The presence and subcellular localization of caspase 3-like proteinases in plant cells

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Abstract Caspases play a very important role in initiating and executing apoptotic processes in animal cells. In this study we show that plant mitochondria were able to initiate the activation of caspase 3 in a *Xenopus* cell free system. Caspase 3-like activity was found to be present in plant cells and could only be inhibited by the specific caspase 3 inhibitor *N*-acetyl-Asp-Glu-Val-Asp-fluoromethylketone (Ac-DEVD-fmk) and not by cysteine protease inhibitors. By micro-injection of the caspase 3 substrate in living *Chara* cells we showed that caspase 3-like activity was mainly present in the cytosol rather than in the vacuole. This is the first time that in vivo caspase 3-like activity has been demonstrated in plants.

Key words: Caspase 3; Mitochondrion; Micro-injection; Apoptosis; Plant; *Chara*

1. Introduction

Programmed cell death (PCD) is a gene-directed program during development and response to environmental stress or pathogenic attack, in which the cell directs its own death. In plants, PCD was found to occur during developmental processes like flower development, embryogenesis, seed germination, vessel and trachea formation and during hypersensitive response (HR) to pathogenic attack [1]. From recent studies it became clear that biochemical and morphological hallmarks of apoptosis, like cytoplasmic shrinkage, nuclear condensation, membrane blebbing, calcium influx and DNA laddering are similar in animal and plant cells ([2] and references therein). A key event in animal PCD is the release of cytochrome *c* from mitochondria into the cytosol, forming a complex together with Apaf1 and dATP and triggering caspase activation [3]. The cytosolic caspase-mediated apoptotic pathway is highly conserved between animal cells but such a death cascade was not found in plant cells thus far. However, recent studies reveal that there are some similarities between plant PCD and the animal caspase-mediated apoptotic program. Plant cells undergoing PCD induced by HR express so-called plant diseases resistance genes like the *N*-gene product [4]. Similar to Apaf1, the *N*-gene product possesses an amino-terminal nucleotide binding domain and a carboxy-terminal domain implicated in protein–protein interactions. In tobacco cells it was demonstrated that caspase 1-like proteases may participate in this *N*-gene mediated HR [5]. Interestingly, Lacomme and Santa Cruz recently showed that murine Bax, a member of the Bcl-2 protein family, triggers a PCD pathway similar to the PCD pathway induced by *N*-gene mediated HR when expressed in tobacco cells [6]. Induction of PCD requires attachment of Bax to the mitochondrial membrane [6]. In yeast, Bax induced PCD could be blocked by expression of the human Bax-inhibitor-1 [7] and recently a plant homologue of Bax-inhibitor-1 was isolated from rice and *Arabidopsis* and its expression in yeast resulted in suppression of cell death induced by mammalian Bax [8]. Together with the findings that Bax stimulates the release of cytochrome *c* from yeast mitochondria [9] and that release of cytochrome *c* from mitochondria to cytosol occurs during heat-induced PCD in cucumber plants [10], these data indicate that plants and animals may share the initial step in the caspase cascade. To further confirm this hypothesis, determination of caspase activity and its localization is the next step.

Caspases belong to a group of conserved cysteinyl-aspartate which cleave very specific peptide substrates after Asp residues [11]. Caspase activation starts with autoprocessing of procaspase 9 to the active caspase 9 which cleaves procaspase 3 to the active caspase 3, the main executioner of the final degradation phase of the animal cell death program.

In spite of the fact that sequences of the highly conserved members of the caspase protein family have not been found in most current genomic databases and expressed sequence tags (ESTs) from plant cells yet, a lot of cysteine and aspartate proteinases are described to be involved in plant PCD occurring during HR [5], senescence [12], differentiation of tracheal elements [13], oxidative stress [2] and seed germination [14,15]. However, proteinase activity with properties similar to apoptotic initiating or executing caspases (e.g. caspase 3) were not found in plant cells thus far although some studies strongly indicate that such caspase-like activities may exist in plant cells. For example, it was shown that poly-(ADP-ribose) polymerase (PARP), a specific substrate for caspase 3 during the apoptotic process in animal cells, is involved in H_2O_2-induced PCD in plant cells [16]. Equally to the animal apoptotic pathway, degradation of plant PARP is dependent on cytochrome

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Abbreviations: Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; Ac-YVAD-AMC, *N*-acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin; APW, artificial pond water; DAF, days after flowering; d-ft, dextran conjugated fluorescein-tetramethylrhodamine; DTT, dithiothreitol; fmk, fluoromethylketone; EDTA, ethylenediaminetetraacetic acid; HR, hypersensitive response; PARP, poly-(ADP-ribose) polymerase; PCD, programmed cell death; PMSF, phenylmethylsulfonyl fluoride; PVP, polyvinylpyrrolidone

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c release into the cytosol and could be inhibited by specific caspase 3 inhibitors [17].

In this study we describe the presence of caspase 3-like proteinase activity in plant cells as judged from specific cleavage of caspase 3 substrates in in vitro as well in in vivo plant systems.

2. Materials and methods

2.1. Plant material

Embryonic cell suspensions were from barley (*Hordeum vulgare* L. cv. Igri) immature in vivo embryo (12 days after flowering (DAF))-derived callus. Cells were grown in dark at 25°C by gently shaking in culture medium I as described by Hoekstra et al. [18] with a few modifications: 1650 mg/l NH_4NO_3 instead of 165; maltose and sucrose were replaced by glucose (60 g/l); benzyl aminopurine was replaced by 5 mg/l 2,4-dichlorophenoxyacetic acid; Ficoll 400 and Agarose HMT were omitted.

Chara corallina was cultured as previously described [19].

2.2. Isolation of mitochondria from embryonic suspension cells

Embryonic suspension cells (about 100 g) were harvested by filtration over 1 layer Whatman paper no. 1. For isolation of mitochondria, the residue was homogenized with 20 ml homogenization medium (20 mM HEPES–KOH pH 7.2, 0.35 M mannitol, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.7% polyvinylpyrrolidone (PVP)-25, 2 mM cysteine–HCl and 0.2% bovine albumin) using an Ultrathurrax (Janke and Kunkel, Staufen, Germany; three pulses at 24 000 rpm of 30 s each with 30 s intervals). The homogenate was centrifuged at $2000\times g$ for 5 min and the supernatant was centrifuged again for 10 min at $10\,000\times g$ to obtain the microsomal and mitochondrial pellet. Fractions enriched in mitochondria were isolated from the pellet fraction by its resuspending in 0.5 ml washing buffer (20 mM HEPES–KOH pH 6.8, 0.35 M mannitol, and 0.1% bovine albumin), followed by purification on 21% (v/v) Percoll in washing buffer by centrifugation at $50\,000\times g$ for 20 min. The mitochondria containing pellet was gently removed by pipette and washed two times in 10 ml washing buffer. The mitochondrial pellet was resuspended in 0.5 ml washing buffer and the presence of mitochondria in the pellet examined by staining with nonyl acridine orange (Molecular Probes Europe B.V., Leiden, The Netherlands). The enrichment of mitochondria in the mitochondrial fraction as compared to the microsomal fraction as well as the integrity of the mitochondria was examined by reduction of exogenous cytochrome *c* by succinate–cytochrome *c* reductase [20].

2.3. Caspase assays

Caspase 3 activity was measured in a *Xenopus* cell free system as described by Kluck et al. [21]. Fractions of 5 μl *Xenopus* egg cytosol were incubated in 100 μl assay buffer (20 mM HEPES–KOH pH 7.5, 250 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$ and 1 mM dithiothreitol (DTT)) in presence or absence of purified plant mitochondria. The reaction was started after addition of 70 μM of the fluorogenic peptide substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC; Clontech, Palo Alto, CA, USA). After incubation for 30 min at 30°C, the reaction was stopped by the addition of 50 μl stop buffer (1% sodium acetate trihydrate in 175 mM acetic acid). The fluorescence was measured after addition of 1 ml H_2O in a Perkin Elmer fluorescence spectrometer LS50B (Perkin Elmer, Norwall, CT, USA) at 380/460 nm.

For in vitro caspase activities in plants, about 10 g barley embryonic suspension cells were homogenized in ice-cold HSDTP buffer (25 mM HEPES–KOH pH 7.5, 10% w/v sucrose, 1 mM DTT, 0.1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF)) using an Ultrathurrax (three pulses at 24 000 rpm of 30 s each with 30 s intervals). The homogenate filtered through three layers of cheesecloth and the filtrate centrifuged at $2000\times g$ for 10 min followed by 30 min $25\,000\times g$ at 4°C. Fractions of 10 μl of the supernatant were diluted to 100 μl with HSDTP buffer without Triton X-100 and incubated for 2 h at 30° in presence of 70 μM Ac-DEVD-AMC or 70 μM *N*-acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin (Ac-YVAD-AMC) (for caspase 1, Calbiochem, San Diego, CA, USA). The reaction was terminated by the addition of 50 μl stop buffer and the fluorescence was measured after addition of 1 ml H_2O in a Perkin Elmer luminescence spectrometer LS50B at 380/460 nm. Non-specific cleavage was determined by addition of 150 μM Ac-YVAD-fluoromethylketone (fmk) (for caspase 1) or 150 μM Ac-DEVD-fmk (for caspase 3).

2.4. Micro-injection

Micro-injection was preceded by overnight incubation of separated *Chara* internodal cells in artificial pond water (APW: 0.1 mM $CaCl_2$, 0.1 mM NaCl, 0.1 mM KCl and 2 mM HEPES, pH 7.1). Prior injection, cells were incubated for about 1 min in a high osmolarity solution (200 mM sorbitol in APW) to induce turgor decrease, then placed on a dry plexi plate, on a stereo microscope table. Injection was performed using a volume injector (Micro1, World Precision Instruments). Glass pipettes without internal filament (Clark Electromedical Instruments, Pangbourne Reading, RG8 7HU-England, type GC150-15) were pulled with a Narishige two-stage puller (Tokyo, Japan, Model PB-7), loaded with mineral oil (DryStrip Cover Fluid, Pharmacia Biotech), and back-filled with the sample. The cannula-shaped pipette tip was fire-polished, the final diameter of the tip being about 3–5 μm. After injection cells were stored in the sorbitol APW-solution for about 5 min, then incubated for at least 90 min in APW before fluorescence microscopy.

2.5. Fluorescence microscopy

Fluorescence microscopy was performed on a Axioplan Microscope (Zeiss, Germany). The filters utilized were 365/450 nm for AMC-fluorescence and 510–590 nm for dextran conjugated fluorescein-tetramethylrhodamine (d-ft) fluorescence. The magnification was 200×. The final concentrations of the injected probes were: 70 μM for Ac-DEVD-AMC, 150 μM for Ac-DEVD-fmk and 300 nM for d-ft.

3. Results and discussion

3.1. Plant components interfere with a caspase 3 activating cascade in a Xenopus cell free system

The *Xenopus* cell free system, based on cytosolic extracts from *Xenopus* eggs, was shown to be an useful tool to study caspase activating cascades [21]. We used this system to find out whether plant mitochondria and plant cytochrome *c* were able to activate caspase 3 in *Xenopus* cytosol equivalent to their animal counterparts. Therefore, we isolated mitochondria from barley embryonic suspension cells by use of a Percoll gradient. As judged from specific activity from the mitochondrial succinate–cytochrome *c* reductase, this purification step resulted in a 40-fold enrichment of mitochondria in the purified fraction (814 nmol cytochrome *c* reduced/min/mg protein in the purified fraction and 20 nmol cytochrome *c* reduced/min/mg protein in the microsomal fraction, respectively) and the presence of mitochondria was further confirmed by nonyl acridine orange staining. Moreover, the succinate–cytochrome *c* reductase assay assessed that about 80% of the isolated mitochondria remained intact during purification. The purified mitochondrial fraction was subjected to one freeze–thaw cycle to damage the mitochondrial membranes and to release their soluble content and subsequently caspase 3 activity was measured. No caspase 3 activity could be detected, as judged from specific cleavage of the caspase 3 substrate Ac-DEVD-AMC (Fig. 1A). However, addition of these freeze-thawed mitochondria to the *Xenopus* cell free system resulted in a three-fold increase of the caspase 3 activity as compared to its basic activity (Fig. 1A). Both, basic activity and its activation by the mitochondrial extract was blocked by addition of the specific caspase 3 inhibitor Ac-DEVD-fmk (Fig. 1A). After addition of the mitochondrial extracts during different experiments, we observed a large variation in the amount of activation of caspase 3 in the *Xenopus* cell free system. Therefore, we tested whether the number of mito-

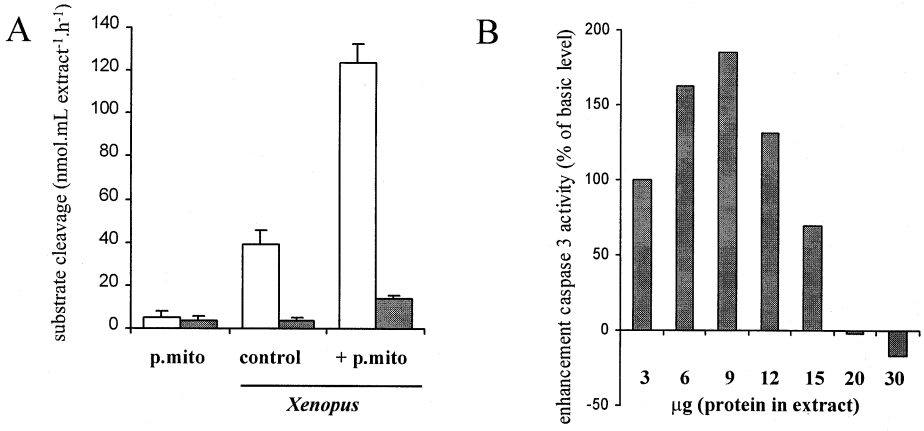

Fig. 1. Activation of caspase 3 in *Xenopus* cell free extracts. A: Activation of caspase 3 activity after addition of plant to *Xenopus* cell free system. p.mito, caspase 3 activity in protein extracts from purified freeze-thawed plant mitochondria; control, basic level of caspase 3 activity in *Xenopus* cell free system; +p.mito, caspase 3 activity in *Xenopus* cell free system after addition of protein extracts from purified freeze-thawed plant mitochondria. The protein content in p.mito was 10 μg. Open bars represent total substrate cleavage, gray bars represent non-specific cleavage after addition the specific caspase 3 inhibitor Ac-DEVD-fmk. Data are means of five independent experiments. Bars represent S.D. B: Relative enhancement of caspase 3 activity in *Xenopus* cell free system after addition of different amounts of plant mitochondrial extracts as compared to its basic level in *Xenopus* cell free system. Values of the amounts of mitochondrial extracts added are in μg total protein in these extracts.

chondria added to the *Xenopus* cell free system was responsible for this variation. In a typical experiment, in which we added different amounts of mitochondrial extracts obtained from one batch of isolated plant mitochondria, we showed that enhancement of the basic caspase 3 was dose- dependent (Fig. 1B). Interestingly addition of high amounts of plant mitochondria (>10 μg total protein in purified extracts) abolished this enhancement and, moreover, addition of concentrations above 20 μg even resulted in inhibition of the caspase 3 activity. It has been demonstrated that endogenous proteins belonging to the inhibitor of apoptosis (IAP) family can block cytochrome *c*-induced apoptosis in cell free extracts from embryonic kidney cells by direct interaction with caspase 9 and thereby preventing the activation of caspase 3 [22]. Also, the baculovirus p35 protein is able to inhibit caspase 3 activity [23]. Although, the presence of such caspase inhibitory proteins is not demonstrated in plant cells thus far, the finding that transgenic expression of the baculovirus p35 protein in tobacco resulted in a delay of HR-induced cell death suggests that such a regulatory mechanism may exist in plant cells [24].

3.2. *Caspase 3-like proteinases in plant cells and their biochemical features*

The fact that mitochondria from barley embryonic suspension cells are able to induce caspase 3 activation in the *Xenopus* cell free system together with the recent findings that cytochrome-*c* was translated from mitochondria to the cytosol during heat-induced PCD in cucumber plants [10] and PARP, a substrate for caspase 3 during animal apoptosis, was found to be involved in plant PCD [16,17], suggest that plants may have a caspase activating pathway and therefore caspase 3-like activity. In order to measure this activity we applied the caspase 3 specific substrated Ac-DEVD-AMC to cytosolic extracts from barley embryonic suspension cells. Indeed addition of the substrate resulted in a fluorescent signal caused by cleavage of the fluorescent moiety after the aspartate residue of the peptide (Fig. 2A, control). In order to test whether the Ac-DEVD-AMC cleavage was executed by regular (cysteine) protease activity or rather by specific caspase 3-like proteins, we included different inhibitors during this caspase assays. As depicted in Fig. 2A, the protease inhibitors PMSF, leupeptin, E64 or a cocktail of several serine, cysteine and metallo protease inhibitors (Complete™, Boehringer Mannheim, Germany) did not inhibit substrate cleavage. However, only when the specific caspase 3 inhibitor Ac-DEVD-fmk was included during the assay the proteolytic activity was suppressed significantly (Fig. 2B). Caspase 1-like activity and its inhibition by the specific caspase 1 inhibitor Ac-YVAD-fmk in plant cells was reported earlier [5]. In our experiments we showed that the caspase 3-like activity was not sensitive for the specific caspase 1 inhibitor, whereas caspase 1-like activity, as judged from cleavage of the caspase 1-substrate Ac-YVAD-AMC, could be suppressed by both caspase 1 and 3 inhibitor (Fig. 2B).

During the performance of the caspase assay two interesting phenomena were observed. First we marked that it was very difficult to detect caspase 3-like activity in buffers with high salt concentration (e.g. buffers with a physiological salt concentration or anion exchange fractions with NaCl concentrations of 100 mM and above). In a caspase assay with an increasing molarity of NaCl we found that caspase 3-like proteinase activity was fairly suppressed at concentrations above 10 mM (Fig. 2C). Similar results were found for caspase 1-like activity (Fig. 2C). The observation that caspase 1-like activity was suppressed in in vitro assays during physiological ionic conditions (e.g. 150 mM NaCl) may explain the discrepancy in literature whether caspase 1-like activities in plant cells could be detected or not [2,5].

Second, addition of stop buffer in the caspase assay, containing 175 mM acetic acid, did not stop the caspase 3-like activity but strongly enhanced the rate of substrate cleavage. Therefore, we tested in a caspase assay the effect of pH on both caspase 1 and 3-like activity. As depicted in Fig. 2D, lowering the pH below the physiological pH enhanced the caspase 3-like activity dramatically, with a pH optimum of 6.5. The caspase 1-like activity was not enhanced by low pH (Fig. 2D). Again, addition of the specific caspase 3 inhibitor Ac-DEVD-fmk completely suppressed the caspase 3-like ac-

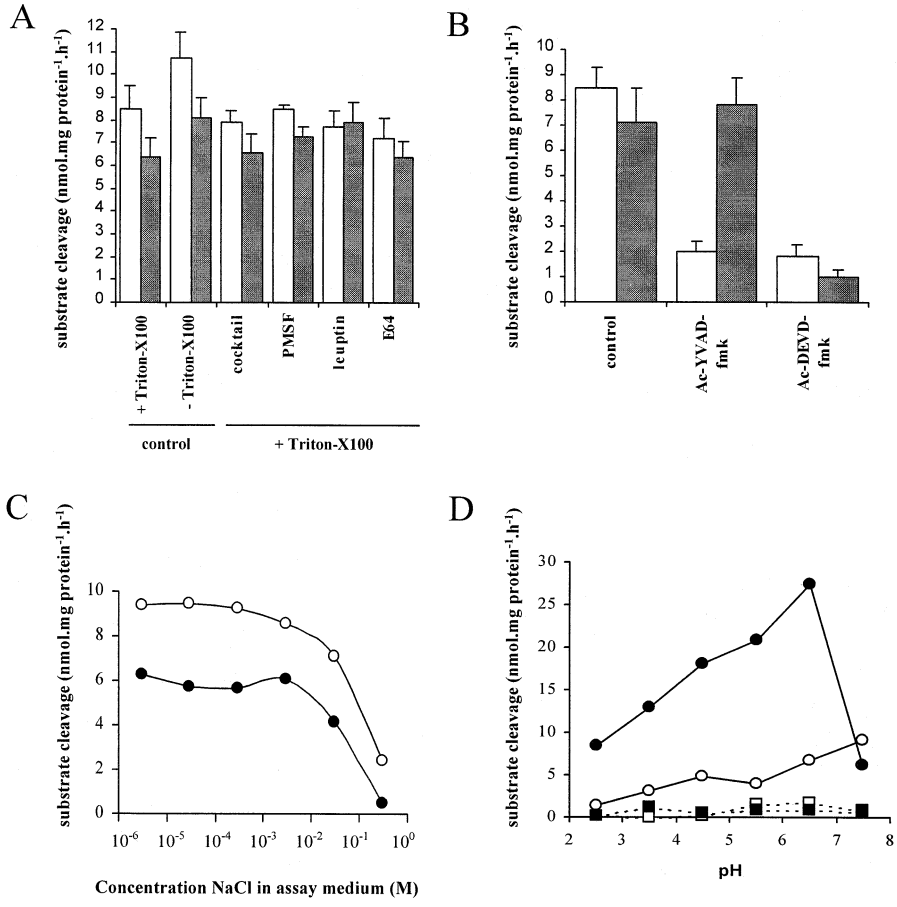

Fig. 2. Biochemical features of plant caspase 1 and 3-like proteinase. A: Effects of proteinase inhibitors on caspase 1 (open bars) and caspase 3 (gray bars) activity in cytosolic extracts of barley embryonic suspension cells. Concentrations of inhibitors cocktail, PMSF, leupeptin and E64 were 0.5 mM. Bars represent +S.D., $n=3$. B. Effects of the specific caspase 1 inhibitor YVAD-fmk and the specific caspase 3 inhibitor DEVD-fmk on caspase 1 (open bars) and caspase 3 (gray bars) activity in cytosolic extracts of barley embryonic suspension cells. Concentrations of both inhibitors were 150 μM. Bars represent +S.D., $n=5$. C: Inhibitory effects of NaCl on both caspase 1 (○) and caspase 3 (●) activities in cytosolic extracts. Values were corrected for non-specific activity measured in presence of Ac-YVAD-fmk (caspase 1) and Ac DEVD-fmk (caspase 3). Measurements were performed in duplo. D: Effects of acidification on caspase 3 (●) and caspase 1 activity (○) during extraction of cytosolic proteins from barley embryonic suspension cells. Both activities were completely blocked by addition of the specific caspase 3 inhibitor Ac-DEVD-fmk (■) or caspase 1 inhibitor Ac-YVAD fmk (□). Cytosolic proteins were extracted in modified CAB buffers containing 25 mM glycine–HCl (pH 2.5 or 3.5), 25 mM acetate/acetic acid (pH 4.5 or 5.5), 25 mM MES–Tris (pH 6.5) and 25 mM Tris–HCl (pH 7.5) instead of HEPES–KOH (7.5). Measurements were performed in duplo. Unless otherwise stated, all cytosolic extracts were made in a buffer containing 0.1% Triton X-100.

tivity. This suggests that the increased activity of the caspase 3-like proteinase at low pH was not caused by spontaneous cleavage of the substrate due to acidification but probably by autocatalization of the caspase 3-like proteinase, by breaking pH sensitive electrostatic interactions between the different domains of the protein. Addition of other protease inhibitors as mentioned in Fig. 2A did not affect the enhanced caspase 3-like activity (data not shown). Furthermore, the fact that in our experiments the caspase 3-like activity but not the caspase 1-like activity was pH sensitive strongly suggests that there are, at least, two different caspase-like proteinases in plants. This suggestion was supported by the observation that the caspase 3-like activity could not be inhibited by the inhibitor specific for caspase 1.

3.3. Subcellular localization of caspase 3-like activity

Now we have demonstrated the presence of caspase 3-like proteinase activity in plant cells, the question arises whether animal and plant cells share the same activation cascade of these proteins. Thus far, subcellular localization studies of cysteine proteases involved in plant PCD showed that these proteins were found to be present in vacuoles or storage organelles [14,15]. However if a caspase 3 activation cascade exists in plant cells and like in animal cells cytochrome *c* released from the mitochondria induces this cascade, one expects to find caspase 3-like activity to be present in the cytosol rather than in vacuolar vesicles or storage organelles. The first indication that the caspase 3-like activity was cytosolic came from our in vitro studies. Cytosolic proteins were extracted from suspension cells in a buffer containing Triton X-100. A concentration of 0.1% Triton X-100 disrupts the membranes and therefore releases the proteins stored in different organelles. These proteins may cause or modulate the caspase 3-like activity. However, if Triton X-100 was excluded from the extraction buffer, no decrease of specific caspase 3 activity was observed (Fig. 2A). The observation that the specific caspase 3-like activity in extracts without Triton X-100 was even higher as compared with extract isolated in presence of Triton

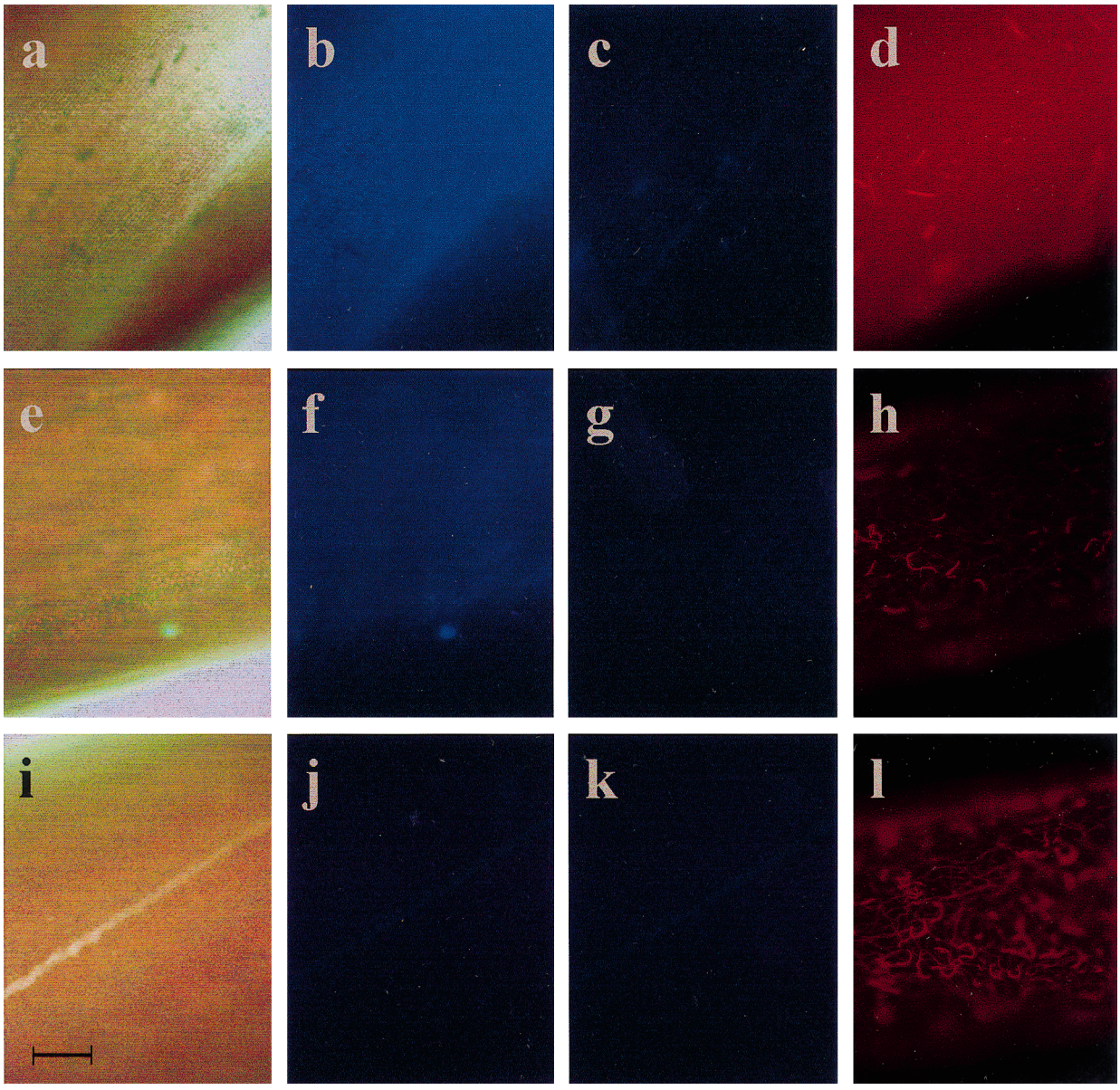

Fig. 3. Subcellular localization of caspase 3-like proteinase activity assayed by micro-injection of the specific substrate Ac-DEVD-AMC in *Chara* cells. a–d: substrates injected into cytosol; e,f: substrates injected in vacuole; i–l: control cells injected in the cytosol with H_2O. Substrates injected were: Ac-DEVD-AMC (b and f); Ac-DEVD-AMC+inhibitor Ac-DECD-fmk (c and g); Ac-DEVD-fmk (k); d-ft (l) and Ac-DEVD-AMC+d-ft (d and h). a,e and i are visible light microscopy images, all the rest are fluorescence microscopy images. Bar represents 100 μm.

X-100 was caused by the difference in the total protein concentration of both fractions. These results point to the fact that plant cells do have a basic caspase 3-like proteinase activity presence in the cytosol and which seems not to be modulated by factors released from organelles. In order to confirm these data obtained from in vitro analysis, we gathered the fluorogenic caspase 3 substrate in giant living *Chara* cells by micro-injection after which the caspase 3-like activity was monitored by fluorescence microscopy. In this in vivo system, we first injected the substrate into the cytoplasm of the cell (Fig. 3a–d). About 1 h after injection fluorescence was visible along the entire cell (Fig. 3b). Co-injection of the specific caspase inhibitor Ac-DEVD-fmk strongly reduced the fluorescence (Fig. 3c) whereas in a control experiment, in which *Chara* cells were only injected with buffer, no fluorescence could be detected at all (Fig. 3i for light microscopy and Fig. 3j,k for fluorescence microscopy). Furthermore we co-injected the high molecular weight conjugate d-ft together with the caspase substrate. The fluorescent signal of this dual labeled conjugate is pH dependent and will be quenched at low (vacuolar) pH. Moreover, the high molecular size of the dextran will prevent distribution to other compartments than the one injected in. By co-injection of this conjugate with the caspase 3 substrate we were able to prove whether the caspase 3 substrate was injected into the cytoplasm or into the vacuole. As depicted in Fig. 3d, a rather strong d-ft fluorescent signal was present in cells in which d-ft together with the caspase 3 substrate was injected. These cells also showed Ac-DEVD-AMC fluorescence. This indicates that our injection system is able to specifically inject substances into the

cytoplasm. Hence, results as shown in Fig. 3b can be attributed to be specific cytoplasmic. If Ac-DEVD-AMC together with d-ft was directly injected in the vacuole of the *Chara* cells (see Fig. 3e–h), only a weak background signal from the d-ft conjugate could be detected (Fig. 3h). This signal was probably caused by autofluorescence of filamentous structures in the *Chara* cells, since the control cells without d-ft conjugate (Fig. 3l) displayed the same signal. In the vacuoles a weak signal caused by specific Ac-DEVD-AMC cleavage was visible (Fig. 3f,g) indicating that next to the cytosol, caspase 3-like activity could be found in the vacuole. However the signal was less strong as compared to the signal found in the cytosol and moreover the low pH of the vacuole may elevate the activity artificially since we demonstrated that low pH increases caspase 3-like activity.

Although these data obtained from in vitro and in vivo measurements clearly demonstrate that caspase 3-like activity is present in plant cells and it is mainly located in the cytoplasm, it is not clear whether or how these proteins are involved in plant PCD yet. The observation that addition of Triton X-100, which disrupts mitochondrial membranes and may release cytochrome *c* during extraction of cytosolic proteins, does not elevate the caspase 3-like activity indicates that the substrate cleavage in these plant cells is caused by a basic level of caspase 3-like proteinase activity, rather than induction of its activity by apoptotic stimuli (e.g. cytochrome *c* release). Moreover, the observation that *Chara* cells injected with the specific caspase 3 substrate were viable for at least 4 weeks after injection, as judged from cytoplasmic streaming as compared to control cells, again indicates that cleavage of the substrate was caused by a basic level of active caspase 3-like proteinases and the presence of an active caspase 3-like proteinase in plant cytoplasm in itself is not ample for executing the PCD process. By co-injection of key components of the animal caspase cascade together with these caspase specific substrates in *Chara* cells we hope to find whether and how this caspase 3-like proteinase activity is involved in executing plant PCD. In addition, micro-injection of *Chara* cells appears to be a very powerful tool for further study of the PCD process in plant cells in vivo and probably will give an answer whether plants and animals may share a common apoptotic pathway.

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