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RESEARCH PAPER

Developmental regulation of a VEIDase caspase-like proteolytic activity in barley caryopsis

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Abstract

Caspases are essential in animal programmed cell death both as initiator and executioner proteases. Plants do not have close caspase homologues, but several instances of caspase-like proteolytic activity have been demonstrated in connection with programmed cell death in plants. It was asked if caspase-like proteases are involved during development of the barley caryopsis. The presence of a caspase-6-like proteolytic activity that preferentially cleaved the sequence VEID was demonstrated. A range of protease inhibitors was tested and only caspase-specific inhibitors showed major inhibitory effects. The profile of VEIDase activity in developing starchy endosperm, embryo, and whole caryopsis was measured and showed a general trend of higher activity in young, rapidly developing tissues. The VEIDase activity was localized *in vivo* to vesicles, shown to be autophagosomes, in randomly distributed cells of the starchy endosperm. The VEIDase activity detected in barley caryopses is similar to activities described previously in mammals, spruce, yeast, and thale cress. In mammals, spruce, and yeast, VEIDase activity has been shown to be positively correlated with the occurrence of programmed cell death. Several manifestations of programmed cell death exist in developing barley caryopsis, indicating a connection between VEIDase activity and developmental programmed cell death in barley.

Key words: Barley, caspase-like activity, programmed cell death, starchy endosperm, VEIDase.

Introduction

Development of the cereal caryopsis is characterized by a number of well-defined phases (Johannsen, 1884; Olsen, 2004). The initial double fertilization results in a diploid ovule and a triploid endosperm. The endosperm undergoes rapid syncytial enlargement forming a coenocyte, a sphere-like structure surrounding a central vacuole. During the two following days, cellularization of the coenocyte occurs. The endosperm differentiates into two main tissues: a central region develops into the starchy endosperm and the peripheral cell layers form the aleurone layer. As the starchy endosperm cells fill up with starch and proteins, they die and the caryopsis desiccates. The aleurone cells remain alive until germination, fulfilling a vital role in mobilizing storage reserves in the starchy endosperm.

Programmed cell death (PCD) is an important part of caryopsis development (Young and Gallie, 2000*a*). Proper orchestration of PCD in various tissues may be essential for the correct development of the caryopsis. Transient tissues and cells are eliminated by PCD enabling correct differentiation and shaping of the remaining tissues and organs. Several tissues of the caryopsis succumb to PCD at different stages of its development (Young and Gallie, 2000*a*). The proper onset and progression of PCD depends on the balance between various phytohormones (Young and Gallie, 2000*b*).

A group of cysteine proteases called caspases are central players in many forms of animal PCD. Caspases have an obligate cleavage preference for an aspartic acid immediately *N*-terminal of the cleavage site (Earnshaw *et al*., 1999). In mammalian PCD, caspases fulfil a role as integrators and propagators of cell death signals (Denault and Salvesen, 2002). Although no close homologues of caspases have been found in plants, several reports show

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Abbreviations: PCD, programmed cell death; VPE, vacuolar processing enzyme; AMC, 7-amino-4-methylcoumarin; dpa, days post-anthesis; FMK, fluoromethylketone; TUNEL, (TdT)-mediated dUTP nick end labelling; MDC, monodansylcadaverine.

the involvement of caspase-like activity in plant PCD (Rotari *et al*., 2005). Recently, two groups of proteases responsible for caspase-like activity in plants have been identified. The first encompasses subtilisin-like serine proteases, termed saspases (Coffeen and Wolpert, 2004) and the second includes vacuolar processing enzymes (VPE) belonging to the same clan of proteases as caspases (Hatsugai *et al*., 2004; Rojo *et al*., 2004).

The role of caspase-like activity in barley caryopsis development is investigated here. It was found that VEIDase is the principal caspase-like activity. This activity is developmentally regulated. Only caspase-specific inhibitors had a major inhibitory effect on the VEIDase activity. *In vivo*, this activity is localized to spherical vesicles randomly distributed throughout the starchy endosperm. The VEIDase-containing vesicles were shown to be autophagosomes, strengthening the link between the VEIDase activity and autophagic PCD.

Materials and methods

Plant material

The barley (*Hordeum vulgare* L. cvs Barke, Midas, and Pongo) plants were grown in a climate chamber (Sun *et al*., 1999). Spikes were individually checked to determine the day of anthesis. Only grains from the central half of the spike were used.

Caspase-like activity assay

Starchy endosperm and embryos were isolated by hand dissection. Due to their smallness it was not possible to isolate embryo and endosperm before 10 d post-anthesis (dpa) and 8 dpa, respectively. Isolated tissues were snap frozen in liquid nitrogen and proteins were extracted (Bozhkov *et al*., 2004). Caspase-like activity was measured as described previously (Bozhkov *et al*., 2004), with minor modification by adding $MgSO_4$ and $CaCl_2$ at 1 mM to the assay buffer. All substrates used were fluorogenic with the general structure, Ac-(X)XXXD-AMC (AMC, 7-amino-4-methylcoumarin; Peptide Institute Inc., Osaka, Japan), where X denotes amino acid residues at the P2 to P5 positions. All measurements were carried out at least three times on each sample.

Inhibition of endosperm caspase-like activity

Protease inhibitors were tested for suppressing VEIDase activity in 10 dpa endosperm extracts. The following concentrations were used: VEID-CHO, 20 μM; LEHD-CHO, 20 μM; YVAD-CHO, 20 μM; TLCK, 10 μM; lactacystin, 20 μM; E-64, 20 μM; calpain inhibitor 1, 20 μM; leupeptin, 10 μM; pepstatin, 10 μM; PMSF, 250 μM; aprotinin, 1 μM; Complete ±EDTA, 1 tablet 50 ml^{-1}. The assays were performed as described above except that inhibitors were added to the reaction mixture. Most inhibitors were obtained from Roche (Basel, Switzerland) except for lactacystin, Ac-VEID-CHO (CHO, aldehyde group), Ac-LEHD-CHO, and Ac-YVAD-CHO (all from Peptide Institute Inc. Osaka, Japan). Dimethylsulphoxide concentration was less than 1% throughout all assays.

In vivo *staining of endosperm caspase-like activity*

VEIDase activity can be visualized *in vivo* with the substrate VEIDN, included in the CyToxiLux®-PLUS kit (OncoImmunin, Inc., Gaithersburg, MD, USA). *In vivo* staining of intact starchy endosperm, cut into halves, was carried out at 10 dpa, and the embryos were adapted from Bozhkov *et al*. (2004). To test the specificity, a set of samples were first incubated in 0.5× MS medium with 2% sucrose containing the VEIDases inhibitors, 50 μM Z-VEID-CHO (Peptide Institute Inc., Osaka, Japan) and 50 μM Z-VEID-FMK (FMK, fluoromethylketone; MP Biomedicals, Irvine, CA, USA) for 30 min prior to staining. VEIDase activity was visualized using a Zeiss LSM 510 confocal microscope with the argon laser 488 nm line and an LP filter of 505 nm. For detection of DAPI stained nuclei, the diode laser 405 nm line and a detection window of 420–480 nm was used. The images were taken with a C-apochromate 40×/1.2W objective lens.

Double staining of autophagic vesicles with MDC and CyToxiLux

Starchy endosperm, isolated as described above, was ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was mixed with an equal volume of a 1:1 mix of 100 μM monodansylcadaverine (MDC; Sigma) solution in PBS and CS solution from the CyToxiLux®-PLUS kit. The sample was mixed and incubated at ambient temperature for 1 h. An aliquot of the supernatant was applied to a microscope slide and viewed using a Zeiss LSM 510 confocal microscope with the blue diode laser 405 nm line and a BP filter of 420–480 nm for MDC and the argon laser 488 nm line and an LP filter of 505 nm for the VEIDase CyToxiLux fluorophore. The images were taken with a C-apochromate 40×/1.2W objective lens.

Double staining of autophagic vesicles with LysoTracker Red and CyToxiLux

Starchy endosperm were prepared and stained with CyToxiLux as described above, followed by a 30 min incubation in 5 μM LysoTracker Red DND-99 (Invitrogen, Carlsbad, Ca, USA) in PBS with 2% sucrose and subsequently by three 10 min washes in PBS with 2% sucrose. The autophagic vesicles were visualized using a Zeiss LSM 510 confocal microscope with the argon laser 488 nm line and a BP filter of 505–530 nm for the VEIDase CyToxiLux fluorophore and the HeNe laser 543 nm line and a BP filter 560–615 for LysoTracker Red. The images were taken with a C-apochromate 40×/1.2W objective lens.

In situ *detection of DNA fragmentation*

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) was performed using the *in situ* Cell Death Detection Kit, TMR red (Roche, Basel, Switzerland). Whole caryopses of five different ages, 4, 9, 13, 19, and 25 dpa, were fixed in 4% formaldehyde (for 12 h in vacuum) and dehydrated through a graded series of ethanol and xylen and embedded in paraplast plus (Sigma). The TUNEL staining was applied to thin sections (6 μm) and images were acquired using the HeNe laser 543 nm line and detection window 585–615 nm. The objective lenses used were plan-neofluar 10×/0.3, plan apochromate 20×/0.75 and C-apochromate 40×/1.2W. Positive controls (pretreating the sections with DNase before TUNEL staining) as well as negative controls (omission of TdT) were included.

Results

Caspase-like activity in the barley endosperm

Assays for caspase-like activities in developing barley caryopses clearly indicated the presence of caspase-like proteases in the starchy endosperm. The highest activity

was detected with a caspase-6 substrate, Ac-VEID-AMC (Fig. 1). The VEIDase activity was considerably higher at 10 dpa compared with 30 dpa.

In a first attempt to characterize the VEIDase caspase-like activity in barley endosperm, its sensitivity towards different protease inhibitors was tested. Only Z-VEID-CHO, a caspase-6 inhibitor, and Z-LEHD-CHO, a caspase-9 inhibitor, showed major inhibitory effects (Fig. 2A). The detected inhibition of Complete Protease Inhibitor Cocktail with EDTA can be attributed to the presence of EDTA, indicating the involvement of a metal ion cofactor. Z-VEID-CHO, Z-YVAD-CHO, and calpain inhibitor I were further tested at different concentrations to determine their inhibitory strength (Fig. 2B). Based on those measurements it was possible to interpolate the IC_{50} for Z-VEID-CHO to 0.34 μM and for calpain I to 61 μM. The substrate and inhibitor specificity exhibited by the barley VEIDase activity is in agreement with other plant caspase-like and animal caspase activities, indicating the presence of a true caspase-like activity during barley caryopsis development.

VEIDase activity in barley endosperm, embryo, and whole caryopses

The profile of VEIDase activity in different tissues of developing caryopses was investigated next. The cleavage activity in the endosperm was highest in the early stages and then fell rapidly and remained at a low level throughout development (Fig. 3). The embryos exhibited high activity levels at the earliest developmental stages followed by a decrease to a steady-state level between 14 and 18 dpa, before it again increased at the end of embryonic development. In whole caryopses the activity peaked at 6 dpa and then decreased. The rise after 20 dpa might be attributed to the increase in embryonic VEIDase activity.

In situ *localization of VEIDase activity in the barley caryopses*

Having proven the existence of a VEIDase caspase-like activity in barley endosperm, the cell permeable fluorescent marker CyToxiLux, containing the substrate VEIDN, was used to investigate if the activity could be localized *in vivo*. In young endosperm tissue, 10 dpa, brightly fluorescent foci were clearly visible after the addition of substrates (Fig. 4A). The foci appeared to be spherical with a diameter of up to 5 μm. Cells containing these fluorescent spheres were randomly distributed throughout the starchy endosperm with no evident pattern. In endosperm that had been pretreated with caspase inhibitors almost no fluorescent signals could be seen (Fig. 4B).

Double staining of VEIDase activity and autophagic vesicles

The presence of the VEIDase activity in vesicular structures within the endosperm cells could be indicative

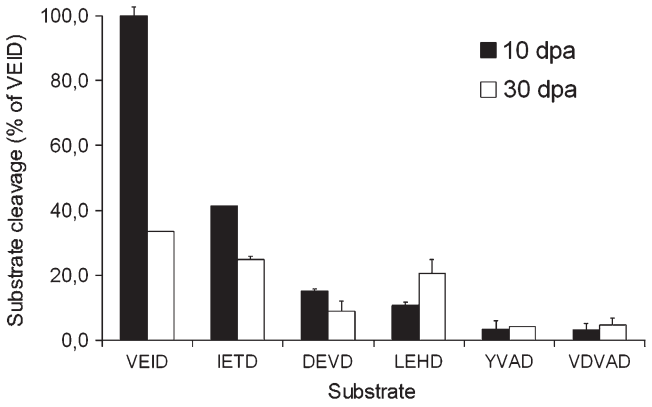

Fig. 1. Caspase-like activity in barley starchy endosperm. Cleavage activity against different caspase substrates is expressed as a percentage of the highest activity (VEIDase). Error bars denote sem.

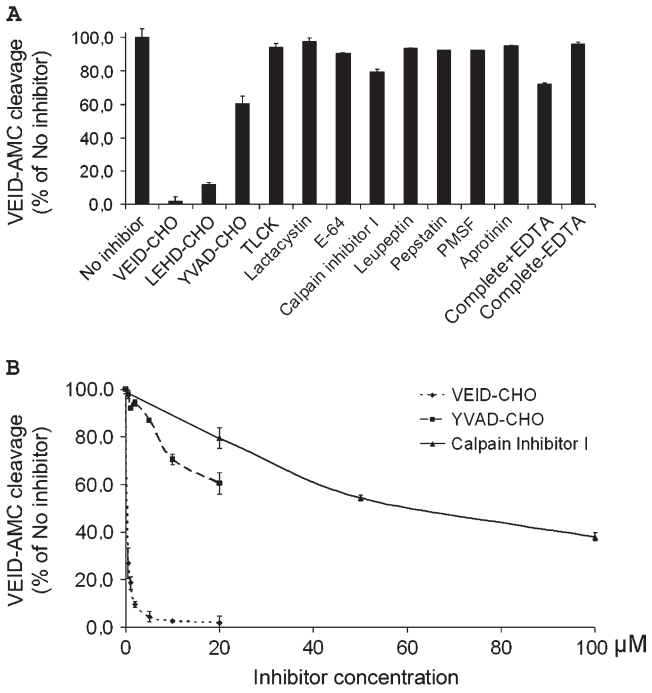

Fig. 2. Effect of protease inhibitors on VEIDase activity. (A) A range of protease inhibitors was tested for their ability to inhibit the VEIDase activity *in vitro*. The concentrations are given in the Material and methods section. Remaining activity is expressed as percentage of activity without inhibitor. Errors denote sem. (B) Inhibitory effect of three inhibitors at various concentrations. Remaining activity is expressed as percentage of activity without inhibitor. Errors bars denote sem.

of an autophagic type of PCD, which is executed through gradual disassembly of the cells by the growing lytic vacuoles (Baehrecke, 2002; Bozhkov *et al*., 2005*a*). This type of PCD is a principal cell suicide strategy in plants devoid of both specialized phagocytic cells and most of the molecular components of the apoptotic machinery. In plant PCD, lytic vacuoles arise from double-membrane vesicles called autophagosomes, which in turn originate from Golgi, proplastids or endoplasmic reticulum (ER; for

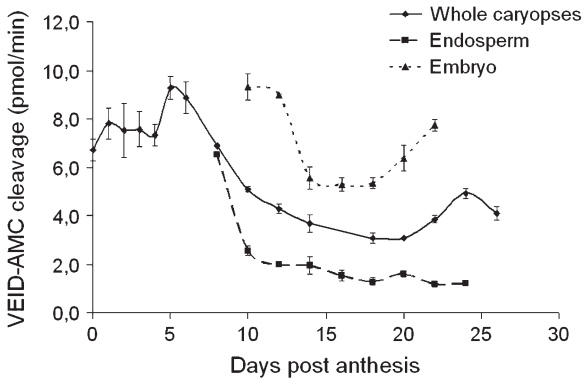

Fig. 3. VEIDase activity during barley caryopsis development. VEIDase activity at various time points during development in starchy endosperm, embryo and whole caryopsis. Errors bars denote sem.

a review, see Bozhkov *et al*., 2005*a*). Due to their acidic nature and membrane lipid composition, protease-containing autophagosomes can be specifically stained with monodansylcadaverine (MDC; Biederbick *et al*., 1995; Contento *et al*., 2005). In order to determine if the VEIDase-containing vesicles in the starchy endosperm were indeed autophagosomes, dual staining of cell contents with CyToxiLux and MDC was performed. The results clearly illustrate the co-localization of VEIDase activity and autophagosomes (Fig. 5A–C). Similar results were obtained by *in vivo* staining using LysoTracker Red DND-99, a red-fluorescent dye that stains acidic compartments of the lytic pathways in living cells (Fig. 5D–F). Taken together, these data demonstrate that the observed VEIDase activity in the barley endosperm exists within autophagosomes, which are an integral part of the plant PCD process.

Nuclear DNA fragmentation in barley caryopses at different developmental stages

As caspase-like activities have been linked to PCD in several plant systems, nuclear DNA fragmentation, as another hallmark of PCD, was investigated in starchy endosperm by subjecting sections of barley caryopses to TUNEL staining. Interestingly, no TUNEL positive nuclei could be detected in the endosperm from samples 4, 9 or 13 dpa, i.e. stages with high VEIDase activity (Fig. 6A, B, and data not shown). As expected, TUNEL positive nuclei could be seen in the nucellus layer at 13 dpa (Fig. 6B) as well as at 4 dpa and 9 dpa (data not shown). In samples of 19 dpa, a small number of TUNEL positive nuclei could be seen in the starchy endosperm (Fig. 6C, D) and the number increased even further in the 25 dpa sample (data not shown). Positive controls treated with DNase prior to TUNEL labelling revealed a large number of nuclei, demonstrating the validity of the procedure, whereas the negative controls, without TdT, showed no staining (data not shown).

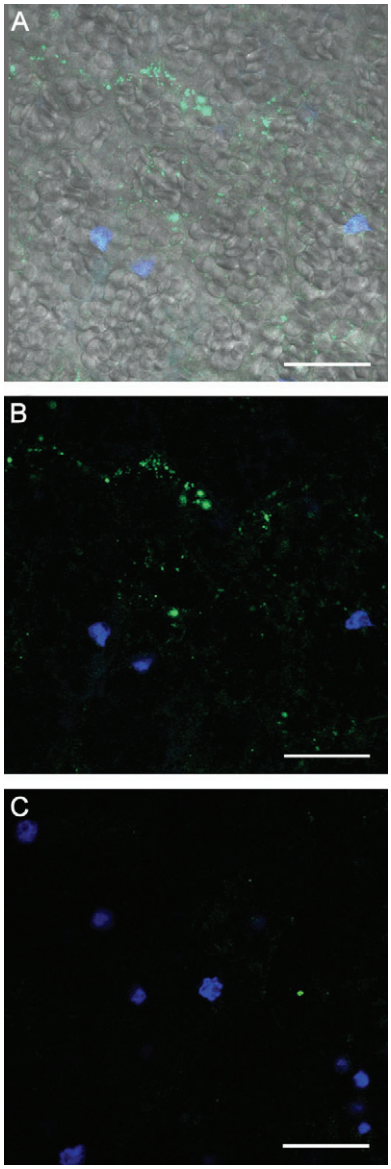

Fig. 4. *In vivo* localization and inhibition of VEIDase activity in central starchy endosperm as visualized by confocal microscopy after cleavage of VEIDN substrate (green fluorescence) included in the CyToxiLux®-PLUS kit. Bars=50 μm. (A) VEIDase activity and nuclei counter-stained with DAPI (violet fluorescence), overlaid by a full spectrum light microscopic image. (B) VEIDase and nuclei counter-stained with DAPI (violet fluorescence). (C) Inhibition of VEIDase activity after incubation with a mixture of VEID-CHO and VEID-FMK. Nuclei were visualized with DAPI (violet fluorescence).

Discussion

VEIDase, a proteolytic activity evolutionary conserved across wide phylogenetic distances

It has been shown here that VEIDase is a principal caspase-like activity in developing barley caryopses. There are several reports in the literature of VEIDase activity in non-mammalian systems. Caspase-like activity detected during embryogenesis in spruce (Bozhkov *et al*., 2004), in

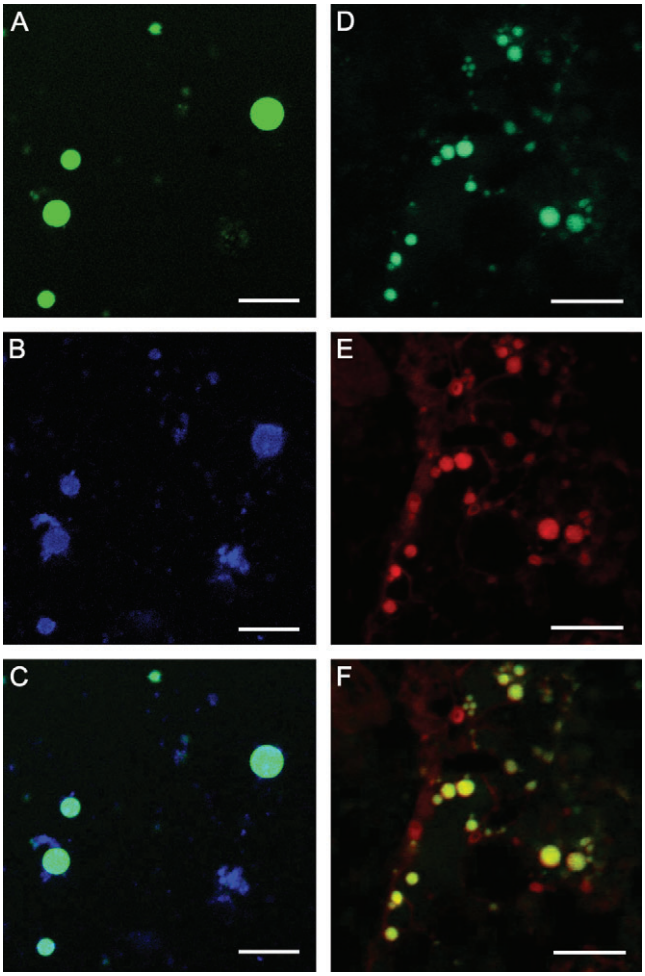

Fig. 5. Double staining of VEIDase activity and autophagosomes in the central starchy endosperm as visualized by confocal microscopy after cleavage of VEIDN substrate (green fluorescence) included in the CyToxiLux®-PLUS kit. Bars=10 μm. CyToxiLux-stained endosperm viewed with excitation and filter settings for LysoTracker red and MDC, respectively, displayed no fluorescence (not shown). (A) Confocal microscopic image of *in vitro* VEIDase activity. Scale bar=10 μm. (B) Confocal microscopic image of autophagosomes using monodansylcadaverine (purple fluorescence). Same area as in (A). (C) Composite confocal microscopic image of (A) and (B). Double-stained areas shown with light green colour. (D) Confocal microscopic image of *in vivo* VEIDase activity. Scale bar=10 μm. (E) Confocal microscopic image of autophagosomes using LysoTracker RED DND-99 (red fluorescence). Same area as in (D). (F) Composite confocal microscopic image of (D) and (E). Double-stained areas shown with yellow colour.

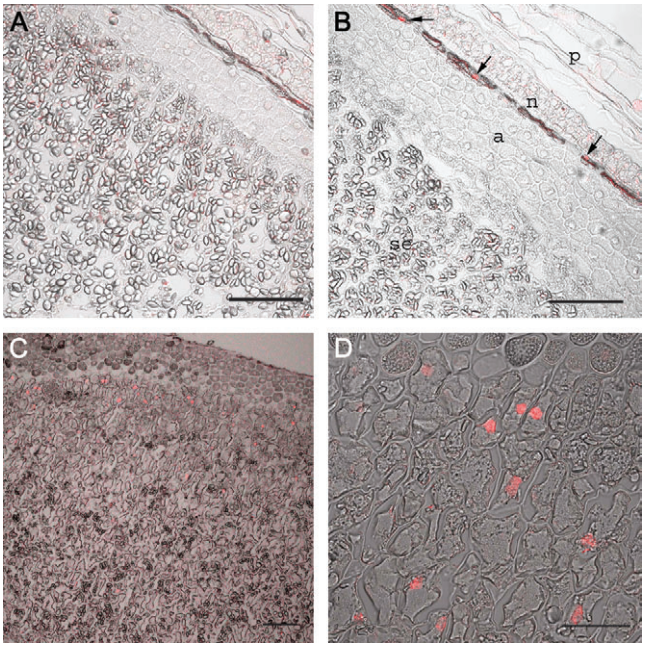

Fig. 6. Nuclear DNA fragmentation in barley caryopsis slices. (A, B) Images showing TUNEL-stained sections of barley caryopsis at 13 dpa. Note a lack of DNA fragmentation in the starchy endosperm. Shown by arrows in (B) are TUNEL positive nuclei in the nucellus. se, starchy endosperm; a, aleurone; n, nucellus; p, pericarp. Scale bar=100 μm. (C) Image of TUNEL-stained section of barley caryopsis at 19 dpa. A few nuclei stain TUNEL positive (red) mostly in the region closest to the aleurone layer but also deeper into the starchy endosperm. Scale bar=100 μm. (D) Image of TUNEL stained section of barley caryopsis at 19 dpa under higher magnification. Scale bar=50 μm.

A. thaliana seedlings (Rotari *et al*., 2005), and in yeast during oxidative stress-and senescence-induced cell death (Madeo *et al*., 2002) all show a similar pattern of relative cleavage rate of different caspase substrates to the caspase-like activity reported in this work. The substrate preference profile of these caspase-like activities are also close to the one specified for mammalian caspase-6 (Talanian *et al*., 1997). Only in mammalian systems has the protease responsible for the VEIDase activity been isolated and shown to be a caspase-6. In yeast (Madeo *et al*., 2002) and spruce (Suarez *et al*., 2004), the VEIDase activity has been shown to correlate with metacaspase expression. However, metacaspases have different substrate specificity than canonical caspases (Vercammen *et al*., 2004; Bozhkov *et al*., 2005*b*), indicating that the VEIDase activity detected *in vivo* is caused by proteases acting downstream to metacaspases in the cell death pathway. It is interesting to note that the four examples of VEIDase activity are separated by long phylogenetic distance. It thus appears that very similar, with regard to activity, proteases involved in PCD, exist in angiosperms, both in dicots (Rotari *et al*., 2005) and monocots (this study) and in gymnosperms (Bozhkov *et al*., 2004), as well as in yeast (Madeo *et al*., 2002), and animals (Foley *et al*., 2004), indicating a striking case of convergent evolution. Further studies are required to identify the protease(s) responsible for the VEIDase activity. Biotinylated caspase inhibitors have previously been used to isolate proteases with caspase-like activity (Faleiro *et al*., 1997). Several proteins in the starchy endosperm interact with the general caspase inhibitor, Biotin-VAD-FMK (M Borén, unpublished data). One of those proteins does not bind after pretreating the sample with the Ac-VEID-CHO inhibitor, indicating a VEIDase specific binding pattern. Unfortunately this protein has so far eluded all attempts at identification.

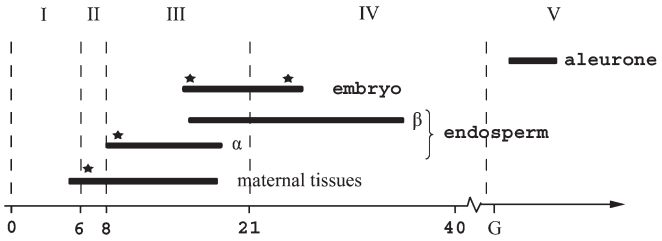

Fig. 7. Instances of programmed cell death during cereal caryopsis development. Black bars denote periods of cell death in the various tissues during the four stages (I–IV) of caryopsis development, representing, respectively, the syncytical, cellularization, differentiation, and maturation phase, and during germination (G; stage V). Periods of VEIDase activity in each tissue is denoted by filled stars. Numbers on the *x*-axis denote dpa. α, the first sign of PCD in the starchy endosperm; β, PCD as defined by TUNEL and Evan's blue staining.

In vivo *localization of VEIDase activity in barley starchy endosperm*

VEIDase activity was localized *in vivo* to spherical bodies throughout the starchy endosperm (Fig. 4). There are several reports of lytic organelles with a similar appearance in different plants during PCD. Those organelles have been shown to arise from ER (Hayashi *et al*., 2001; Schmid *et al*., 2001), Golgi or proplastids (Filonova *et al*., 2000). As has been shown, using double staining with known markers for lytic organelles (Fig. 5), the VEIDase-containing spherical shapes detected in the barley starchy endosperm can be classified as autophagosomes. There is growing evidence linking the protease-containing lytic organelles to PCD in plants (for a review see Hatsugai *et al*., 2006). It was recently shown that VPE posseses caspase-1-like activity (YVADase) as well as being localized to lytic vacuoles (Hatsugai *et al*., 2004; Rojo *et al*., 2004), thus providing a mechanistic link between caspase-like activity and lytic compartments during the execution of plant PCD. Isolation of plant proteases with VEIDase activity will be important for a better understanding of the mechamisms of autophagic PCD in plants.

Role of PCD during cereal caryopsis development.

Development of the barley caryopsis from fertilization to ripening involves several instances of PCD (Cejudo *et al*., 2002), which has been divided into four stages: (I) syncytical, (II) cellularization, (III) differentiation, and (IV) maturation (Bosnes *et al*., 1992). A fifth stage, (V) germination (G), needs to be added to cover all aspects of PCD during caryopsis development. To sketch an overview of PCD in the developing cereal caryopsis, available data from barley, maize, rice, and wheat have been superimposed onto the developmental timeline of the barley caryopsis (Fig. 7). Programmed cell death is first observed in the maternal nucellar and pericarp tissues as early as 5 dpa in wheat (Dominguez *et al*., 2001). The maternal tissues are progressively degraded, to supply nutrients and provide space, until the last specialized transfer cells in the nucellar projection finally denucleate around 18 dpa in wheat (Dominguez *et al*., 2001) and 24 dpa in maize (Kladnik *et al*., 2004). The high VEIDase activity correlates well with high levels of PCD in the young caryopses (Figs 3, 7). The first signs of PCD in the starchy endosperm, as indicated by the bar labelled α in Fig. 7, are manifested by denucleation starting as soon as cellurization is completed in rice (Lan *et al*., 2004) and by loss of membrane integrity as detected by electron spin resonance (ESR) in wheat (Golovina *et al*., 2000). In endosperm, the highest levels of VEIDase activity correspond with this early period of cell death (Figs 3, 7). This is interpreted to mean that VEIDase activity and PCD participate in early endosperm differentiation. Since PCD at this stage was not indicated by TUNEL staining it appears that the VEIDase activity detected is uncoupled from DNA fragmentation during early seed development. PCD, as indicated by TUNEL and Evan's blue staining, represented by the bar labelled β in Fig. 7, can be seen from 12 dpa in rice (Lan *et al*., 2004) and 16 dpa in wheat and maize (Young and Gallie, 1999). The suspensor cells are the first cells of the embryo committed to PCD (Bozhkov *et al*., 2005*a*), starting at 14 dpa in maize. A VEIDase activity has been shown to be essential for suspensor cell PCD during spruce embryo development (Bozhkov *et al*., 2004). In late embryonic development, *c*. 27 dpa in maize, a few cells in the scutellar node are still TUNEL positive as a result of vascular differentiation (Giuliani *et al*., 2002). In the present study, the two waves of embryonic PCD are reflected by the two peaks of VEIDase activity in the barley embryo (Figs 3, 7). A few days after germination the aleurone cells enter PCD (Fath *et al*., 2000). All the times indicated in Fig. 6 should be considered approximate. Based on available evidence it is our belief that all cereals have a conserved sequence of PCD during caryopsis development. It differs in details and timing but the overall pattern and sequence of PCD events appear conserved between cereals. Based on the results, in combination with the available literature, we would like to suggest that the VEIDase activity found in barley starchy endosperm and in the embryo plays an important role during caryopsis development.

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