM) and effectively abolished by the administration of protease inhibitors and lanthanum. The results indicated that apoptotic cell death occurs at

Pseudomonas inoculated normal Nevrokop 1164 and ttr transformed tobacco lines, and in fumonisin treated tomato cells.

Materials and Methods

Chemicals: Fumonisin B1 was obtained from ICN Biochemicals and the caspase-inhibiting peptides from Bachem AG (Bubendorf, Switzerland). All other chemicals were obtained from Sigma.

Plant material and *Pseudomonas* infection

Tobacco plants, cv. Nevrokop and ttr transformed line were cultivated in pots at controlled environment (16h light/8 h dark, light intensity 4000 lux, 26°C, RH 60-70%).

Tabtoxin resistance gene (*ttr*) has been cloned by Anzai, Yoneyama and Yamaguchi (1989) from the bacterium *Pseudomonas syringae* pv. *tabaci* and established that the expression of the gene confers the resistance to wildfire pathogen. Commercial cultivar of oriental tobacco Nevrokop 1146 was transformed using the leaf disc method of Horsch et al. (1985). The plasmid pARK 21 carrying *ttr* and NPT II genes in *Agrobacterium tumefaciens* LBA 4404 was used for transformation. The resistance to *P. syringae* pv. *tabaci (*American Type Culture Collection, No. 17914*)* was studied in greenhouse conditions by multiple mechanical inoculation and detached leaf bioassay. We have obtained a homozygous line from Nevrokop 1146 tolerant to the American strain of *P. syringae* pv. *tabaci* and fully resistant to all Bulgarian isolates (7).

Leaves from 3rd position from the apex were inoculated with *Pseudomonas syringae* pv. *tabaci* (ATCC 17914), by the method of detached leaf bioassay. Bacteria was grown on a solid Nutrient Agar media overnight, washed with distilled water to a concentration 10^6 cfu ml^{-1} (OD_{600} of 0.6). Dilutions 10 and 100 times in water were done for obtaining 10^5 cfu and 10^4 cfu of the bacteria. Bacterial suspension was hand-infiltrated into the mesophyll of leaves. Before inoculation chemical inhibitors were applied at the point of infection. For inhibition of leason formation the following inhibitors were tested: (Ná-p-Tosyl-L-lysine chloromethylketone (TLCK), acyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK), benzyloxycarbonyl-Asp-2,6-dichlorobenzoyloxymethylketone (Z-Asp-CH2-DCB) and $LaCl_3$. Following inoculation, the leaves were kept in climatic cabinet (KBWF 240) at 29°C and >90% humidity under 16h light/8 h dark. Disease severity was assessed by the formation or lack of lesions.

Cell culture

Tomato (*Lycopericon esculentum* Mill.) cell suspension culture, line Msk8 (40), kindly provided by T. Boller, Botanisches Insitut, Universitat Basel, Ewitzerland, was grown on Murashige-Skoog type liquid medium supplemented with 5 μM a-naphthalene acetic acid, 1 μM N^6-benzyladenine and vitamins as described by (1). Cells were subcultured every 7 d by making 1:4 dilution in 25 ml of fresh medium in 100-ml flasks with alluminum caps.

Cell death induction and inhibition

Cells were used for experiments 5 d after subculture. Cell death inducer FUM and inhibitors (TLCK, Ac-YVAD-CMK or $LaCl_3$) were added simultaneously to 5 ml of suspension culture in 30-ml flasks with screw-caps. FUM and $LaCl_3$ were dissolved in water. The peptides were applied in dimethylsulfoxide (final solvent concentration 0.1% v/v). Dimethylsulfox-

ide had no effect on the viability of the cells. FUM and the inhibitors were tested in at least three independent experiments. Cell viability was determined by staining with 0.002% fluorescin diacetate (FDA).

MDA and hydrogen peroxide assay

A 0.3 g leaf tissue was homogenized in 3 ml 0.1 % TCA on ice, centrifuged at 10000 g for 20 min and supernatant was used for the assay. MDA was determined according to (16) including TCA (trichloroacetic acid)/TBA (thiobarbituric acid) addition and a heat/cool cycle. Absorption was read at 532 nm and 600 nm. MDA concentration was calculated using its extinction coefficient 155 $mM^{-1}.cm^{-1}$ (30). The endogenous level of H_2O_2 was determined specrophotometrically at 390 nm after incubation of leaf extracts with 1 mol/L KJ. Hydrogen peroxide content was calculated using a standard curve and expressed in $\mu M.g^{-1}$ FW.

Protease assay

Protease activity was determined following the method of Sarath (1990). The part of leaves with lesions of infection were grounded on ice with extraction buffer containing 100 mM_NaPi pH 7.5, centrifuged twice at 10000 x *g* for 15 min and the supernatant was used for the assay. A substrate azoalbumin (2% in the extraction buffer) and an aliquot of the extract were added to the reaction mixture. The samples were incubated for 45 min at 37° C and the reaction was terminated with 10% TCA. After 5 min centrifugation at 10000 rpm the pellet was discarded and 1 M NaOH added to the supernatant. Absorbency was measured at 440 nm with reference to a blank. Also, enzyme blank with azoalbumin, TCA and extract (added in this order) was used.

Results and Discussion

Caspase-like enzyme activity has been detected following the infection of tobacco with *Pseudomonas syringae* pv. *phaseolicola* (15a) and a role of caspase-like proteases has been established at apoptotic cell death of tomato suspension cells at camptothecin elicitation (13). To determine whether caspase-like proteases are involved in pathogenesis-induced and fumonisin-induced cell death, caspase-specific peptide inhibitors were co-infiltrated with *P. syringae* pv *tabac*i in tobacco leaves or applied together with fumonisin B1 in

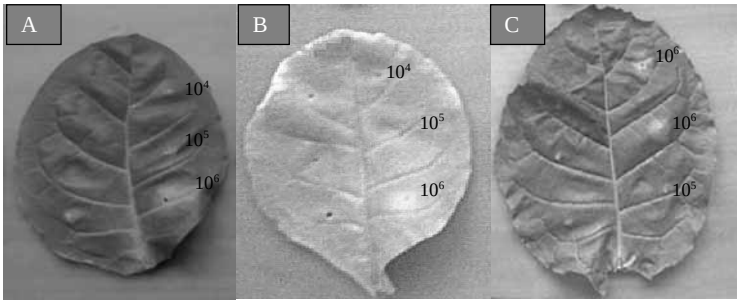

Fig. 1. Effect of protease inhibitors on lesion development in response to *P. syringae* pv. *tabaci* 7 dpi. Leaves were infiltrated with bacteria (right part of the leaf) and co-infiltrated with bacteria and an inhibitor (left part of the leaf). A - Nevrokop 1164 + 0.1 mM Ac-YVAD-CMK; B – *ttr* line + 0.1 mM Z-Asp-DZB; C - Nevrokop 164 + 1 mM TLCK. Bacteria concentration is indicated in cfu.

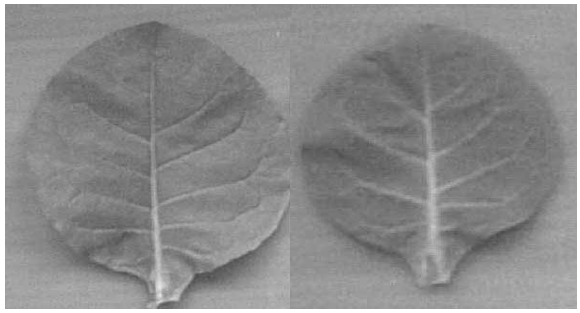

Fig. 2. Control tobacco leaves after 7 days in climate chamber: left - Nevrokop 1165, right - *ttr* line.

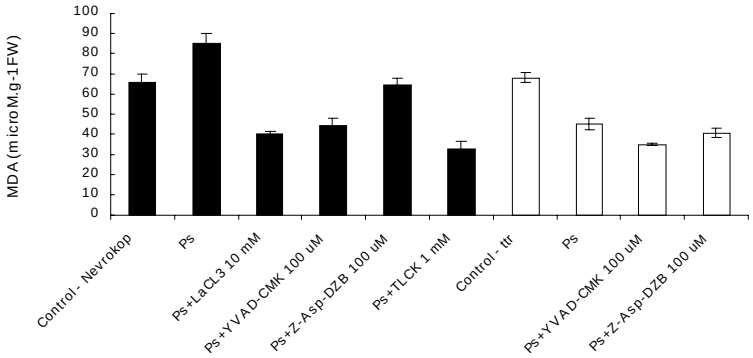

Fig. 3. Amount of H_2O_2 in lesions of tobacco leaves infected with *P. syringae*, pv. *tabaci*, (cv. Nevrokop 1146 and *ttr* transformed line). Data are means of three independent experiments. Error bars indicate SEM (n=12).

tomato cell culture. The irreversible caspase-1 (ICE)-inhibitor Ac-YVAD-CMK was tested for cell death-inhibiting activity in tomato suspension and both Ac-YVAD-CMK, and a broad spectrum caspase inhibitor Z-Asp-CH2-DCB were tested for their ability to inhibit lesion formation in *Pseudomonas syringae* pv *tabac*i infected tobacco leaves. In Nevrokop 1164 the development of lesions was significantly restricted at the application of 0.1 mM Ac-YVAD-CMK. In *ttr* tobacco line the appearance of lesions was completely suppressed by 0.1 mM Z-Asp-CH2-DCB (**Fig. 1**). Strong inhibition of infection was also observed when the inoculated leaves were pre-treated with 1 mM TLCK. (Fig.1). Concentration of bacteria that caused distinct lesions was 10^6 cfu. Lower concentrations 10^5 and 10^4 cfu did not or induced restricted lesion formation in both tobacco lines. Higher concentrations of tested caspase and protease inhibitors showed toxicity and caused leaf chlorosis. Contrary, the lower concentrations did not inhibit the infection. Lesion formation was inhibited and at the treatment of leaves with 10 mM $LaCl_3$ (data not included). During the incubation in the climate chamber, the control leaves from both tobacco lines stayed fresh without damages (**Fig. 2**).

Bacteria growth was tested *in vitro* in the presence of Ac-YVAD-CMK, Ac-YVAD-

CMK, TLCK or $LaCl_3$ and no reduction of the growth was found. Also, bacteria grown for 24 hrs in the presence of inhibitors, washed and applied into the leaves sustained the ability to elicit lesion formation (data not shown). Our data are complementary with for a reduced cell death in tobacco, susceptible to *Pseudomonas syringae* pv *tabac*i, where the leaves have been infiltrated with caspase inhibitor Ac-DEVD-CHO (60).

Plants perceive chemical signals from pathogens and translate them into suitable biochemical responses. ROS generation, including hydrogen peroxide production occurs typically at HR of plant-pathogen interactions (44). In recent papers the involvement of oxidative stress in the signal transduction and plant defense response at pathogen invasion and chemical elicitation has been reported (12, 14, 68, 70). Abiotic elicitors, such as ozone (O_3) in concert with ethylene cause formation of HR-like lesions and enhanced production of ROS (57). Evidence also exist that O_3 triggers HR and it is accompanied by H_2O_2 accumulation in Bel W3 tobacco plants (58). Ethylene signalling is found to play a role in the cell death induced by the mycotoxin fumonisin B1 in Arabidopsis and tomato (3, 52).

In order to establish whether the oxidative stress is involved in the HR of studied normal and transgenic tobacco line, we have measured the production of H_2O_2. The inoculation of line Nevrokop 1164 caused a higher production of H_2O_2 in the affected areas and the amount of H_2O_2 was about 40% less in the infected *ttr* line (**Fig. 3**). An inhibition of H_2O_2 accumulation was established in response to the applied inhibitors, the effect being better pronounced in tabtoxin transformed line. Regulatory role of caspase-like proteases in the oxidative burst has peen suggested earlier and efficient inhibition of H_2O_2 production and cell death has been achieved by application of caspase inhibitors to CPT-treated tomato cells (14). H_2O_2 has been found to activate mammalian NF-*k*B (necrosis factor kappa-B protein) in the induction of inflammatory, immune, and acute phase responses by diverse stress-stimuli and is thought to be activated by secondary signals generated by the reaction of membrane lipids with H_2O_2 (44).

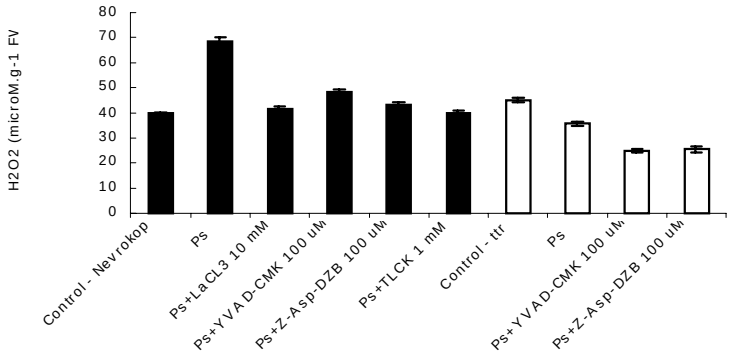

Fig. 4. MDA amount in lesions of tobacco leaves infected with *P. syringae*, pv. *tabaci*, (cv. Nevrokop 1146 and *ttr* transformed line). Data are means of three independent experiments. Error bars indicate SEM (n=12).

The isolation of a PCD-induced homologue of human protein PIRIN in tomato provides some clue about the role of putative NF- *k*B-associated pathways in plant defense mechanisms (56). Although plant homologues to NF-kB have not been described, as H_2O_2 is a mobile signal in plants, the signal system may involve a more stable product generated by further metabolism of ROS with a cellular substrate. Cell death in the tobacco leaves infected with *Pseudomonas* was prevented by cysteinyl-aspartic and serine protease inhibitors and also H_2O_2 release was diminished together with the less extend of membrane damage detected by the amount of MDA. H_2O_2 is a signal molecule and apart of its involvement in membrane oxidation it can activate other metabolic events directly leading either to cellular damage or to activation of defense responses (10, 14).

Ion leakage has been used to compare the relative amount of cell death in *Pseudomonas* infected plant tissues (4, 5, 15a). At the final stage cell death involves membrane destruction and metabolites such as malondialdehyde (MDA) can give an indication for membrane condition. MDA is a widely used stress indicator of plant membrane damage and is a product of peroxidation of polyunsaturated fatty acids (41). We have measured the amount of MDA in the lesions of bacteria infection and in the leaf areas with same size where inhibitors and bacteria were applied together. In response to the treatment with inhibitors the amount of MDA was reduced in comparison to its amount in the lesions caused by the infiltration of leaves with *P. syringae* pv *tabac*i without an inhibitor (**Fig. 4**) and this coincided with the restricted lesion formation. The bacteria infection of *ttr* transformed line caused lower increase of MDA than in non-tolerant Nevrokop 1164. Caspase inhibitors Ac-YVAD-CMK and Z-Asp-CH2-DCB and serine protease inhibitor TLCK remarkably diminished the level of MDA in treated leaf areas. The lower amount of MDA in the tolerant tobacco line is an indication that HR involves lipid peroxidation of membranes. Evidence about the apoptotic nature of membrane peroxidation is also that the MDA production was inhibited by caspase inhibitors. For improvement the interpretation of the results for lipid peroxidation, probably the determination of other compounds in parallel with MDA are necessary. Interestingly, a certain level of MDA and H_2O_2 were detected in control non-treated leaves. In comparison to the controls, the infection caused an increase of MDA and H_2O_2 in Nevrokop 1164 and a decrease of the two metabolites in *ttr* line. This result might be due to the tolerance of *ttr* line to bacteria, where the HR is better pronounced than in the non-tolerant tobacco line.

Calcium is an important element in elicitor-mediated cell suicide signaling and is a second messenger involved in apoptotic cell death, including HR (46, 73). Calcium is also an essential component of cell death pathway in CPT-triggered programmed cell death in tomato suspension cells (14). In mammalian systems elevated levels of intracellular Ca^{2+} can activate programmed cell death and DNA digestion through direct stimulation of endonucleases or via Ca-dependent proteases, phosphatases and phospholipases (49, 54). In bacteria elicited suspension tobacco cells the increased Ca^{2+} influx and HR response have been prevented by La^{3+} (6). An inhibition of ROS production and extracellular medium alkalinization has been reported at the use of calcium channel blockers (La^{3+},

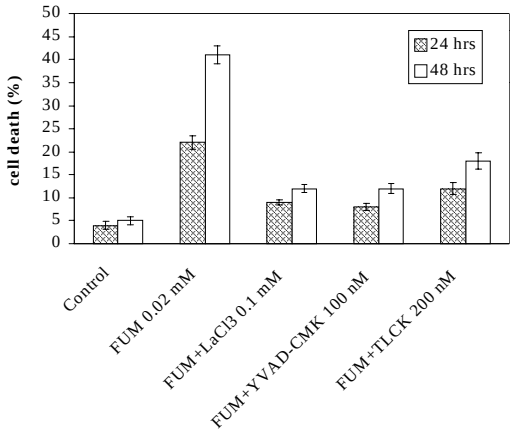

Fig. 5. Inhibition of FUM-induced cell death in tomato cell culture after 24 and 48 hrs. Data are the means and standard errors of at least three independent experiments using different batches of cells.

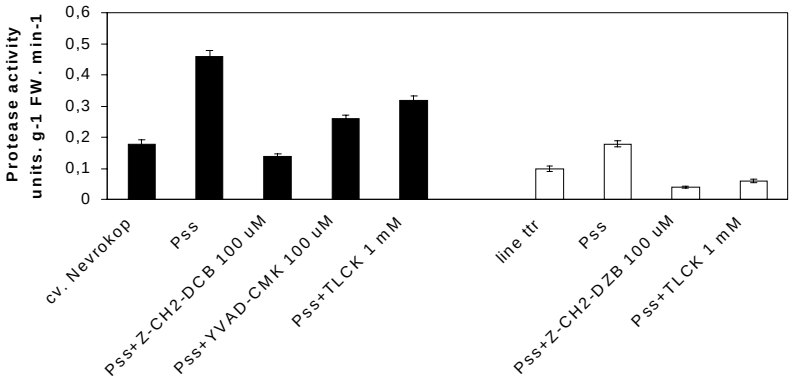

Fig. 6. Effect of protease inhibitors on protease activity in detached tobacco leaves infected with *P. syringae* pv. *tabaci*. Data are means of three independent experiments. Error bars indicate SEM (n=12).

ruthenium red, verapamil, nifedipine) in elicited tobacco suspension cells (37). Other chemical modulators that are able to decrease cytosolic free Ca^{2+}, such as W-7 (N-[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide) and Ca^{2+} chelator EGTA can also inhibit the programmed cell death in plants (27). To study the role of Ca^{2+} in PCD triggering we have assayed the effect of $LaCl_3$ on lesion formation and on the amount of hydrogen peroxide and MDA in tobacco leaves inoculated with *P. syringae* pv. *tabaci*. Also, the effect of $LaCl_3$ on the cell death in tomato suspension cells elicited by FUM was tested. Five fold reduction of FUM-induced tomato cell death was found at the treatment with 1 mM $LaCl_3$ (**Fig. 5**). 10 mM lanthanum inhibited the H_2O_2 production and reduced the content of MDA when it

was co-infiltrated with the bacteria in tobacco leaves (Fig. 3, 4).

The depletion of Ca^{2+} reduces the formation of ROS in cultured spruce cells (62). In tomato suspension La^{3+} effect on H_2O_2 decrease was suggested to cause a blockage of Ca channels, thus preventing the activation of enzymes involved in ROS production (14). Other authors have shown that inhibitors of elicitor-stimulated ion fluxes block the oxidative burst (36). Our results are another indication that calcium is implicated in pathogenesis-triggered deadly signal and plays a role in the control of oxidative burst.

Here we have shown that FUM-induced cell death can be inhibited by serine protease inhibitor and also by caspase inhibitor. In response to 20 μM FUM five fold increase of cell dead over non-treated control cells after 24 hrs and eight-fold after 48 hrs was established. The application of 100 nM Ac-YVAD-CMK to FUM elicited tomato cells reduced the number of dead cells by 65 % after 24 hrs and 71 % after 48 hrs **(**Fig. 5)

Serine protease inhibitor TLCK (200 nM) was assayed for its ability to inhibit FUM-induced cell death and was found to inhibit the cell death by about 46 % and 56 % after 24 hrs and 48 hrs respectively (Fig. 5). These results together with the inhibition of protease activity in bacteria induced lesions and restricted development of the infection indicated that proteolysis is a part of the chemical and bacteria-induced cell death pathway both in tobacco leaves and tomato suspension cells. At the application of peptide inhibitor Z-Asp-CH2-DCB, the activity of proteases was significantly diminished in response to *Pseudomonas* infection (Fig. 6). Lower, but also significant inhibition was measured in response to Ac-YVAD-CMK. Protease activity was inhibited by the caspase inhibitors both in the wild type and transgenic ttr line. Stronger inhibition of proteases was found in *Pseudomonas* infected *ttr* leaves when TLCK was used. This is an indication that serine proteases play a role in the leason formation in the tobacco line tolerant to *P. syringae* pv *tabaci*, where the HR was better pronounced. Our results showed that protease activity in infected tobacco leaves was more effectively suppressed in the presence of Z-Asp-CH2-DCB. Although we have determined only the total protease activity, the effect of Z-Asp-CH2-DCB might be attributed to the participation of broad spectrum caspase-like proteases in *Pseudomonas*-induced cell death. During PCD associated with HR and senescence different caspase-like protease activities have been detected and is has been suggested that this may reflect differences in the mechanism of signal transduction pathway (42). By inhibitory study and determination of protease activity it has been shown that different classes of proteases, including serine, cysteine and caspase-like proteases are implicated in the signal cascade of programmed cell death in megagametophyte cell of white spruce seeds (28).

In summary, our results illustrate that cell death in normal Nevrokop 1164 and *ttr* transgenic tobacco at sites infected with *P. syringae* pv *tabaci* is apoptotic-like and can be abolished by caspase specific inhibitors and serine protease inhibitor. The cell death in the lesions was accompanied with enhanced H_2O_2 accumulation and MDA production in the wild type and with reduced levels of both metabolites in the transgenic line. Lanthanum, caspase inhibitors and TLCK reduced the amount of H_2O_2 and MDA in the affected lesions. FUM-induced cell death in tomato suspen-

sion cells was greatly suppressed by the application of protease inhibitors and lanthanum.

The caspases are highly conserved among animal cell types and to date no true homologues have been identified in plants. Recently, homology searches have revealed a new group of caspase-related cystein proteases in fungi and plants, designated metacaspases (39, 67). Modelling of the three-dimensional protein structure of metacaspases indicates significant tertiary structure homology to animal caspases (the caspase-hemoglobinase fold) (67). Mutational studies in *Trypanosoma brucei* first suggested that metacaspases indeed function as cystein proteinases (66). Recently, it was shown that the only metacaspase present in *Saccharomyces cerevisiae* displays caspase-like proteolytic activity that is activated when yeast is stimulated by hydrogen peroxide to undergo apoptosis (48). The functions of the different plant metacaspases are still unknown. Considering their structural and evolutionary relationship to caspases some of them may exhibit caspase-like activity and may play a role in plant cell death. Alternatively, other, caspase-unrelated, proteases in plants may recognise caspase substrates and may be responsible for apoptotic phenotype in dying plant cells (71). A cell death pathway with similarities to animal apoptosis exists in plants (18, 33, 42) and the dying plant cells show apoptotic hallmarks indicating that a similar death machinery may operate. The striking effect of synthetic and macromolecular caspase inhibitors on cell death in plants and the existence of caspase-related proteases suggest an involvement of caspase-like activity in plant cell death (71). The recent findings that ROS play an important role in animal as well as plant cell death further support the view of functional conservation of cell death pathways between animals and plants.

The interest for improving the resistance of important crops to pathogens has remarkably stimulated the research on the identification of signals produced in plant-pathogen interaction and the biochemical and molecular steps required for the activation of defense mechanisms. Apoptotic machinery designated to impair pathogen spread appear as plant hypersensitive response. Genetically modified resistant plants and suspension cultures represent model systems where apoptotic-like cell death can be induced by phytopathogens and by bacterial and fungal toxins and inhibitors of PCD can be tested. The identification and characterization of genes and metabolic pathways involved in the HR provides a clue to better understanding the process of apoptosis in plants and to establish the evolutionary aspects of PCD similarities between the living creatures. Bacteria and fungi cause serious damage on the plant health and diminish the yield. At pathogen invasion the process of PCD is linked to initiation of resistance (74). HR is involved in incompatible plant-pathogen interactions but besides its role in the infected cells, the HR may coordinate the defense response in neighboring cells. Since the cell death associated with disease symptoms and HR are proposed to share common events, the investigation on underlying biochemical pathways and molecular mechanisms in wild type and transgenic plants may shed more light into resistance mechanisms in plants.

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