Correspondence

Metacaspase-dependent programmed cell death is essential for plant embryogenesis

Maria F. Suarez[1], Lada H. Filonova[1], Andrei Smertenko[2], Eugene I. Savenkov[1], David H. Clapham[1], Sara von Arnold[1], Boris Zhivotovsky[3] and Peter V. Bozhkov[1]

In plants, as in animals, programmed cell death (PCD) is a key process responsible for the elimination of unneeded structures and for overall shape remodeling during development [1]; however, the molecular mechanisms remain poorly understood. Despite the absence of canonical caspases in plants, dying plant cells show an increased proteolytic caspase-like activity [2]. Moreover, the cell death can be suppressed using synthetic [2] or natural [3] caspase inhibitors. This raises the question of whether plants have specific cysteine proteases with a role similar to metazoan caspases in the execution of PCD. Metacaspases are the best candidates to perform this role, because they contain a caspase-specific catalytic diad of histidine and cysteine as well as conserved caspase-like secondary structure [4,5]. Here we show the first experimental evidence for metacaspase function in the activation and/or execution of PCD in plants, and also demonstrate the fundamental requirement of plant metacaspase for embryogenesis.

We explored the role of plant metacaspases in PCD using a model system of somatic embryogenesis of Norway spruce (*Picea abies*), where the pathway of embryo development (Figure 1A) resembles zygotic embryogeny, even though the embryo origin is different in each case (i.e., somatic cells in proembryogenic mass (PEM) versus zygote) [6]. In this developmental pathway autophagic PCD ablates PEMs at the time of their differentiation to embryos and then eliminates terminally differentiated embryo suspensor as the embryos enter late embryogeny [6,7] (Figure 1A).

We have isolated a 1687 bp cDNA sequence from the embryogenic cell cultures (EMBL database accession number AJ534970). The encoded protein shows a significant degree of conservation with metacaspases and falls into the type II plant metacaspase subfamily (Figure S1A). The protein was named mcII-Pa. The predicted secondary structure of mcII-Pa contains conserved domains and motifs present in all members of the caspase/metacaspase/paracaspase superfamily [5] (Figure S1B). The putative mcII-Pa catalytic diad of cysteine and histidine is placed in the α/β fold characteristic for the caspase-hemoglobinase fold (CHF)-containing proteins [5]. The predicted mcII-Pa protein lacks both the death-effector domain and the caspase-activating recruitment domain found in classical initiator caspases, but has a p20-like domain including the active-site pentapeptide DXCHS (where X is A or S) shared by all metacaspases [5] (Figure S1B). This domain is fused to the 268 amino acid carboxy-terminal region consisting of a large insert of approximately 180 amino acids and a p10-like domain.

In situ hybridization analysis has revealed restricted accumulation of the *mcII-Pa* mRNA in those embryonic tissues and structures that are committed to death (Figure 1B). In the early embryos, the accumulation of the transcript was found in the embryo suspensor, with no hybridization signal detected in the embryonal masses composed of proliferating cells that will further differentiate to form mature embryos [6] (Figure 1B). In the mature embryos, the expression of *mcII-Pa* was associated with procambial strands (Figure 1B), the early stage of xylem differentiation.

To investigate the role of this metacaspase in the embryonic PCD, a hairpin RNA construct was designed to stably suppress *mcII-Pa* gene by RNA interference (Figure S2). Transformation of embryogenic cell cultures with this construct reduced the level of metacaspase mRNA by 97–99% for different clones (Figure 1C). In somatic embryogenesis of Norway spruce, withdrawal of the plant growth regulators (PGR) auxin and cytokinin is required to switch from the proliferation of PEMs to embryo development (Figure 1A) [6,7]. Accordingly, for untransformed lines and the clones transformed with the control plasmid pAHC25, withdrawal of PGR led to early embryo development through the establishment of embryonal mass and terminal differentiation of embryo suspensor (Figure 1D). In contrast, the clones silenced for the metacaspase gene continued to proliferate without any signs of embryonic pattern formation (Figure 1D). Silencing of metacaspase also resulted in a significant reduction of both VEIDase activity (the principal caspase-like activity associated with embryo development [2]) and the frequency of TUNEL-positive cells (Figure 1C), demonstrating the cell death-protective effect of metacaspase silencing. A strikingly similar PCD-deficient phenotype was obtained through downregulation of metacaspase expression using an RNA antisense approach (data not shown) and by specific *in vivo* inhibition of the VEIDase activity [2]. Our results suggest that mcII-Pa is a molecular component of the developmental cell death machinery, essential for maintaining the proper balance between cell death and proliferation required for plant embryogenesis.

Discovery of CHF-containing proteins, including meta- and paracaspases [4,5], has resulted in a rapid explosion of research on the function of these proteins in diverse organisms. To date, the yeast protein YCA1 is the only metacaspase identified to mediate PCD [8], whereas the cell death function of paracaspases is doubtful [9]. The discovery of metacaspase-dependent PCD in a higher plant and demonstration of its developmental role in

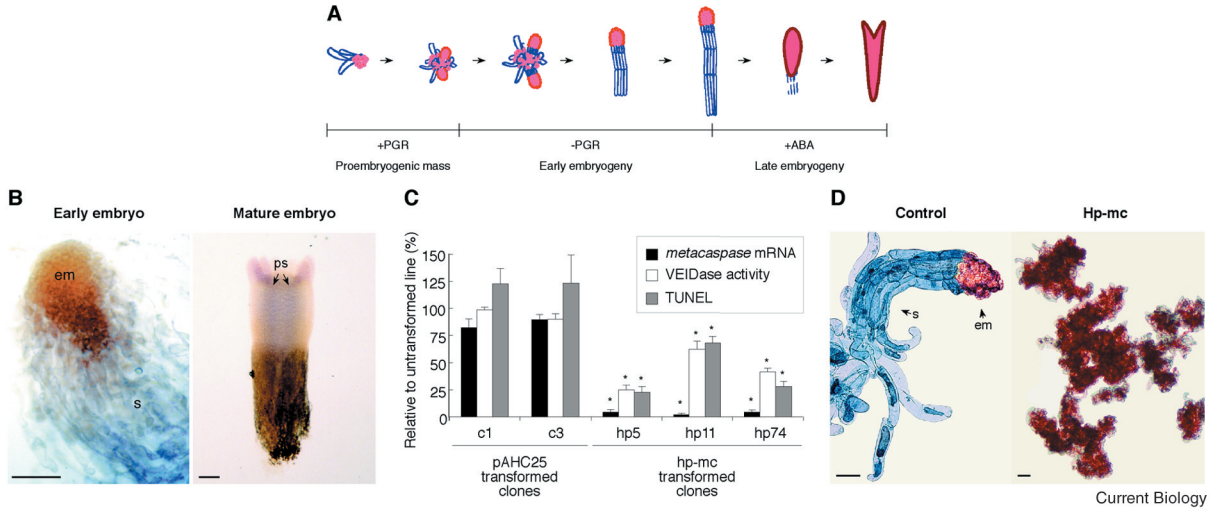

Figure 1. Role of metacaspase in PCD during plant embryogenesis.
(A) Developmental pathway of somatic embryogenesis in Norway spruce (not drawn to scale), including proliferation of PEMs (stimulated by addition of PGR), early embryogeny (stimulated by withdrawal of PGR) and late embryogeny (stimulated by abscisic acid, ABA). Blue and red colors denote dying cells (Evans blue-positive) and living cells (acetocarmine-positive), respectively [6]. (B) *mcII-Pa* transcripts accumulate in the embryo suspensor (no expression detected in the embryonal mass) during early embryogeny and in the procambial strands during late embryogeny. *In situ* hybridization signal is shown in purple. (C) Effects of RNAi on the levels of metacaspase mRNA, VEIDase activity and TUNEL (all three assayed 4 days after withdrawal of PGR). Values plotted represent the means (± SEM) of triplicate experiments. *, $p < 0.01$ versus untransformed cell line; Student's t-test. Similar results have been obtained for all five clones carrying the hairpin construct. (D) Representative examples of the phenotypes of the clones transformed with control and hp-mc-containing plasmid. The clones were grown for 4 days in PGR-free medium and stained with acetocarmine and Evans blue to assess cell viability. em, embryonal mass; ps, procambial strands; s, suspensor. Scale bars, 100 μm.

multicellular pattern formation illustrate functional similarities between an ancient family of caspase-related proteins (metacaspases) and canonical caspases conserved throughout evolution. Emerging knowledge of the diverse functions of meta- and paracaspases, along with increasing evidence that canonical caspases may participate in non-apoptotic processes [10], call for further investigation of the signaling pathways regulated by distinct members of the caspase/metacaspase/paracaspase superfamily.

Acknowledgments
We thank U. Lagercrantz, M. Elfstrand, M. Medina, S. Orrenius and H. Ronne for advice and comments on the manuscript. This work was supported by the Spanish Ministry of Education, the Swedish Foundation for International Cooperation in Research and Higher Education, Carl Tryggers Foundation and Troedssons Fund.

Supplemental Data
Supplemental Data are available at http://www.current-biology.com/supplemental

References
1. Lam, E. (2004). Controlled cell death, plant survival and development. Nat. Rev. Mol. Cell Biol. *5*, 305-315.
2. Bozhkov, P.V., Filonova, L.H., Suarez, M.F., Helmersson, A., Smertenko, A.P., Zhivotovsky, B., and Von Arnold, S. (2004). VEIDase is a principal caspase-like activity involved in plant programmed cell death and essential for embryonic pattern formation. Cell Death Differ. *11*, 175–182.
3. Lincoln, J.E., Richael, C., Overduin, B., Smith, K., Bostock, R., and Gilchrist, D.G. (2002). Expression of antiapoptotic baculovirus p35 gene in tomato blocks programmed cell death and provides broad-spectrum resistance to disease. Proc. Natl. Acad. Sci. USA *99*, 15217–15221.
4. Uren, A.G., O'Rourke, K., Aravind, L., Pisabarro, M.T., Seshagiri, S., Koonin, E.V., and Dixit, V.M. (2000). 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.
5. Aravind, L., and Koonin, E.V. (2002). Classification of the caspase-hemoglobinase fold: detection of new families and implications for the origin of the eukaryotic separins. Proteins *46*, 355–367.
6. Filonova, L.H., Bozhkov, P.V., Brukhin, V.B., Daniel, G., Zhivotovsky, B., and Von Arnold, S. (2000). Two waves of programmed cell death occur during formation and development of somatic embryos in the gymnosperm, Norway spruce. J. Cell Sci. *113*, 4399–4411.
7. Smertenko, A.P., Bozhkov, P.V., Filonova, L.H., Von Arnold, S., and Hussey, P.J. (2003). Re-organisation of cytoskeleton during developmental programmed cell death in *Picea abies* embryos. Plant J. *33*, 813–824.
8. Madeo, F., Herker, E., Maldener, C., Wissing, S., Lächelt, S., Herlan, M., Fehr, M., Lauber, K., Sigrist, S.J., Wesselborg, S., et al. (2002). A caspase-related protease regulates apoptosis in yeast. Mol. Cell *9*, 911–917.
9. Ruefli-Brasse, A.A., French, D.M., and Dixit, V.M. (2003). Regulation of NF-(kappa)B-dependent lymphocyte activation and development by paracaspase. Science *302*, 1581–1584.
10. Algeciras-Schimnich, A., Barnhart, B.C., and Peter, M.E. (2002). Apoptosis-independent functions of killer caspases. Curr. Opin. Cell Biol. *14*, 721–726.

[1]Department of Plant Biology and Forest Genetics, Swedish University of Agricultural Sciences, Box 7080, SE-750 07 Uppsala, Sweden. E-mail: peter.bozhkov@vbsg.slu.se [2]The Integrative Cell Biology Laboratory, School of Biological Sciences and Biomedical Sciences, University of Durham, South Road, Durham DH1 3LE, UK. [3]Institute of Environmental Medicine, Karolinska Institutet, Box 210, SE-171 77 Stockholm, Sweden.