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BRIEF COMMUNICATION

# Protein changes during programmed cell death in tobacco

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## Abstract

Programmed cell death (PCD) was induced by the Yariv reagent in *Nicotiana tabacum* cv. Bright Yellow-2 cell suspension. The analyses of proteins extracts by 2-D electrophoresis clearly show massive protein degradation which was mainly due to cysteine protease activity. In contrast, some proteins remained unchanged up to 72 h after PCD induction. Peptide mass fingerprints of these proteins, obtained by MALDI-TOF, identified calreticulin, heat shock protein (HSP) 60, HSP70, malate dehydrogenase and mitochondrial ATP synthase β-subunit.

*Additional key words*: heat shock proteins, calreticulin, *Nicotiana tabacum* BY-2 suspension cells, Yariv reagent.

Programmed cell death (PCD), which occurs in animal and plant cells, is an active cellular suicide process involved in development and stress responses to both biotic and abiotic stimuli (Levine *et al.* 1996). In plants, PCD plays an essential role in senescence (Xie *et al.* 2008), xylogenesis (Demura and Fukuda 1993, Fukuda 1997), aerenchyma formation (Lenochova *et al.* 2009), flower (Sin and Chye 2004) and endosperm development (Young and Gallie 2000), hypersensitive reaction (Del Pozo and Lam 1998, Heath 2000) and heat shock (Fan and Xing 2004). The study of PCD in whole plants can be difficult because this process often occurs in a small group of cells buried in the non-affected tissues. Several plant cell suspensions, *e.g.*, apple (Mizuno *et al.* 2005), *Arabidopsis* (Danon and Gallois 1998, Gao and Showalter 1999, Duval *et al.* 2005, Townley *et al.* 2005), petunia (Beers and Freeman 1997), potato (Mizuno *et al.* 2005), tobacco (Koukalová *et al.* 1997, Chen *et al.* 2000, Herbert *et al.* 2001, Houot *et al.* 2001, Víteček *et al.* 2007), tomato (De Jong *et al.* 2000, Hoeberichts *et al.* 2001) and maize (Stein and Hansen 1999) have been used as model systems to study PCD induced by a wide range of treatments. Our pathogen-free experimental system for PCD induction in tobacco Bright Yellow-2 (BY-2) suspension cells makes use of the $(\beta\text{-D-galactosyl})_3$ Yariv reagent (Chaves *et al.* 2002).

The major systems responsible for proteolysis during animal apoptosis are the caspases, a family of cysteine proteases. Although caspase homologues have not been found in plants, a caspase-like activity was detected in association with the hypersensitive response (Del Pozo and Lam 1998). Also a caspase-related family of proteases, termed metacaspases, was found by plant genomic analysis (Uren *et al.* 2000) and more recently, functional homologues of animal caspases have been identified in plants (Hara-Nishimura *et al.* 2005).

In spite of efforts to elucidate the molecular mechanisms underlying PCD, many of the proteins involved in this process remain unknown. Furthermore, it is known that the information obtained using genomic

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*Abbreviations*: AGPs - arabinogalactan-proteins; ATPase - ATP synthase β-subunit; BY-2 - Bright Yellow-2; CRT - calreticulin; 2;4-D - 2;4-dichlorophenoxyacetic acid; 2-DE - 2-dimensional electrophoresis; IEF - isoelectric focusing; HSP - heat shock protein; MALDI-TOF - matrix assisted laser desorption/ionization-time of fight; MS - mass spectrometry; MDH - malatedehydrogenase; MS medium - Murashige and Skoog medium; PCD - programmed cell death; TBP - tributylphosphine; TCA - trichloroacetic acid.

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tools does not fully reflect the expression of genes at the protein level. In fact, of the eighty cDNAs and ten proteins identified in these studies, only three proteins matched the cDNAs and only the levels of one transcript and its protein varied in a similar way (Swidzinski *et al.* 2002, 2004). These observations encouraged us to focus our attention on a proteomic analysis of PCD induced in tobacco BY-2 cells by the Yariv (1962) reagent [1,3,5-tris(4-β-d-galactopyranosyloxyphenylazo)-2,4,6-trihydroxybenzene].

The *Nicotiana tabacum* L. BY-2 suspension cells were cultured in Murashige and Skoog (MS) medium supplemented with 20 g $dm^{-3}$ sucrose, 2 mg $dm^{-3}$ 2,4-dichlorophenoxyacetic acid (2,4-D, *Duchefa Biochemie*, The Netherlands) and kept under weak irradiance of 9 μmol(photon) $m^{-2}$ $s^{-1}$ at temperature of 24 ºC on a rotatory shaker. In flasks of 250 $cm^3$, 30 $cm^3$ of 4-d-old cultures, at a density of $2 \times 10^6$ cells $cm^{-3}$, were sub-cultured to fresh MS medium with or without β-D-galactosyl Yariv reagent (100 μM), at a final volume of 100 $cm^3$ (Chaves *et al.* 2002). The $(\beta\text{-D-galactosyl})_3$ Yariv reagent was synthesized as previously described (Yariv *et al.* 1962). Cells were collected after 30 min, 1, 2, 4, 6, 12, 24, 48 and 72 h on a *Whatman No. 1* filter paper, immediately frozen in liquid nitrogen and stored at -70 ºC until extraction. From each collected sample an aliquot was stained with either propidium iodide (PI) or Toluidine blue O and observed under the microscope in order to determine the percentage of dead cells, and to check the characteristic cell morphology of cells undergoing PCD. The cell death of about 40 %, was observed 72 h after the beginning of the treatment (Fig. 1) and it reached to 90 % 96 h after PCD induction.

The protein patterns of untreated and of 100 μM Yariv reagent treated cells were analysed by 2-DE. Sample preparation for 2-DE was based on the procedure described by Tsugita and Kamo (1999). Frozen BY-2 cells (2 g) were ground in liquid nitrogen in a cooled mortar. The homogenate was suspended in 3 $cm^3$ of 10 % trichloracetic acid (TCA), 0.07 % β-mercaptoethanol in acetone (-20 ºC), incubated for 2 h at -20 ºC and subsequently centrifuged at 22 000 *g* for 10 min at 4 ºC. The pellet was recovered and washed three times with a solution of 0.07 % β-mercaptoethanol. After centrifugation, the supernatant was discarded and the pellet dried under vacuum at 4 ºC. Proteins were solubilised in rehydration buffer (9 M urea, 4 % (m/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.5 % (v/v) *Triton X-100*, 3 mM tributyl phosphine (TBP) and 0.5 % (v/v) ampholyte-containing buffer (IPG buffer), pH 4 - 7, from *Amersham Biosciences,* Uppsala, Sweden) overnight at 25 ºC and quantified using the *PlusOne 2-D Quant* kit (*Amersham Biosciences*). Isoelectric focusing (IEF) was performed in 24 cm IPG strips (pH 3 - 7, nonlinear) using a *IPGphor* isoelectric focusing unit (*Amersham Biosciences*) at 20 ºC, with the current limited to 50 μA per strip, according to the following protocol: 9 h of rehydration, followed by 10 h at 50 V, 30 min at 300 V, 3 h gradient 300 - 8 000 V, and 13 h at 80 000 V. Prior to the second dimension, the focused IPG strips were equilibrated for 20 min in 15 $cm^3$ of equilibration buffer [6 M urea, 30 % (v/v) glycerol, 2 % (m/v) sodiumdodecyl sulphate (SDS), 300 mM Tris-HCl pH 8.8, 2 mM TBP and a trace of Bromophenol blue]. SDS-PAGE was performed on 12 % polyacrylamide gels (255 × 205 × 1 mm) in the *Ettan DALT* large vertical system (*Amersham Biosciences*) at 15 mA per gel and 15 ºC.

2-DE gels were stained with colloidal Coomassie Blue according to (Neuhoff *et al.* 1988), scanned using the *ImageScanner*™ (*Amersham Biosciences*) and analysed by the Image *Master-Platinum* software *v. 5.0* (*Amersham Biosciences*). For each sample, four gels were run (two different protein extractions and two gel

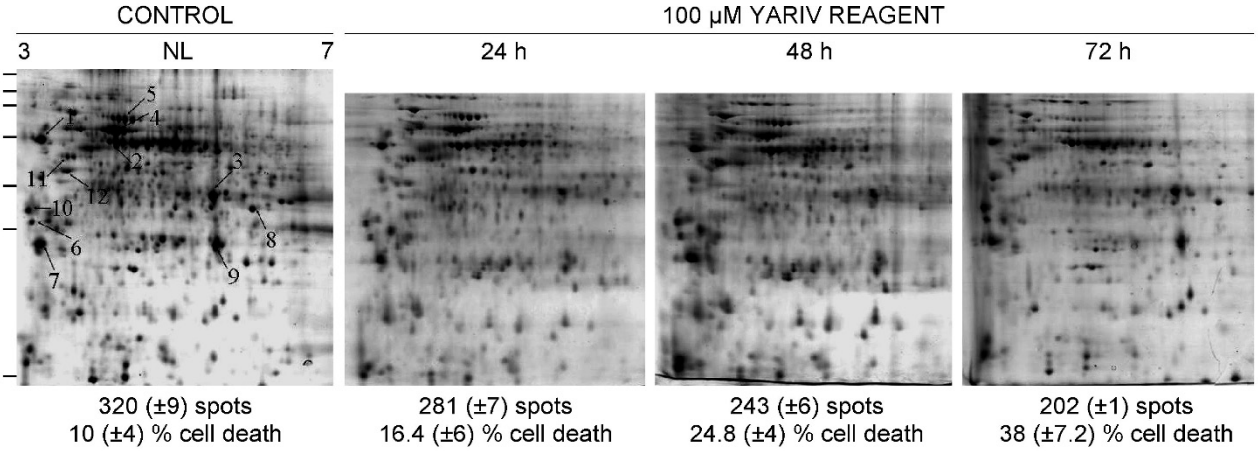


Fig. 1. 2-DE gels of BY-2 cells undergoing PCD induced by 100 μM Yariv reagent, for 24, 48 and 72 h. Total cellular proteins (750 μg) were loaded in 24 cm IPG strips, pH 3 - 7; NL - nonlinear. The second dimension was performed in 12 % SDS-PAGE gels and the gels were stained with Coomassie Blue. Molecular markers are indicated on the left side of the control gel (150, 100, 75, 50, 35, 25 and 15 kDa). The spots excised for protein identification are numbered from 1 to 12. The average number of spots (± SD) present in each gel is indicated. The percentage of cell death over time was evaluated by PI-positive staining.

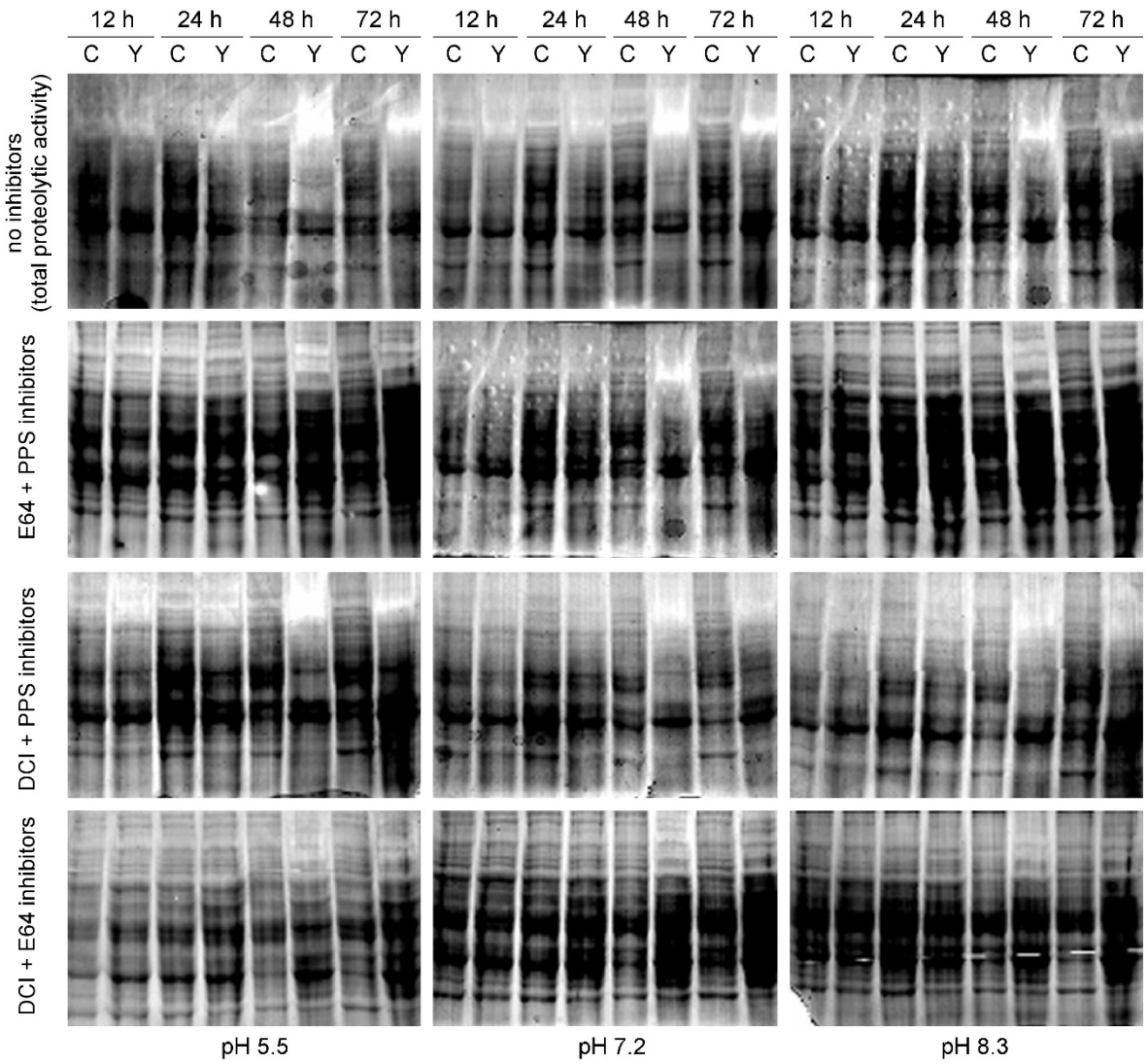


Fig. 2. In-gel protease activity assayed at pH 5.5, 7.2, 8.3 with azoalbumin as substrate in soluble protein fraction from control (C) and Yariv treated (Y) cells for 12, 24, 48 and 72 h. Different protease inhibitors were used to detect activities of specific protease classes (see text for details). The same amount of protein (20 μg) was loaded per lane.

replicates) and only the spots present in every replicate were considered.

Changes in the protein patterns due to the Yariv reagent were only detected for treatments longer than 12 h (Fig. 1). The observed changes consisted in a significant decrease in the total number of polypeptide spots, although many spots were maintained and differently affected by PCD. Surprisingly, no new spots were evident in the gels. Even if the synthesis of new proteins would not be expected during PCD (Elbaz *et al.* 2002), the cleavage of proteins due to increased proteolytic activity could lead to the disappearance of some spots and appearance of new ones. Furthermore, several authors reported that caspase activation involves cleavage of procaspases, and consequently one spot should give rise to two new ones (Li *et al.* 1997, Susin *et al.* 1999, Sutton *et al.* 2003). It is well known that protein cleavage does not occur randomly during apoptosis. The selective resistance of some proteins to degradation during PCD can be indicative of their involvement in the process. Therefore, our approach was focused in the identification of proteins that remained in the gels during PCD induced in BY-2 suspension cells, since they may play a role in this process. After comparing the 2-DE protein patterns, 12 spots were considered relevant for analysis (Fig. 1), excised from the gel and subject to *MALDI-TOF/MS* identification.

Protein identification was performed by NCBInr data base searches, using *MASCOT* software (http://www.matrixscience.com). Accepted significant threshold was at the $P < 0.05$ level. Possible post-translational modifications, such as oxidation of methionine, acetylation of the N-terminal, and carbamidomethylation of cysteines were considered. Five proteins were

identified: calreticulin (spot 1), mitochondrial ATP synthase β-subunit (spot 2), malate dehydrogenase (MDH, spot 3), HSP70 (heat-shock protein) (spot 9) and HSP60 (spot 10). By measuring the volume relative percentage of these spots it was found that calreticulin, HSP60 and ATPase β-subunit did not change up to 72 h from PCD induction. HSP70 remained constant up to 48 h, decreasing afterwards, and malate dehydrogenase remained constant up to 48 h, increasing afterwards. The connection between the identified proteins resistant to proteolysis and the PCD induced in BY-2 cells by the Yariv reagent is still fragmentary, but there appears to be a connection between the subcellular localization of these proteins either in mitochondria (for HSP60, ATPase β-subunit and MDH), or in ER (for calreticulin and HSP70) and the known involvement of these cellular compartments in PCD. Indeed, it is well known the involvement of mitochondria in PCD, and several studies emphasize the important role of ER as a subcellular compartment implicated in the apoptotic execution (Nakagawa *et al.* 2000, Nakamura *et al.* 2000, Rao *et al.* 2004). Additionally, some of the identified proteins (calreticulin and probably MDH) are related to $Ca^{2+}$ which is known to be involved in cell signalling and considered one of the main agents of the PCD process (Krebs 1998, Malhó 1999, Ferri and Kroemer 2001, Giorgi *et al.* 2008).

The decrease in HSP70, 48 h after PCD induction, and the maintenance of HSP60, up to 72 h after PCD induction, may be correlated with the observed acceleration of cell death 72 h after Yariv PCD induction. In animal cells, several studies strongly indicate that HSP70 delays the PCD progression, suggesting an anti-apoptotic role for this protein (Sapozhnikov *et al.* 2002, Jaattela 2004, Sreedhar and Csermely 2004, Joo *et al.* 2005, Stankiewicz *et al.* 2005, Chakraborty *et al.* 2008). Furthermore, in plants it has been reported that the increase in the expression of HSP70 in salicylic acid-mediated apoptosis also correlates with reduced apoptosis (Cronjé *et al.* 2004). HSP60 has a complex role in apoptosis, either preventing or promoting it depending on their subcellular localization (Samali *et al.* 1999, Shan *et al.* 2003, Sreedhar and Csermely 2004). The fact that HSP60 did not change up to 72 h after PCD induction in BY-2 cells, suggests that in this system it may function as an inhibitor of PCD. Indeed, in animal cells cytoplasmic HSP60 was found to sequester several antiapoptotic molecules such as Bax and Bak (Kirchhoff *et al.* 2002). Taken together, these results suggest that the presence of HSP60 and HSP70 during the PCD progress can delay PCD execution in BY-2 cells induced by the Yariv reagent.

Considering the decrease in the number of polypeptide spots observed in the gels, we decided to analyze protein degradation in association with the Yariv treatment. We performed an assay of protease activity in gel at pH 5.5, 7.2 and 8.3, for 12, 24, 48 and 72 h, using azoalbumin as the protease substrate (Fig. 2).Two grams of frozen BY-2 cells were ground in liquid nitrogen using a mortar and pestle. The powder was suspended in 4 $cm^3$ 100 mM Tris-HCl pH 7.2, 30 mM EDTA, 30 mM DTT and incubated for 4 h at 4 ºC and centrifuged at 14 000 *g* for 30 min at 4 ºC. The proteins from the supernatant, precipitated at -20 ºC by acetone in range of 20 - 80 %, were dried under vacuum at 4 ºC, resuspended in 0.8 $cm^3$ of 100 Tris-HCl (pH 7.2) buffer for 3 h at 4 ºC and quantified by the Bradford (1976) method using the *BioRad* (Hercules, USA) kit.

Extracts (20 μg of protein) were subjected to electrophoresis at 4 ºC in a 10 % SDS-PAGE gel (without reducing agents) copolymerized with 0.5 % (m/v) azoalbumin according Henssen and Dowdle (1980). SDS was removed from the gels by incubation the in 2 % Triton X-100, for 30 min at room temperature, followed by an overnight incubation at 37 ºC in either 50 mM Tris-Mes (pH 5.5), 50 mM Tris-HCl (pH 7.2), or 50 mM Tris-HCl (pH 8.3) supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 mM DTT. Different cofactors and pH-values of the incubation buffer were used, to avoid underestimation of the protease activities, since each class of protease requires different cofactors and optimum pH. Since azoalbumin is copolymerised in the gel, bands corresponding to proteases appear unstained after gel staining with Coomassie blue, due to azoalbumin degradation (Fig. 2). In the absence of inhibitors we have observed a unique band of proteolytic activity. Although this band is present in every lane, it was more evident in the extracts of cells undergoing PCD, especially 48 h after induction and at pH 5.5.

To analyse the different classes of proteases the extracts were incubated prior to electrophoresis, for 30 min at 4 ºC, in the presence of the class-specific inhibitors. After the electrophoresis, and SDS/Triton X-100 exchange the gels were incubated as previously described but in the presence of class-specific inhibitors at the following concentrations: 0.1 mM 3,4-dichloroisocumarin (DCI) for serine proteases, 0.03 mM trans-epoxisuccinyl-L-leucilamid-(4-guanidin) butane (E-64) for cysteine proteases and 0.03 mM pepstatin A (PPS) for aspartic proteases (Domínguez and Cejudo 1995). Each gel had a combination of class-specific protease inhibitors in order to detect one class of protease activity at a time. So, for serine protease activity, E64 + PPS, for cysteine protease activity, DCI + PPS and for aspartic protease activity, DCI + E64, were used. When the inhibitor for cysteine proteases (E-64) was absent, a clear band of protease activity was detected, which is more intense in the gel lane of samples treated for 48 h with the Yariv reagent. In the presence of the inhibitors for cysteine and aspartic proteases (E-64 + PPS), as well as in the presence of inhibitors for serine and cysteine proteases (DCI + E-64), this clear band appeared to be located in the same position in all gels, but with lower intensity. The conjugation of these observations suggests that the band observed in the several gels corresponds to only one protease belonging to the specific class of the cysteine proteases, which reach the maximum at 48 h after the beginning of the PCD process. Since cysteine proteases

were the most significant protease activity in the PCD process, the band of activity was more intense at pH 5.5.

We conclude that cysteine proteases are the most active proteases for the protein degradation during the PCD, but this degradation is not random. Furthermore, to our knowledge this is the first work reporting the persistence of HSP60 and HSP70 during plant PCD. These results can be regarded as a starting point for further studies to evaluate the putative involvement of these and other proteins in plant PCD.

## References


Beers, E.P., Freeman, T.B.: Proteinase activity during tracheary element differentiation in *Zinnia* mesophyll cultures. - Plant Physiol. **113**: 873-880, 1997.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Chakraborty, P.K., Mustafi, S.B., Ganguly, S., Chatterjee, M., Raha, S.: Resveratrol induces apoptosis in K562 (chronic myelogenous leukemia) cells by targeting a key survival protein, heat shock protein 70. - Cancer Sci. **99**: 1109-1116, 2008.

Chaves, I., Regalado, A.P., Chen, M., Ricardo, C.P., Showalter, A.M.: Programmed cell death induced by (b-D-galactosyl)$_3$ Yariv reagent in *Nicotiana tabacum* BY-2 suspension-cultured cells. - Physiol. Plant. **116**: 548-553, 2002.

Chen, H.-M., Zhou, J., Dai, Y.-R.: Cleavage of lamin-like proteins in vivo and in vitro apoptosis of tobacco protoplasts induced by heat shock. - FEBS Lett. **480**: 165-168, 2000.

Cronjé, M.J., Weir, I.E., Bornman, L.: Salicylic acid-mediated potentiation of Hsp70 induction correlates with reduced apoptosis in tobacco protoplasts. - Cytometry A **61**: 76-87, 2004.

Danon, A., Gallois, P.: UV-C radiation induces apoptotic-like changes in *Arabidopsis thaliana*. - FEBS Lett. **437**: 131-136, 1998.

De Jong, A.J., Hoeberichts, F.A., Yakimova, E.T., Maximova, E., Woltering, E.J.: Chemical-induced apoptotic cell death in tomato cells: involvement of caspase-like proteases. - Planta **211**: 656-662, 2000.

Del Pozo, O., Lam, E.: Caspases and programmed cell deah in hypersensitive response of plants to pathogenes. - Curr. Biol. **8**: 1129-1132, 1998.

Demura, T., Fukuda, H.: Molecular cloning and characterization of cDNAs associated with tracheary element differentiation in cultured *Zinnia* cells. - Plant Physiol. **103**: 815-821, 1993.

Domínguez, F., Cejudo, F.J.: Pattern of endoproteolysis following wheat grain germination. - Physiol. Plant. **95**: 253-259, 1995.

Duval, I., Brochu, V., Simard, M., Beaulieu, C., Beaudoin, N.: Thaxtomin A induces programmed cell death in *Arabidopsis thaliana* suspension-cultured cells. - Planta **222**: 820-831, 2005.

Elbaz, M., Avni, A., Wiel, M.: Constitutive caspase-like machinery executes programmed cell death in plant cells. - Cell Death Differentiation **9**: 726-733, 2002.

Fan, T., Xing, T.: Heat shock induces programmed cell death in wheat leaves - Biol. Plant. **48**: 389-394, 2004.

Ferri, K.F., Kroemer, G.: Organelle-specific initiation of cell death pathways. - Nat. cell. Biol. **3**: E255-263, 2001.

Fukuda, H.: Tracheary element differentiation. - Plant Cell **9**: 1147-1156, 1997.

Gao, M., Showalter, A.M.: Yariv reagent treatment induces programmed cell death in *Arabidopsis* cell cultures and implicates arabinogalactan protein involvement. - Plant J. **19**: 321-331, 1999.

Giorgi, C., Romagnoli, A., Pinton, P., Rizzuto, R.: Ca2+ signaling, mitochondria and cell death. - Curr. mol. Med. **8**: 119-130, 2008.

Hara-Nishimura, I., Hatsugai, N., Nakaune, S., Kuroyanagi, M., Nishimura, M.: Vacuolar processing enzyme: an executor of plant cell death. - Curr. Opin. Plant Biol. **8**: 404-408, 2005.

Heath, M.C.: Hypersensitive response-related death. - Plant mol. Biol. **44**: 321-334, 2000.

Herbert, R.J., Vilhar, B., Evett, C., Orchard, C.B., Rogers, H.J., Davies, M.S., Francis, D.: Ethylene induces cell death at particular phases of the cell cycle in the tobacco TBY-2 cell line. - J. exp. Bot. **52**: 1615-1623, 2001.

Hoeberichts, F.A., Orzaez, D., van der Plas, L.H., Woltering, E.J.: Changes in gene expression during programmed cell death in tomato cell suspensions. - Plant mol. Biol. **45**: 641-654, 2001.

Houot, V., Etienne, P., Petitot, A.-S., Barbier, S., Blein, J.-P., Suty, L.: Hydrogen peroxide induces programmed cell death features in cultured tobacco BY-2 cells, in a dose-dependent manner. - J. exp. Bot. **52**: 1721-1730, 2001.

Jaattela, M.: Multiple cell death pathways as regulators of tumour initiation and progression. - Oncogene **23**: 2746-2756, 2004.

Joo, M., Chi, J.G., Lee, H.: Expressions of HSP70 and HSP27 in hepatocellular carcinoma. - J. korean med. Sci. **20**: 829-834, 2005.

Kirchhoff, S.R., Gupta, S., Knowlton, A.A.: Cytosolic heat shock protein 60, apoptosis, and myocardial injury. - Circulation **105**: 2899-2904, 2002.

Koukalová, B., Kovarik, A., Fajkus, J., Siroký, J.: Chromatin fragmentation associated with apoptotic changes in tobacco cells exposed to cold stress. - FEBS Lett. **414**: 289-292, 1997.

Krebs, J.: The role of calcium in apoptosis. - Biometals **11**: 375-382, 1998.

Lenochová, Z., Soukup, A., Votrubová, O.: Aerenchyma formation in maize roots. - Biol. Plant. **53**: 263-270, 2009.

Levine, A., Pennell, R.I., Alvarez, M.E., Palmer, R., Lamb, C.: Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. - Curr. Biol. **6**: 427-437, 1996.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., Wang, X.: Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. - Cell **91**: 479-489, 1997.

Malhó, R.: Coding Information in plant cells: the multiple roles of $Ca^{2+}$ as a second messenger. - Plant Biol. **1**: 487-494, 1999.

Mizuno, M., Tada, Y., Uchii, K., Kawakami, S., Mayama, S.: Catalase and alternative oxidase cooperatively regulate programmed cell death induced by beta-glucan elicitor in potato suspension cultures. - Planta **220**: 849-853, 2005.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B.A., Yuan, J.: Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-

beta. - Nature **403**: 98-103, 2000.

Nakamura, K., Bossy-Wetzel, E., Burns, K., Fadel, M.P., Lozyk, M., Goping, I.S., Opas, M., Bleackley, R.C., Green, D.R., Michalak, M.: Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis. - J. cell. Biol. **150**: 731-740, 2000.

Neuhoff, V., Arold, N., Taube, D., Ehrhardt, W.: Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. - Electrophoresis **9**: 255-262, 1988.

Rao, R.V., Ellerby, H.M., Bredesen, D.E.: Coupling endoplasmic reticulum stress to the cell death program. - Cell Death Differentiation. **11**: 372-380, 2004.

Samali, A., Cai, J., Zhivotovsky, B., Jones, D.P., Orrenius, S.: Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of jurkat cells. - EMBO J. **18**: 2040-2048, 1999.

Sapozhnikov, A.M., Gusarova, G.A., Ponomarev, E.D., Telford, W.G.: Translocation of cytoplasmic HSP70 onto the surface of EL-4 cells during apoptosis. - Cell. Prolif. **35**: 193-206, 2002.

Shan, Y.X., Liu, T.J., Su, H.F., Samsamshariat, A., Mestril, R., Wang, P.H.: Hsp10 and Hsp60 modulate Bcl-2 family and mitochondria apoptosis signaling induced by doxorubicin in cardiac muscle cells. - J. mol. cell. Cardiol. **35**: 1135-1143, 2003.

Sin, S.F., Chye, M.L.: Expression of proteinase inhibitor II proteins during floral development in *Solanum americanum*. - Planta **219**: 1010-1022, 2004.

Sreedhar, A.S., Csermely, P.: Heat shock proteins in the regulation of apoptosis: new strategies in tumor therapy: a comprehensive review. - Pharmacol. Theor. **101**: 227-257, 2004.

Stankiewicz, A.R., Lachapelle, G., Foo, C.P., Radicioni, S.M., Mosser, D.D.: Hsp70 inhibits heat-induced apoptosis upstream of mitochondria by preventing Bax translocation. - J. biol. Chem. **280**: 38729-38739, 2005.

Stein, J.C., Hansen, G.: Mannose induces an endonuclease responsible for DNA laddering in plant cells. - Plant Physiol. **121**: 71-79, 1999.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Brenner, C., Larochette, N., Prevost, M.C., Alzari, P.M., Kroemer, G.: Mitochondrial release of caspase-2 and -9 during the apoptotic process. - J. exp. Med. **189**: 381-394, 1999.

Sutton, V.R., Wowk, M.E., Cancilla, M., Trapani, J.A.: Caspase activation by granzyme B is indirect, and caspase autoprocessing requires the release of proapoptotic mitochondrial factors. - Immunity **18**: 319-329, 2003.

Swidzinski, J.A., Leaver, C.J., Sweetlove, L.J.: A proteomic analysis of plant programmed cell death. - Phytochemistry **65**: 1829-1838, 2004.

Swidzinski, J.A., Sweetlove, L.J., Leaver, C.J.: A custom microarray analysis of gene expression during programmed cell death in *Arabidopsis thaliana*. - Plant J. **30**: 431-446, 2002.

Townley, H.E., McDonald, K., Jenkins, G.I., Knight, M.R., Leaver, C.J.: Ceramides induce programmed cell death in *Arabidopsis* cells in a calcium-dependent manner. - Biol. Chem. **386**: 161-166, 2005.

Tsugita, A., Kamo, M.: 2-D electrophoresis of plant proteins. - Methods mol. Biol. **112**: 95-97, 1999.

Uren, A.G., O'Rourke, K., Aravind, L.A., Pisabarro, M.T., Seshagiri, S., Koonin, E.V., Dixit, V.M.: Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. - Mol. Cell **6**: 961-967, 2000.

Víteček, J., Wunschová, A., Petřek, J., Adam, V., Kizek, R., Havel, L.: Cell death induced by sodium nitroprusside and hydrogen peroxide in tobacco BY-2 cell suspension. - Biol. Plant. **51**: 472-479, 2007.

Xie, Q.E., Liu, I.D., Yu, S.X., Wang, R.F., Fan, Z.X., Wang, Y.G., Shen, F.F.: Detection of DNA ladder during cotyledon senescence in cotton - Biol. Plant. **52**: 654-659, 2008.

Yariv, J., Rapport, M.M., Graf, L.: The interaction of glycosides and saccharides with antibody to the corresponding phenylazo glycosides. - Biochem. J. **85**: 383-388, 1962.

Young, T.E., Gallie, D.R.: Programmed cell death during endosperm development. - Plant mol. Biol. **44**: 283-301, 2000.