RESEARCH PAPER

Caspase-like activity in the seedlings of *Pisum sativum* eliminates weaker shoots during early vegetative development by induction of cell death

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Abstract

Activation of aspartate-specific cysteine proteases (caspases) plays a crucial role in programmed cell death (PCD) in animals. Although to date caspases have not been identified in plants, caspase-like activity was described in tobacco during a hypersensitive response to pathogens and in *Arabidopsis* and tomato cell cultures during chemical-induced PCD. Caspase-like activity was also detected in the course of plant development during petal senescence and endosperm PCD. It is shown here that caspase-like proteases play a crucial role in the developmental cell death of secondary shoots of pea seedlings that emerge after removal of the epicotyl. Caspase-like activity was induced in senescing secondary shoots, but not in dominant growing shoots, in contrast to the papain-like cysteine protease activity that was stronger in the dominant shoot. Revitalization of the senescing shoot by cutting of the dominant shoot reduced the caspase-like activity. Injection of caspase or cysteine protease inhibitors into the remaining epicotyl tissue suppressed the death of the secondary shoots, producing seedlings with two equal shoots. These results suggest that shoot selection in pea seedlings is controlled by PCD, through the activation of caspase-like proteases.

Key words: Apical dominance, cysteine proteases, epicotyl, senescence, shoot development.

Introduction

Pea seedlings constitute the classical model for studying the process of shoot selection in early development of the apical meristem. Original work by Snow showed that when a 7-d-old pea epicotyl is cut off, two new shoots develop below the cut surface. Initially, they are of equal size, but one of them soon establishes apical dominance by a presently unknown mechanism, while the secondary shoot stops growing and rapidly ages (Snow, 1931, 1932). The apical dominance of the more robust shoot represents an extreme case of dominance, which invariably results in the death of the secondary shoot. However, if the dominant shoot is removed before death occurs, then the weak shoot is revitalized and starts growing. Importantly, it was shown that the death of the weak shoot does not depend on endogenous lack of nutrients, suggesting an active process regulated by internal stimuli (Sachs, 1966). This process, particularly its initiation, is subject to regulation by many environmental and autonomous (internal) factors. In general, conditions that are unfavourable for growth accelerate the selection of the dominant shoot and the senescence and cell death of the secondary shoot.

Senescence is a final phase of plant vegetative and reproductive development, preceding the widespread death of cells and organs. Leaf senescence is the final stage of leaf development. Physiological, biochemical, and molecular studies show that, during senescence, leaves undergo highly co-ordinated changes in cell structure, metabolism, and gene expression (Gan and Amasino, 1997). The requirements for new RNA and protein synthesis imply that leaf senescence is a genetically programmed cell death (PCD) process (Crafts Brandner et al., 1996). Leaf senescence can be considered as the archetype of PCD, and, in fact, it has been shown to exhibit the hallmarks of apoptotic cell death (Greenberg, 1996; Caccia et al., 2001). PCD or apoptosis significantly differ from necrotic cell death in the biochemical mechanism and

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in the subsequent biological effects, particularly in pathological implications (Steller, 1995; Beers, 1997).

The earliest and most significant change in leaf cell structure is the breakdown of the chloroplast, the organelle that contains up to 70% of the leaf protein. During this process carbon assimilation (photosynthesis) is replaced by catabolism of chlorophyll and other macromolecules such as proteins, lipids, and RNA. Senescence is a tightly regulated developmental process that involves turnover and recapture of cellular material, ultimately resulting in cell death (Beers, 1997; Noodén et al., 1997). It is influenced by many hormones, environmental conditions, and signalling molecules (Quirino et al., 2000).

Apoptosis, which is a highly regulated form of PCD, is a major form of cell death in animals, plants, and even in yeast (Raff et al., 1994; Pennell and Lamb, 1997; Frohlich and Madeo, 2000). In animals, PCD is characterized by several morphological and biochemical hallmarks, such as shrinkage of the cytoplasm, nuclear condensation, membrane blebbing, and induction of proteases and endonucleases (Steller, 1995). While these processes have been also observed in plant PCD, they are not associated with every type of PCD in plants (Pennell and Lamb, 1997). Frequently, only some of the hallmarks accompany plant PCD, suggesting the existence of several alternative cell death pathways (Greenberg, 1996). Variations in the mechanisms of PCD were also described in animal systems.

Molecular studies in nematodes and animals have found that apoptosis is controlled by a proteolytic cascade that involves a set of conserved cysteine proteases, caspsases, which cut target proteins after an aspartate residue (Thornberry and Lazebnik, 1998). Cysteine proteases also play a crucial role in many forms of plant PCD, including senescence (Solomon et al., 1999; Heath, 2000). The induction and the function of true caspase-like activities in plants has been recently reviewed in Woltering et al. (2002). The predominant class of senescence-induced proteases belongs to the cysteine protease family (Valpuesta et al., 1995; Drake et al., 1996; Ye and Varner, 1996). In castor beans, papain-like cysteine protease was shown to accumulate in senescing plant tissue, concomitant with the appearance of DNA cleavage, indicating the induction of PCD (Schmid et al., 2001). Moreover, inhibition of the cysteine protease activity by chemical inhibitors suppressed the leaf senescence process (Beers, 1997; Noodén et al., 1997).

The high specificity of caspases towards their substrates makes them ideal enzymes for the regulation of crucial decisions in the cell. Indeed, the caspase-dependent pathway is the major type of apoptosis in many animal systems, although a caspase-independent pathway was also described (Cohen, 1997). Although caspase homologues were not found in plants, the complete sequence of the Arabidopsis genome revealed the presence of several metacaspase genes (Uren et al., 2000). Recently, caspase-like activity was detected in protein extracts from tobacco plants during a hypersensitive reaction, which is regarded as a form of PCD (del Pozo and Lam, 1998). Caspase-like activity was also detected in Arabidopsis and tomato cell cultures undergoing PCD triggered by nitric oxide or by other chemicals that also induce PCD in animals (Clarke et al., 2000; De Jong et al., 2000). Specific peptide inhibitors of caspases suppressed camptothecin-induced PCD in tomato suspension cultures, supporting possible caspase(s) activity in plants (Orzaez et al., 2001).

Moreover, tomato plants that expressed the antiapoptotic baculovirus p35 gene, which interacts with the active site of the target caspases, blocked PCD (Lincoln et al., 2002). It is shown here that the induced senescence and cell death of the secondary shoots in pea seedlings is regulated by a caspase-like activity, which occurs concurrently with increased oxidation. Inhibition of the caspase-like activity affected the natural morphogenesis after epicotyl removal, and resulted in the growth of plants with two equal-sized shoots.

### Materials and methods

#### Chemicals

Protease substrates were from Calbiochem-Novabiochem (La Jolla, CA, USA). Other chemicals were from Sigma (St Louis, MO, USA). Peptides were dissolved in DMSO and diluted in water before addition to the reaction mix or injection into seedlings. Amastatin was dissolved in ethanol.

#### Plant material

Pea seeds (Pisum sativum var. arvense Poir. cv. Dun) were sown in vermiculite. The plants were grown at 21 °C and 12 h of light (275 μmol m⁻² s⁻¹ light intensity). One week after germination, plants were transferred into hydroponic conditions with Hoagland solution, and the seminal shoots were cut off. Two new shoots developed within 7 d, one of which became dominant and the other senesced and started to die unless the dominant shoot was removed.

#### Protease assays

Proteins were extracted from shoots frozen in liquid nitrogen by grinding 1 g of tissue in a cold mortar and then adding 5 ml of 200 mM phosphate buffer pH 7, 1 mM EDTA, and 1% polyvinylpoly-pyrolidone. Samples were centrifuged for 10 min at 14 000 g, 4 °C and the supernatant was used for the protease assay. Protein concentration was determined according to Bradford (1976), and 20 μg of proteins were used for the protease activity assay. The reactions were adjusted to 100 μl volume in buffer (20 mM MES pH 6.6, 0.25 mM dithiothreitol (DTT), 100 mM NaCl, 2.5 mM EDTA, and 2.5 μg ml⁻¹ fluorogenic substrate). The reactions with inhibitors were incubated for 20 min at 30 °C prior to addition of the substrates.

Proteolytic activity was measured in a FL600 fluorometer (BioTek, VI, USA; excitation 360/40; emission 460/40, sensitivity 100). The blank fluorescence readings (minus substrate) were subtracted. The following fluorogenic substrates were used: [t-benzoyloxy carbonyl (CBZ)-Gly-Gly-Arg-AMC (GGA) and [t-benzoyloxy carbonyl (CBZ)-Arg-Arg-AMC (AA) for testing general cysteine protease activities; [t-butyloxycarbonyl (Boc)-Gly-Lys-Arg-AMC (GLA) for analysis of the trypsin activity; caspase-like
activity was assayed with 4-(dimethylaminophenylazo)benzoic acid (DABCYL)-Tyr-Val-Ala-Asp-Ala-Pro-Val-5-((2-aminoethyl)-lamine)naphthalene-1-sulphonic acid (EDANS) and with Ac-YVAD-AMC substrates. All substrates were dissolved in DMSO and diluted in water to the final concentration. Caspase inhibitor (Ac-Aasp-Glu-Val-Asp-CHO) was used at 0.2 mM or 20 nM concentrations [which were found to be effective in plants (Wottering et al., 2002)] for in vitro (see Figs 4 and 5 in the Results) and in vivo (see Fig. 6 and Table 1 in the Results) experiments, respectively. The general cysteine protease inhibitor trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E-64) was used at a 10 µM concentration throughout this study.

In gel protease assay

Proteins (20 µg) were incubated for 5 min at 30 ºC in sample buffer and separated in a 10% SDS-polyacrylamide gel. The gel was washed twice with 50 mM TRIS, pH 7.5, and 0.25% Triton X-100 for 45 min each and renatured overnight at 30 ºC in 10 mM TRIS, pH 7.5, and 0.25% Triton X-100. Activity was detected in a solution of 20 mM MES pH 6.6, 0.25 mM dithiothreitol (DTT), 100 mM NaCl, 2.5 mM EDTA, and 2.5 µg ml⁻¹ Ac-YVAD-AMC substrate. The fluorescence in the gel was followed in real time by impregnating the ICE substrate into the gel and viewing with an ImageMaster VDS system (Pharmacia) equipped with a 360 nm excitation lamp above the gel and a 460 nm emission filter.

Lipid peroxidation

Lipid hydroperoxides were measured with a Lipid Hydroperoxide (LPO) Assay Kit (Cayman Chemicals, USA), according to the manufacturer’s instructions. The total cell extract from the pea shoot was normalized according to the protein concentration. Equal amounts of extraction buffer, saturated in methanol, were added and the samples were vortexed for 2 min. One ml of cold chloroform was added and the samples vortexed again. After centrifugation for 5 min at 1500 g at 4 ºC, the bottom chloroform layer was collected and mixed with 450 µl of chloroform:methanol 2:1 (v:v) mix and 50 µl of chromogen (as supplied in the kit). The reactions were done in 300 µl by adjusting the volume with water. The absorbance was read against chloroform:methanol (without chromogen) at 500 nm and compared with a standard curve.

Northern blot analyses

Total RNA was extracted from the pea shoot at different times using the RNeasy Plant Mini Kit (QIAGEN) as described by the manufacturer. Five micrograms of total RNA were separated on 1.5% (w/v) agarose denaturing formaldehyde gels, and RNA was visualized by staining the gels with ethidium bromide (1 mg ml⁻¹). The separated RNAs were subsequently blotted onto Hybond N⁺ membrane (Amersham Pharmacia Biotech), according to the manufacturer’s instructions, and cross-linked by UV irradiation. The Arabidopsis SAG12 gene was used as a probe and radioactive probe.

Chromatography

Cleared protein extracts were loaded onto a Biologic (Bio-Rad) medium pressure chromatography system with an anion exchange column of 1 ml UNO-Q. Proteins were eluted with a linear NaCl gradient at 1 ml min⁻¹.

ROS measurements

The amount of ROS in shoots of pea plants was measured according to (Lu and Higgins, 1998). Briefly: the samples (at least in triplicate) were washed for 90 min in water with vigorous shaking, and then placed into the reaction buffer (10 mM TRIS pH 8, 10 units horseradish peroxidase, 10 µM 2′,7′-dichlorofluorescein diacetate) in a final volume of 200 µl. The samples were kept in the dark for 10 min and read using a BioTek FL600 fluorometer with the following conditions: excitation 485/40; emission 530/40; sensitivity 100. The fluorescent probe was prepared by diluting 2 mg ml⁻¹ ethanol stock solution. A standard curve was prepared before each experiment using different H₂O₂ concentrations in fresh medium. The measure was normalized according to the dry weight.

Results

Developmental and physiological changes in establishment of shoot dominance

To study the process of shoot selection in early development of pea seedlings the apical meristems were cut off 7 d after germination. Two new shoots emerged below the cut. The growth of the shoots was disproportionate, resulting in a larger leading branch and a small secondary one (Fig. 1A). The secondary shoot died within 21 d when left untouched. However, cutting off the dominant shoot stopped the rapid senescence of the secondary shoot, and re-established its growth rate, indicating that the process was reversible (Fig. 1B).

To see whether the deterioration of the secondary shoot was associated with the expression of senescence-associated genes from other systems, the RNA from each shoot was probed with a genetic marker of the SAG12 gene that was shown to be completely senescence-specific. This gene was first isolated from naturally senescencing Arabidopsis leaves (Lohman et al., 1994), but homologous genes were found in other plants as well (Ori et al., 1999; Pontier et al., 1999). Northern analysis of SAG12 gene expression showed that it was present predominantly in the dying branch. Interestingly, its transcription stopped after cutting off the dominant shoot, indicating that the SAG12 gene expression was.reversibly stopped by cutting the shoots.
gene expression depended on the continuation of the senescence programme (Fig. 2).

In some species leaf senescence is accompanied by a respiratory burst, resulting in development of an oxidative stress (Casano et al., 1994; Merzlyak and Hendry, 1994; Heber et al., 1996). Production of reactive oxygen species (ROS) during the senescence of the dying (secondary) branch was examined by measuring oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) in the dominant and the secondary shoots (Lee et al., 1999; Murata et al., 2001). Shoot cuttings were incubated in DCFH-DA solution for 10 min in the dark and the amount of oxidized dye measured by spectrofluorometry. The dying shoot produced consistently more ROS (Fig. 3A). The increase in ROS production was detected 11 d after removal of the apical meristem, coinciding with the beginning of dominance establishment. If not counteracted by a corresponding increase in antioxidant activity, oxidative stress leads to increased lipid peroxidation. More than 3-fold higher levels of lipid hydroperoxides were detected in the dying shoot, in line with increased ROS in that branch (Fig. 3B).

**Proteolytic activity during induction and reversal of senescence**

Caspase induction is a central step in animal PCD, including the ageing-associated cell death (Wang et al., 1998). Recently, caspase-like activity was also discovered in plants. Protease involvement in shoot death was analysed 1 week after cutting off the primary bud by assaying the proteolytic activity in the dominant and secondary shoot extracts by measuring the cleavage of several fluorogenic peptide substrates. Cysteine proteases were assayed by using two different substrates, t-benzoyloxycarbonyl (CBZ)-Gly-Gly-Arg-AMC (GGA) and Nα-benzoyloxycarbonyl (CBZ)-Arg-Arg-AMC (AA). Trypsin-like activity was assayed with t-butyloxycarbonyl (BOC)-Gly-Lys-Arg-AMC (GLA), which is cut preferentially by trypsin (Wilson et al., 2002). Caspase-like activity was measured with a relatively long substrate, 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL)-Tyr-Val-Ala-Asp-Ala-Pro-Val-5-([2-aminoethyl]amino)naphthalene-1-sulphonic acid (EDANS), that is specific for interleukin-1 beta converting enzyme (ICE) as well as the ICE homologue apopain/CPP-32/Yama (Pennington and Thornberry, 1994; Hugunin et al., 1996).

Proteolytic activity using the two broad-range cysteine protease substrates or the trypsin-type peptide revealed a rather similar pattern in the short and in the long shoots (Fig. 4A). Furthermore, their activities were not significantly altered after cutting off the leading shoot. By contrast, the caspase-specific substrate showed a strong activity only in the short secondary shoot, suggesting the induction of a senescence-associated protease (Fig. 4A). Inclusion of an aminopeptidase inhibitor, amastatin (10 μM), did not alter the activity, indicating that the fluorescent signal was dependent on endopeptidase activity (data not shown). Exclusive activity in the dying secondary

![Fig. 2. Northern blot analysis of a senescence-associated gene, SAG12 in developing pea shoots. Pea seedlings were grown as described in the legend of Fig. 1. Total RNA was extracted after 2 weeks from the dominant long shoot (L), and from the short (secondary) shoot (S I), and 1 d later the dominant shoot was cut off. One week after cutting off the dominant shoot total RNA was also prepared from the recovering (formerly) secondary shoot (S II). An equal amount of total RNA (8 μg per lane) was probed with the SAG12 gene from Arabidopsis. RNA loading was checked by reprobing the same filter with 18 S rRNA probe.](image1)

![Fig. 3. Development of oxidative stress during senescence in the short shoot. (A) Accumulation of ROS after the cutting of the primary shoot was assayed at the indicated days in the secondary (short) and dominant (long) shoots. Similar-sized samples were cut off with a scalpel and the results were normalized according to the dry weight of the samples. (B) Lipid peroxidation in the dominant and the secondary shoots was measured 3 weeks after cutting off the epicotyl.](image2)
shoot was also observed with short caspase-specific peptide substrates, benzoxycarbonyl (Cbz) Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin (YVAD) or Ac-Asp-Glu-Val-Asp-AMC (DEVD) that are preferentially cleaved by caspases 1 and 3, respectively, although it should be mentioned that YVAD also acts as a substrate for caspases 3, 4, and 7 (Villa et al., 1997) (data not shown). Importantly, the proteolysis of the ICE substrate was suppressed by very low concentrations (0.2–0.5 nM) of reversible caspase inhibitors (Ac-Asp-Glu-Val-Asp-CHO or Ac-Tyr-Val-Ala-Asp-CHO). A relatively high dosage of 10 μM of a general cysteine protease inhibitor, E-64, inhibited both the aspartate- and the arginine-specific proteases. Taken together, the substrate specificity and the inhibition profile of the proteases support the involvement of a cysteine protease with caspase-like target specificity.

The time-course of caspase-like activity was studied in protein extracts from the leading and the senescing shoots, beginning at 3 d after cutting of the epicotyl (which was the earliest time that enough biological material could be collected for protein analysis in the small shoot). Proteins were concentrated and assayed with the long ICE substrate. Increased caspase-like activity was already detected in the short shoot 3 d after cutting, and it remained high throughout the duration of the experiment (Fig. 4B). By contrast, when the same samples were assayed with the general substrate of cysteine proteinases (GGA), stronger activity was observed in samples from the long shoot, indicating that the increased proteolysis of the caspase substrate in the dying shoot was not due to a general up-regulation of proteinases (Fig. 4C).

**Partial purification of the caspase protein from the dying secondary shoot**

To characterize the caspase-like activity, total protein extracts were prepared from the senescing shoot. The caspase-like proteolytic activity was recovered by precipitation with 30% ammonium sulphate. The precipitate was dialysed and loaded onto a cation exchange column and eluted with a NaCl gradient. The caspase-like activity that appeared in the flowthrough (data not shown) was loaded on an anion exchange column. A clear difference was seen in the profile of proteins eluted with a linear gradient of NaCl, indicating major changes in protein expression during shoot senescence (Fig. 5A). Each fraction was analysed by testing the activity with the long ICE substrate.

![Fig. 4. Protease activity in the dominant and secondary (before and after removal of the dominant branch) shoots. (A) Total protein extracts were prepared from the dominant and the secondary shoots (before or after cutting off the dominant branch). Protease activity was assayed 1 week after cutting off the epicotyl in the dominant (black bars) and secondary shoots (white bars), and the secondary shoots were assayed again 1 d after cutting off the dominant branch (hatched bars). The following fluorogenic substrates were used: t-benzoyloxycarbonyl (CBZ)-Gly-Gly-Arg-AMC (GGA) and the Nα-benzoxycarbonyl (BOC)-Gly-Lys-Arg-AMC (GLA), the 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL)-Tyr-Val-Ala-Asp-Val-5-(2-aminoethyl)amino)naphthalene-1-sulphonic acid (EDANS) (ICE). The ICE inhibitor, Ac-Asp-Glu-Val-Asp-CHO (ICE + inh), was used at a very low concentration of 0.2 nM. (B) Time-course of caspase-like activity after cutting off the epicotyl. Total proteins extracts were prepared 3 (the earliest possible), 4, 5, 6, and 7 d after the cutting off the epicotyl and the proteolytic activity against the caspase substrate was determined as in (A). (C) Time-course of proteases activities against a general cysteine protease substrate. Protein extracts were prepared as in (B), and assayed with the t-benzoxycarbonyl (CBZ)-Gly-Gly-Arg-AMC (GGA) substrate.**
substrate. The proteolytic activity was only recovered in two adjacent fractions towards the end of the NaCl gradient, between 0.6–0.7 μS, indicating the acidic nature of the protease (Fig. 5B).

Proteolytic activity was also detected in these fractions using the short caspase-1 (Ac-YVAD-AMC) or caspase-3 (Ac-DEVD-AMC)-specific substrates (data not shown). To obtain information on the molecular size of the caspase-like protease, the eluted fractions were concentrated and analysed in a polyacrylamide gel following SDS-PAGE. Proteolytic activity was assayed by impregnation of the short fluorogenic substrate (Ac-YVAD-AMC), which acts as a substrate for caspases 3, 4, and 7. The activity was visualized in an ImageMaster VDS system (Pharmacia). The active bands corresponded to apparent molecular weight of 55 kDa (Fig. 5C).

**Inhibition of caspases leads to morphological changes in seedling development**

The above results implied a possible involvement of caspase-like proteins in the death of the secondary shoot. In order to test whether caspases play a shaping role in vivo in the death of the shoot, various protease inhibitors were injected into the stem of the epicotyl, immediately after the removal of the apical meristem. The subsequent additions were done by direct injections into the secondary shoot. Application of the inhibitors with anti-cysteine protease activity reversed the death process in the secondary shoot.

**Table 1. Shoot length in plants injected with protease inhibitors**

<table>
<thead>
<tr>
<th>Length</th>
<th>Water</th>
<th>ICE inhibitor</th>
<th>E-64</th>
<th>AEBSF</th>
<th>Aprotinin</th>
<th>AAF-CMK</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1 cm</td>
<td>50 (37–62)</td>
<td>0 (0–7)</td>
<td>0 (0–6)</td>
<td>0 (0–6)</td>
<td>52 (33–71)</td>
<td>56 (38–73)</td>
</tr>
<tr>
<td>2–3 cm</td>
<td>50 (0–6)</td>
<td>28 (17–42)</td>
<td>26 (16–38)</td>
<td>22 (13–35)</td>
<td>0 (0–12)</td>
<td>0 (0–11)</td>
</tr>
<tr>
<td>&gt;3 cm</td>
<td>0 (0–6)</td>
<td>24 (14–37)</td>
<td>21 (12–33)</td>
<td>11 (6–23)</td>
<td>0 (0–12)</td>
<td>0 (0–11)</td>
</tr>
</tbody>
</table>

In brackets is the number of plants that fall within each group according to statistical analysis using 90% (±5%) confidence level for binomial distribution. AAF-CMK, alanyl-alanyl-phenylalanine-chloromethylketon.

**Fig. 5.** Partial purification of caspase-like activity. (A) Anion exchange chromatography of proteins from the dominant (dotted line) and the secondary (dashed line) shoots. Proteins were extracted 1 week after cutting off the epicotyl and precipitated with 30% ammonium sulphate. The pellet was dialysed and loaded onto a cation exchange column; the activity, which eluted in flowthrough, was loaded on a 1 ml UNO-Q column (Bio-Rad, Hercules, CA) and eluted with a continuous gradient of NaCl (solid line). (B) The eluted fractions were tested with the ICE (caspase-1) substrate 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL)-Tyr-Val-Ala-Asp-Ala-Pro-Val-5-[[2-aminoethyl]amino]naphthalene-1-sulphonic acid (EDANS). (C) In-gel protease assay of the eluted fractions with the caspase substrate. Fractions from (B) were precipitated with 30% (NH₄)₂SO₄ and separated by SDS-PAGE. Protease activity was assayed by impregnating the Ac-YVAD-AMC substrate into the gel as described in the Materials and methods. The numbers above each lane indicate the eluted frations.

**Fig. 6.** Inhibition of caspase-like activity reverses the senescence imposed by the dominant branch. Water (A) or caspase-3 inhibitor (Ac-Asp-Glu-Val-Asp-CHO) (B) (0.2 mM) were injected into the stub of the epicotyl, immediately after removal of the sprout, followed by injections into the short (secondary) branch every 3–4 d.
branch. Injection of the caspase-3 inhibitor strongly inhibited the death of the secondary branch, resulting in the almost equal growth of both shoots (Table 1). A slightly lower protection was also observed by the general cysteine protease inhibitor, E-64, and by the serine-cysteine protease inhibitor, AEBSF (Fig. 6). It should be noted that AEBSF was shown to inhibit cysteine proteases in plants and hypoxia-reoxygenation-induced apoptosis in rat kidney cells by the inhibition of caspase-9 activity (Dong et al., 2000). By contrast, the addition of serine protease inhibitors (25 μM alanyl-alanyl-phenylalaninechloromethylketone or 2 μM aprotinin) had no effect on secondary shoot development (Table 1).

Discussion

Apical dominance is defined as the inhibition of lateral buds by the dominant apex. The inhibition may be partial, causing slower development of the secondary branches or alteration of their growth direction, or it can completely stop their growth. Pea plants exhibit an extreme case of apical dominance, whereby the weaker shoot is actually killed (Snow, 1931). Interestingly, removal of the epicotyl causes both branches to grow equally well. However, once one of the shoots gains advantage due to some stochastic event, such as shading, pathogen attack, or insect-caused damage the other shoot rapidly establishes dominance and induces death of the weaker branch. The death process can be rapidly and completely reversed by removing the dominant cotyledonary bud (Fig. 1). The activation of different proteases and the reversibility of the death process suggests an active cell death process that is associated with senescence.

In animal systems, caspase activation has emerged as a key step in the regulation of developmental apoptosis (Martin and Green, 1995; Xue and Horvitz, 1995). Although non-caspase-dependent PCD also operates in animals, caspases are critical for developmentally induced apoptosis (Cohen, 1997). For example, in nematodes, mutations in the caspase gene, Ced-3, prevent cell death of specific cells, while ectopic expression of caspase genes causes additional cell death (Xue and Horvitz, 1995). In plants, developmentally induced PCD occurs in aleurone and in unpollinated ovules, during aerenchyma and formation of tracheary elements. In some plants PCD causes leaf lobes and perforations (Pennell and Lamb, 1997). To date none of these processes has been associated with caspasas or caspase-like activity. However, cysteine proteases were shown to be activated during xylogenesis and in flower and leaf senescence (Minami and Fukuda, 1995).

On the other hand, caspase-like activity was reported for several stress-triggered PCD processes, such as hypersensitive reaction and chemical treatments (del Pozo and Lam, 1998; Sun et al., 1999; De Jong et al., 2000). The involvement of caspase-like activity for execution of the PCD in plants is also supported by inhibitor studies, whereby addition of either caspase-3 inhibitor (Ac-DEVD-CHO) or caspase-1 inhibitor (Ac-YVAD-CMK) suppressed hypersensitive cell death from avirulent bacteria infection or from mycotoxin induced PCD (Woltering et al., 2002). The caspase-specific inhibitors also effectively blocked PCD from chemical and heat shock treatments (Sun et al., 1999; De Jong et al., 2000). These results show that caspase-like activity operates in developmental PCD in pea seedlings, that is associated with organ senescence.

The process of senescence is tightly associated with oxidative stress (del Rio et al., 1992; Smirnoff, 1993; Pell et al., 1997). Increased H₂O₂ concentration and lipid peroxidation occur in leaves during senescence (Jimenez et al., 1998; Berger et al., 2001). ROS also play a major role in PCD induction in plants (Levine et al., 1994; Noctor and Foyer, 1998; Mazel and Levine, 2001). Treatment of soybean cells with H₂O₂ induced cysteine proteases, which were instrumental in their PCD (Solomon et al., 1999; Heath, 2000). In Arabidopsis cells, H₂O₂-triggered PCD could be suppressed by cysteine protease inhibitors including caspase-specific inhibitors (Tiwari et al., 2002). In the present study, ROS production in the dying shoot was correlated with protease activation, suggesting that ROS may be involved in the signalling of PCD in the secondary branch (Fig. 3).

Several factors point to the possible involvement of caspase-like proteases in the death of the weaker secondary shoot. The proteolytic caspase-like activity was only induced in the dying shoot (Fig. 4). Importantly, the papain-like activity, on the contrary, was even slightly stronger in the dominant shoot. Moreover, the molecular weight of the caspase-specific activity (Fig. 5C) was much larger than that of the papain-like cysteine proteases. Judging by the molecular weight of the proteolytic activity, the pep protein may be similar to human initiator caspases-8 and -10. However, direct comparison will only be possible after cloning of the pep protease. The caspase-like activity was inhibited by subnanomolar concentrations of a caspase inhibitor. Inhibition of the protease activity completely blocked the death process and produced plants with equal shoots, indicating that the control exerted by the dominant shoot is mediated via a caspase-like protease (Fig. 6; Table 1).

To date the caspase-like activity in plants has been detected only in cases associated with either severe stresses, such as heat shock (Tian et al., 2000), treatment with toxic chemicals (Sun et al., 1999; De Jong et al., 2000), pathogen infection (del Pozo and Lam, 1998), or with specific developmental events, such as petal senescence (Xu and Hanson, 2000) or endosperm cell death during germination (Schmid et al., 1999). A relatively broad range of caspase-specific substrates has been
observed in the present study and in the other plant systems that have been analysed with several substrates (Woltering et al., 2002). This could be a result of a simultaneous induction of several different caspase-like proteases, or alternatively, it could stem from a relaxed stringency in substrate recognition in plants. Both these phenomena have been described in the well characterized animal systems (Cohen, 1997; Margolin et al., 1997; Rano et al., 1997; Bae et al., 2001).

In summary, it has been shown that protease(s) with caspase-like activity play a determinative role in the early vegetative development of pea seedlings. Since the sequencing projects of model plant genomes have not yet identified true caspases, it is difficult to attribute the cleavage of the various caspase-specific substrates to specific enzymes. Since the pea genome is not expected to be sequenced soon, the isolation of the caspase-like activity is necessary for analysing the substrate specificity. Unfortunately, the activity is present only in the dying shoots, which constitutes a very small amount of available biological material for purification.

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