Ricinosomes Predict Programmed Cell Death Leading to Anther Dehiscence in Tomato

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Successful development and dehiscence of the anther and release of pollen are dependent upon the programmed cell death (PCD) of the tapetum and other sporophytic tissues. Ultrastructural examination of the developing and dehiscing anther of tomato (Solanum lycopersicum) revealed that cells of the interlocular septum, the connective tissue, the middle layer/endothecium, and the epidermal cells surrounding the stomium all exhibit features consistent with progression through PCD. Ricinosomes, a subset of precursor protease vesicles that are unique to some incidents of plant PCD, were also present in all of these cell types. These novel organelles are known to harbor KDEL-tailed cysteine proteinases that act in the final stages of corpse processing following cell death. Indeed, a tomato KDEL-tailed cysteine proteinase, SlCysEP, was identified and its gene was cloned, sequenced, and characterized. SlCysEP transcript and protein were restricted to the anthers of the senescing tomato flower. Present in the interlocular septum and in the epidermal cells surrounding the stomium relatively early in development, SlCysEP accumulates later in the sporophytic tissues surrounding the locules as dehiscence ensues. At the ultrastructural level, immunogold labeling localized SlCysEP to the ricinosomes within the cells of these tissues, but not in the tapetum. It is suggested that the accumulation of SlCysEP and the appearance of ricinosomes act as very early predictors of cell death in the tomato anther.

Successful reproduction in the vast majority of angiosperms is dependent on the proper development and release of the male gametophytes, the pollen, from the anthers. A thorough understanding of the processes of pollen formation and release is useful for agricultural practices and maintenance of both agriculturally and ecologically important genetic banks. In those agricultural species that are normally self-crossing, the artificial induction of male sterility can facilitate cross-pollination and the production of hybrids, allowing for an increase in the pool of genetically diverse individuals. Cross-pollination also allows for the flow of genetic information between closely related species and is receiving a great deal of public attention with the advent and use of transgenics in agriculture. Understanding of the processes leading to pollen production and release is of great importance given the potential ecological significance of the release and transmission of transgenes from agricultural crop plants into native wild relatives (Goldberg et al., 1993; Ma, 2005).

Interestingly, the successful production of viable pollen is dependent on the death of sporophytic tissues of the anther. Microsporogenesis, microgametogenesis, and the resulting formation of viable pollen within the locules of the anther are dependent on nutritive contributions from the surrounding sporophytic tissues (detailed in Ma, 2005, and refs. therein). As an essential part of anther development and pollen formation, cells of the tapetum are sacrificed through programmed cell death (PCD). With continued development of the microgametophytes, the cellular constituents resulting from tapetal PCD provide nutrition, act in exine sculpting, and are deposited as the materials characteristic of the pollen wall (Wu and Cheung, 2000; Varnier et al., 2005; Vizcay-Barrena and Wilson, 2006). PCD then extends radially to cells of the middle layer and connective tissues nearest the locular chambers, the digested cellular contents presumably providing additional nutritional resources in support of the anabolic metabolism of the microgametophytes during reserve accumulation (Wetzel and Jensen, 1992; Varnier et al., 2005). Finally, cells of the endothecium and epidermal cells surrounding the stomium undergo PCD just prior to dehiscence (Varnier et al., 2005). Disruption of PCD in the sporophytic tissues of the anther can lead to male sterility. Either premature or aborted death of tapetal cells results in the disruption of the nutrient supply to the microgametophytes, resulting in their death (Ku et al., 2003; Kawanabe et al., 2006). Similarly, PCD in the outer sporophytic tissues is required for pollen release, and disruption of the timing of PCD in the endothecium
and epidermal cells surrounding the stomium also results in male sterility (Beals and Goldberg, 1997; Sanders et al., 2005, and refs. therein). Pollen may be viable, but its efficient release from the anther is compromised (Ge et al., 2005). Thus, the timely and controlled death of cells of the sporophytic tissues of the anther is necessary for production of the male gametophyte.

PCD is common to all multicellular organisms (for review, see Zakeri and Lockshin, 2008). Indeed, some of the biochemical hallmarks of apoptosis, a paradigm of PCD in animals, are also seen during anther maturation. These include DNA “laddering,” or the digestion of genomic DNA into internucleosomal fragments, and cytochrome c release from the mitochondria (Balk and Leaver, 2001; Varnier et al., 2005). However, apoptosis in animals is dependent on the activity of key cytosolic Cys proteinases, the caspases, as initiators, and a resultant caspase cascade to continue and complete the apoptotic process. Plants do not have traditional caspases but seem to rely more heavily on the activity of vacuolar, and other, Cys proteinases as potential activators and terminal players (Trobacher et al., 2006, and refs. therein). Indeed, Koltunow et al. (1990) and Xu and Chye (1999) have documented the accumulation of particular transcripts encoding individual Cys proteinases in various tissues of the maturing anthers of tobacco (Nicotiana tabacum) and eggplant (Solanum melongena), respectively. Xu and Chye (1999) predict a role for one encoded Cys proteinase in the PCD of the cells of these anther tissues. Although Koltunow et al. (1990) do not refer to PCD directly, transcripts encoding one proteinase increase dramatically and in a sequential manner in the circular central cells, the stomium, and subsequently in the connective tissue and endothecium cells surrounding the locules. All of these tissues undergo PCD and play a role in anther dehiscence and pollen release (Bonner and Dickinson, 1989; Varnier et al., 2005), suggesting a role for the encoded proteinase in PCD. It should be mentioned, however, that neither the study by Koltunow et al. (1990) nor Xu and Chye (1999) dealt with the actual Cys proteinase, but only with relative transcript abundance during development.

Among the terminal players in some specific incidents of PCD are KDEL-tailed Cys proteinases that reside in rough endoplasmic reticulum-derived organelles, the precursor protease vesicles and ricinosomes. First documented by Mollenhauer and Totten (1970), the ricinosomes and the enzymes within these unique organelles have been implicated in PCD of the cells of the postgerminative castor (Ricinus communis) seed endosperm and senescent day lily (Hemerocallis spp.) petal (Gietl et al., 1997; Schmid et al., 1999) and in PCD of the nucellus during castor seed development (Greenwood et al., 2005). More recently, ricinosomes, but not the enzymes within, have been implicated in PCD of the endosperm of postgerminative tomato (Solanum lycopersicum) seed (DeBono and Greenwood, 2006). The collapse of the central vacuole essentially defines the point of death in plant cells (Jones, 2001) and results in the acidification of the cytoplasm. In those incidents of PCD in which ricinosomes are involved, it is believed that the acidification of the cytosol results in the autocatalytic processing of the enzyme to its mature active form. Concomitantly, the ricinosomes swell and break open, releasing these very active enzymes, which then act in the final processing of the cell corpse. It should be noted that Rogers (2006) distinguishes ricinosomes from precur sor protease vesicles, in that the former deliver their enzymic contents directly to the cytosol following vacuolar collapse whereas the latter fuse with the central vacuole, delivering vacuolar processing enzymes responsible for the processing of a number of proteins. When present, the occurrence of ricinosomes in cells seems to infallibly predict that those cells are going to die (Gietl and Schmid, 2001).

The histological details of anther development and some cytological details of cell death in the sporophytic tissues, particularly the tapetum (Papini et al., 1999; Wang et al., 1999; Wu and Cheung, 2000), have been documented for a number of species, including members of the Solanaceae (Bonner and Dickinson, 1989; Koltunow et al., 1990; Goldberg et al., 1993; Sanders et al., 2005; Varnier et al., 2005). However, with limited exceptions (Sanders et al., 2005; Varnier et al., 2005), ultrastructural details of the progression of PCD in anther tissues other than the tapetum have been largely ignored and limited to documenting mitochondrial and nuclear changes that occur as the cells approach death. Common ultrastructural features consistent with the progression of PCD in plant cells have been provided (Gunawardena et al., 2004, and references therein; DeBono and Greenwood, 2006) and include the following: progressive condensation of chromatin with nuclear shrinkage, invagination, and lobing; aberrant morphologies and changes in electron density of mitochondria and plastids; vesiculation and vacuolization of the cytoplasm with changes in electron density of the tonoplast and cytoplasm; shrinkage of the plasma membrane from the cell wall; and vacuolar collapse. Ricinosomes may or may not be present.

Tomato is a very important crop plant. With a wide range of documented cultivars, varieties, and commercially important mutants available, as well as a plethora of other genetic resources, tomato proves an excellent experimental subject. This is especially true as the International Tomato Genome Sequencing Project nears completion (Mueller et al., 2005). These attributes, accompanied by the relatively large size of the plants, organs, and seeds, may make tomato very amenable to studying developmental PCD processes. In this study, we investigate the ultrastructural details of the progression of PCD in the connective, middle layer/endothelial tissues and the epidermal cells defining the stomium in developing anthers of tomato. The observation that ricinosome-like organelles are common during PCD in these tissues prompted an
investigation into the existence and patterns of expression of a gene or genes encoding a KDEL-tailed Cys proteinase and to further examine its location and potential role during anther development and dehiscence.

RESULTS

Morphology of the Developing Tomato Anther

The tomato androecium consists of six anthers arranged in a ring around a central cavity through which the style protrudes (Fig. 1A). Individual anthers are bithecal and tetrasporangiate (Fig. 1B). Pollen development occurs within the four locules, each of which is surrounded by the sporophytic cell layers of the tapetum, the middle layer, and epidermal layers (Fig. 1B). The endothecium may not be apparent but, when present, is usually more obvious in the wall surrounding the adaxial locules (Fig. 1B). The pair of locules within each theca is divided by four to six cell layers of connective tissue, with the interlocular septum marking the region separating the connective tissue from the stomium (Fig. 1B). There is a progressive loss of cells of the sporophytic layers with development (Fig. 1, C–G). The inner and outer tapetum, which line the locules at stage 9 (see Brukhin et al., 2003, for tomato flower development schedule), are reduced by stage 11 and are almost completely absent from anthers of stage 13 through 20 flowers (Fig. 1, C–G). The connective tissue and stomia are continuous in stage 9 anthers, but by stage 13 to 16 the interlocular septum and cells of the connective tissue degrade, creating the interlocular channel. The number of cell layers in the middle layer/endothecium continue to be reduced with development. By stage 16, the region adjacent to the stomium consists of a single layer of epidermal cells (Fig. 1, E and F). By stage 20, the septa are completely degraded and the stomia have opened to release the pollen, completing the dehiscence process (Fig. 1G).

Ultrastucture of the Stomial, Middle Layer, and Connective Tissue Cells during Anther Maturation to Dehiscence

The ultrastructure of the epidermal cells surrounding the stomium (Fig. 2; henceforth referred to as

![Figure 1. Structure and development of the tomato androecium and anther. A, Whorl of anthers constituting the androecium. Bar = 200 μm. B, Tissues/structures of the anther. c, Connective tissue; e, epidermis; en, endothecium; is, interlocular septum; l, locule; m, middle layer; st, stomium; t, tapetum; v, vasculature. Bar = 100 μm. C to G, Developmental series illustrating the progressive loss of sporophytic cell layers/structures leading up to dehiscence. Tapetal layers apparent at stage 9 (C) and 11 (D) begin to be lost by stage 13 (E), the interlocular septum being close to disruption at that time (E), and loss of the septum is seen by stage 16 (F). There is a progressive loss of cell layers of the connective tissue and the middle layer/endothecium from stage 13 (E) through stage 16 (F) leading to dehiscence (G), with the cells surrounding the stomium separating at stage 20 (G), marking the completion of the dehiscence process. Bar in G = 100 μm. [See online article for color version of this figure.]
Figure 2. Stomial cells of the developing tomato anther display features consistent with the progression of PCD. A to F, Developmental series roughly equivalent to that seen in Figure 1, C to G. Stages 9 (A), 11 (B), 15 (C and D), 18 (E), and near 20 (F) are shown. A, Stage 9 epidermal cells surrounding the stomium appear normal with the exception of the presence of ricinosomes (stars). Cellular material in the central vacuole suggests that autophagy is occurring. The cell illustrated has recently divided, with new cell wall forming between adjacent sibling cells (cw, with white arrow). Ricinosomes are also apparent in the interlocular septal cell lying immediately beneath the epidermal cells (bottom). B, Stage 11 epidermal cell showing the accumulation of autophagic vesicles/vacuoles (av) in the cytoplasm. Ricinosomes are evident (stars) in the stomial cells. Features of the cytoplasm...
The accumulation of large calcium oxalate organelles are also seen in these cells prior to death quickly (Figs. 2, A–C, and 3, A–C). Ricinosome-like amorphous contents with a more electron-translucent approximately 0.5 to 2 that of the rough endoplasmic reticulum, were approxi-mately early in development of the anther, surrounding the stomium. These organelles were ev-ident relatively early in development of the anther, even being present in cells that had recently divided (Fig. 2A), and persisted in the cells with progression to dehiscence (Fig. 2, B–D and G–J). The organelles were often associated with the rough endoplasmic reticulum, were surrounded by a membrane comparable to that of the rough endoplasmic reticulum, were approximately 0.5 to 2 µm in diameter, and usually had dense amorphous contents with a more electron-translucent region near the periphery (Fig. 2, A–D and G–J).

In comparison with the cells of the stomium, PCD in the underlying interlocular septum occurs very quickly (Figs. 2, A–C, and 3, A–C). Ricinosome-like organelles are also seen in these cells prior to death (Fig. 2A). The accumulation of large calcium oxalate crystals in the vacuoles of the interlocular septum cells, lost during sectioning and resulting in voids, are characteristic of anthers of the Solanaceae and provide a landmark for orientation during ultrastructural studies (Fig. 2F, lower cell, and Fig. 3, A–C, upper cells).

Features consistent with PCD as observed in the stomial cells were also seen in the cells of the connective tissue and middle layer/endothecium (Fig. 3, A–D and E–H, respectively). Chromatin condensation and irregularities in nuclear shape were more prevalent than in the stomial cells, and cell wall integrity was increasingly compromised with progression through to dehiscence. In addition, paracrystalline structures, presumably peroxisomes, were often seen in cells of the middle layer/endothecium. Ricinosome-like organelles, however, were not easily observed in either connective tissue or middle layer/endothecial cells until later stages. Close observation revealed that ricinosome-like organelles did exist in these cells early in the progression to death but that the organelles were small compared with those found in the stomial cells and had contrast similar to the cytoplasm (compare Fig. 3, I–L, with Fig. 2, G–J, noting the differences in magnification). Size and contrast increased as death became imminent (Fig. 3, H and M). Interestingly, ricinosome-like organelles were never observed in the tapetum at any stage through the progression to dehiscence (data not shown).

The occurrence of the ricinosome-like organelles in the cells of the stomium, middle layer/endothecium, interlocular septum, and connective tissue, similar to those found in cells of castor oil seed endosperm and nucellus that are destined to die, strongly suggested that the terminal steps of PCD in these cells and tissues involved a KDEL-tailed Cys proteinase(s). Further research was conducted to substantiate this.

**Tomato Has a Gene Encoding a KDEL-Tailed Cys Proteinase**

Castor CysEP encodes a KDEL-tailed Cys proteinase that accumulates specifically in ricinosomes and is known to be a predictor of some incidents of PCD in that species (Schmid et al., 1999). A BLAST search of the National Center for Biotechnology Information database was conducted using the CysEP mRNA.
sequence (GenBank accession no. AF050756), and a highly homologous, uncharacterized cDNA from tomato fruit was identified (GenBank accession no. BT014302). Reverse transcription (RT)-PCR of stage 13 tomato flower bud RNA using primers complementary to regions of the BT014302 mRNA sequence resulted in the amplification of a 1,198-bp cDNA identical to BT014302. Amplification of the corresponding genomic sequence resulted in a 1,700-bp fragment, and comparison with the cDNA sequence revealed that the gene contains three introns. Genome walking culminated in the cloning of a 4,270-bp ge-
nominal DNA fragment, designated SlCysEP for Solanum lycopersicum Cys endopeptidase (GenBank accession no. EU122386). Two genes were identified within the 4,270-bp sense strand of the genomic sequence: SlCysEP in the plus orientation and Methionine Sulfoxide Reductase A, a fruit-ripening gene (National Center for Biotechnology Information accession no. S44898), in the minus orientation (data not shown).

In silico translation of the open reading frame of SlCysEP predicts a 360-amino acid papain-like pro-pro-Cys proteinase containing a hydrophobic N-terminal signal peptide and having a C-terminal KDEL endoplasmic reticulum-retrieval motif, among other features (Fig. 4). Alignment of the predicted SlCysEP amino acid sequence to those of CysEP (castor), SH-EP (mung bean [Vigna mungo]), TPE4A (pea [Pisum sativum]), and SEN11 (Hemerocallis species) demonstrates that there is a significant amount of amino acid identity between SlCysEP and these senescence-related proteinases (Fig. 4). CysEP and the SlCysEP translation product both contain 360 amino acids with 76% identity, and this comparison allowed a prediction of the SlCysEP cleavage sites based on the known cleavage sites within CysEP (Fig. 4). The signal peptide is likely cleaved between amino acids 20 and 21, and the propeptide between amino acids 125 and 126, producing a 235-amino acid mature enzyme (amino acids 126–360). The predicted molecular masses of unprocessed SlCysEP, the proprotein, and mature enzyme are 40.6, 38.3, and 25.5 kD, respectively.

Southern-blot analysis, using a digoxigenin-labeled cDNA probe complementary to 479 bp of exon 1, against restriction-digested genomic DNA of tomato suggests that SlCysEP is present as a single copy in the tomato genome (Fig. 5). Recombinant pro-SlCysEP undergoes self-hydrolysis at pH 4.8, with complete conversion from an isoform of about 45 kD to an isoform of about 28 kD occurring within 12 min; no processing occurs at pH 7.0 (Fig. 6C). At pH 4.8, an intermediate cleavage product of 43 kD was observed immediately upon the addition of the acid, and two intermediate cleavage products of 32 and 30 kD could be seen after 3 min. Only the 28-kD isoform was present after 12 min of incubation (Fig. 6C).
SlCysEP Is Restricted to Specific Tissues of the Anthers during Floral Development and Senescence

Northern-blot analyses determined that SlCysEP transcripts were abundant in stage 13 to 15 flowers and that expression in these flowers was restricted to the stamen (Fig. 7A). Western immunoblot analyses confirmed that SlCysEP was minimally expressed in stage 1 to 12 flowers (Fig. 7B). Three peptides were detected in stage 13 to 15 flowers: a 44-kD peptide, slightly larger than the predicted molecular mass of the entire SlCysEP protein (40.6 kD); a smaller peptide of approximately 43 kD that could be a processed form lacking the signal peptide (predicted at 38.3 kD); and a 29-kD peptide similar to the predicted molecular mass of the mature enzyme (25.5 kD; Fig. 7B). The 29-kD peptide alone was detected in stage 18 to 20 flowers. Accumulation of the proteinase was restricted to the stamens (Fig. 7B).

To address the possibility that the affinity-purified anti-SlCysEP antibodies were cross-reacting with other Cys proteinases, an immunoprecipitation experiment was performed using total protein extracted from stage 18 to 20 anthers. A single peptide corresponding to the 29-kD peptide in Figure 7B was pulled down (Supplemental Fig. S1). That the antibodies did not detect any proteins in other senescing floral tissues, at the time when the anthers are becoming competent to dehiscence (Fig. 7B), is also good evidence that there is minimal cross-reactivity with other Cys proteinases.

Immunohistochemistry revealed that SlCysEP is restricted to the interlocular septum in anthers from stage 11 flowers (Fig. 8A) but later accumulates in the cells of the stomium, the middle layer/endothecium, and the connective tissues (Fig. 8B). With progression to dehiscence, SlCysEP becomes restricted to the cells of the stomium (Fig. 8C), with some remaining in the epidermal cells that surround the stomium even after dehiscence is complete (Fig. 8D). No signal was seen in controls using preimmune serum (Fig. 8, E–H).

SlCysEP Localizes to Ricinosomes in Cells Undergoing PCD

Immunogold labeling using rabbit anti-SlCysEP localized SlCysEP to the ricinosome-like organelles in the stomial, interlocular septum, and middle layer/endothecium cells during development and dehiscence.

Figure 7. Accumulation of SlCysEP transcript and protein in developing tomato flowers. A, Northern-blot analysis using total flower RNA from whole flowers at different developmental stages and from floral tissues of stage 13 to 15 flower buds. 25S ribosomal RNA is shown as a loading control. B, Western blot of total flower protein from different developmental stages and different floral tissues of stage 13 to 15 flowers detected with affinity-purified anti-SlCysEP rabbit polyclonal antibodies. Pd, Pedicel; Se, sepal; Pe, petal; St, stamen; Ca, carpel.
(Fig. 9), confirming that the organelles are indeed ricinosomes. Accumulation of the enzyme begins as early as stage 9 in the stomial and interlocular septum cells (Fig. 9, A and E) and by approximately stage 13 in the cells of the middle layer/endothecium as tapetal degeneration proceeds (Fig. 9). Labeling intensity over the ricinosomes increased as the ricinosomes became more electron dense with increased maturity of the cells and tissues (Fig. 9, B, F, G, K, and L). At death, the ricinosomes rupture, in some cases evidently in conjunction with the loss of tonoplast integrity (Fig. 9C), releasing SlCysEP to the cytoplasm (Fig. 9, C, D, I, and M, asterisks with arrows), which has shrunk away from the cell wall (Fig. 9, D, I, L, and M).

**Multiple Cys Proteinase Genes Are Expressed throughout Anther Development**

Given that multiple Cys proteinases are often expressed during PCD (for review, see Trobacher et al., 2006), there is a strong possibility that other Cys proteinases act either before or concurrent with SlCysEP. To investigate this possibility, gene-specific primer sets were designed to amplify fragments of transcripts from several tomato Cys proteinase genes or unigenes. RT-PCR performed on total RNA extracted from anthers at stages 1 to 13, 13 to 18, and 18 to 20 confirmed that multiple Cys proteinase genes are expressed in tomato anthers (Fig. 10). SlCysEP expression in stages 1 to 13 and 13 to 18 is similar, but it decreases in stages 18 to 20. TDI-65, encoding a drought-inducible 65-kD Cys proteinase found in the nucleus, chloroplasts, and some cytoplasmic regions of mesophyll cells (Tabaeizadeh et al., 1995), is expressed evenly in all stages examined. Expression of SICYSPRO, a member of the C1A family of Cys proteinases, is similar to TDI-65. SGN-U321596, a unigene predicted to encode a 42.7-kD C1A family Cys proteinase similar to Arabidopsis (Arabidopsis thaliana) XBCP3 (Zhao et al., 2000), is also evenly expressed in anthers at all stages examined. Transcripts encoding a putative 40.3-kD meta-caspase, SIMCA2, belonging to the C14B family of Cys peptidases, are detectable in stages 1 to 13; however, expression increases through stages 13 to 18 and 18 to 20. Expression of another putative C1A family member unigene, SGN-U321072, could not be detected despite EST data suggesting that it is expressed in flower buds (data not shown).

**DISCUSSION**

The progression of PCD in the nontapetal sporophytic tissues of the tomato anther, from mid development leading up to dehiscence, has been documented using light and electron microscopy. Our observations of the morphological and cytological features of anther development and dehiscence are consistent with those observed previously for tomato (Bonner and Dickinson, 1989, and refs. therein) and tobacco, an-
Figure 9. Immunogold localization of SlCysEP in sporophytic tissues of tomato anther. A to D, Stominal cells. E to I, Cells of the connective tissue. J to M, Middle layer/endothecium cells. A, E, and J, Early development, approximately stages 9 to 11. B, F to H, and K to L, Mid to late development, approximately stages 15 to 18. C, D, I, and M, Late development/dehiscence following vacuolar collapse.
other member of the Solanaceae (Koltunow et al., 1990; Goldberg et al., 1993; Sanders et al., 2005). The general features of anther development, as described by Goldberg et al. (1993), apply very well to tomato, with minor exceptions. Unlike tobacco, in which the stoma- mium consists of multiple cell layers (Sanders et al., 2005) and the endothecium is easily identified, the stoma- mium in tomato is only one cell layer thick and the endothecium is not well-defined and may be absent in large portions of the mature anther (Bonner and Dickinson, 1989).

The majority of previous cytological studies of an- ther development fail to detail the changes in cellular ultrastructure that accompany the progression to dehiscence. Although a number have provided ultra- structural details of tapetal PCD during pollen development (Brighigna and Papini, 1993; Papini et al., 1999), these tend to ignore other anther tissues that play a fundamental role both in providing additional nutrition for the pollen and in the dehiscence process itself. By documenting changes in nuclear and mitochondria- l structure, Varnier et al. (2005) demonstrated that, in Lilium, PCD extends radially out from the tapetum to involve the other sporophytic tissues. Our observations here extend those of Varnier et al. (2005), demonstrating that the cells of the stomium, connective tissue, and middle layer/endothecium ex- hibit features consistent with the progression of PCD as put forward by Gunawardena et al. (2004). These features include the progressive condensation of chromatin with nuclear shrinkage, invagination, and lob- ing. Although not performed here, accompanying these, nuclei become positive for terminal UTP nick end labeling of digested genomic DNA ends, and genomic DNA is internucleosomal fragmented, pro- ducing the electrophoretic laddering pattern typical of apoptotic cell death (Varnier et al., 2005). Other fea- tures include the development of aberrant morphol- ogies and changes in electron density of mitochondria and plastids, with the former possibly indicative of cytochrome c release that has been implicated in plant PCD (Varnier et al., 2005). Vesculation and vauco- lization of the cytoplasm are accompanied by shrinkage of the plasma membrane from the cell wall, changes in electron density of the tonoplast, and development of a flocculent appearance of the cytoplasm. Increased cytoplasmic electron density and disorganization and vauco- lation collapse mark cell death (Gunawardena et al., 2004, and refs. therein). An additional feature, again consistent with the entry and progression of a cell death program in at least some instances of PCD, are the appearance and accumulation of ricinosomes, which lyse upon the collapse of the central vacuole (Gietl and Schmid, 2001).

Figure 9. (Continued.)
Tapetum is seen on the right side of J. SICysEP is localized to ricinosomes (stars) in all cases (A, B, E to H, and J to L) until the central vacuole collapses, after which SICysEP is released to the cytosol (C, D, I, and M). The inset in C shows a control using anti- SICysEP antibodies preabsorbed with rSICysEP. Bars = 0.5 μm. cw, Cell wall; m, mitochondria; n, nucleus; p, plastid; v, vacuole.

Figure 10. Transcript analysis of several Cys proteinase genes during anther development. Gene-specific primers were used to amplify regions of Cys proteinase transcripts via RT-PCR on total RNA from stages 1 to 13, 13 to 18, and 18 to 20 anthers. Actin is included as a loading control.

The timing of the appearance of these characteristic- s, however, is dependent on cell type. The cells of the middle layer and connective tissue show a steady progression of those features commonly associated with PCD, with nuclear invaginations and some condensation of chromatin occurring early in the process. Although present, ricinosomes are not well developed until late in the death program in these cells. In contrast, modifications to nuclear structure in the cells immediately surrounding the stomium occur relatively late in the process, but ricinosomes are well developed very early, potentially being present even in dividing cells. The cells surrounding the stomium perform a number of functions. They form the first line of defense against pathogen invasion and insect attack during pollen development and maturation, yet they present the most vulnerable point of entry, as the anther wall in that area is only one cell thick after the loss of the intersporangial septum. The stomium must remain impenetrable until the pollen is mature, but it is also the site of dehiscence, which itself requires a weakening of cell wall adhesion at the point of the stomium. This weakening of the adhesion between adjacent cells is a late event and is dependent on the activity of wall-degrading enzymes produced by the cells themselves (Jenkins et al., 1999). The cells sur- rounding the stomium are fated to die very early but must remain alive and be actively synthesizing and exporting wall-degrading enzymes; thus, they would likely have to maintain functional transcriptional and translational machinery. Our observation that there is little evidence of nuclear invagination and chromatin condensation in these cells until relatively late in the dehiscence process supports this notion.

Perhaps the most novel finding of this study was that ricinosomes were common to all of the incidents of PCD in the sporophytic tissues followed during tomato anther development and dehiscence, with the
exception of that occurring in the tapetum. In agreement with the notion of Rogers (2006), the term “ricinosome” is preferred, as the organelles are operationally different from precursor protease vesicles. Unlike the latter, ricinosomes do not fuse with the central vacuole; instead, they lyse upon the collapse of the central vacuole at death, releasing their contents. The appearance of ricinosomes has been correlated with the occurrence of PCD in the nucellus of developing, and in the endosperm of postgerminative, castor oil seed (Schmid et al., 1999; Gietl and Schmid, 2001; Greenwood et al., 2005), and the term ricinosome is indeed derived from the genus name Ricinus (Mollenhauer and Totten, 1970). Outside of these, ricinosomes have been observed and linked to the PCD of endosperm cells in postgerminative tomato seed (DeBono and Greenwood, 2006) and, perhaps, with the senescence of day lily flower petals (Schmid et al., 1999). To our knowledge, no other instances of ricinosome involvement in plant PCD have been documented until this study.

In castor oil seed, ricinosomes harbor CysEP, an inactive pro-Cys proteinase having a KDEL endoplasmic reticulum-retrieval motif. The enzyme functions in the final stages of corpse processing, during PCD of the nucellar and endospermic cells during development, and following germination (Schmid et al., 1999; Gietl and Schmid, 2001; Greenwood et al., 2005) and may be involved additionally in the digestion of extensins in the walls of the dead cells (Helm et al., 2008). The tomato genome contains a single copy of SlCysEP, a gene encoding an enzyme homologous to CysEP that localizes to the same organelle, and our results suggest that it has a similar function. Interestingly, the developmental accumulation of SlCysEP mimicked almost precisely the pattern of expression seen for the TA56 transcript in tobacco anthers (Koltunow et al., 1990), which encodes a Cys proteinase, recently shown to be KDEL tailed (accession no. EU429306).

The proteolytic processing of purified native CysEP (Schmid et al., 2001) and SH-EP (Okamoto et al., 1999), a KDEL-tailed Cys proteinase from mung bean, are almost identical to that of acidified, recombinant SlCysEP. Unprocessed SlCysEP, some processed intermediates, and mature proteinase occur simultaneously in anther tissues as the dehiscence process proceeds and the sporophytic cells are in various

Table I. Sequences of primers, cycling conditions, and expected product sizes for RT-PCR

| Gene or Unigene and Accession No. | Sequence 5′ → 3′ | Annealing Temperature °C | No. of Cycles | Product Size bp |
|----------------------------------|------------------|--------------------------|---------------|-----------------|
| SlCysEP                          | TGCAATGGAGGATTGATGGACCC | 56 | 30 | 724 |
| EU122386                         | GTAAGAGACTAAAATAAGACGACGAGTAGG | 56 | 30 | 651 |
| TDI-65                           | GAGGATCTCGGGAAAACGTACGCC | 56 | 30 | 651 |
| AF172856                         | GAGGATCTCGGGAAAACGTACGCC | 56 | 30 | 651 |
| SICYSPRO                         | GAGGATCTCGGGAAAACGTACGCC | 56 | 30 | 651 |
| BT014429                         | GAGGATCTCGGGAAAACGTACGCC | 56 | 30 | 651 |
| SGN-                             | GAGGATCTCGGGAAAACGTACGCC | 56 | 30 | 651 |
| U321596                          | GAGGATCTCGGGAAAACGTACGCC | 56 | 30 | 651 |
| SIMCA2                          | GAGGATCTCGGGAAAACGTACGCC | 56 | 30 | 651 |
| Actin                            | GAGGATCTCGGGAAAACGTACGCC | 56 | 30 | 651 |

Table II. Sequences of primers used to amplify SlCysEP cDNA and genomic DNA and to synthesize the digoxigenin-labeled probe used for Southern and northern blotting

| Primer Name | Sequence 5′ → 3′ |
|-------------|-----------------|
| SlCysEP F1  | TGCAATGGAGGATTGATGGACCC |
| SlCysEP R1  | GTAAGAGACTAAAATAAGACGACGAGTAGG |
| SlCysEP R2  | GAGGATCTCGGGAAAACGTACGCC |
| SlCysEP 5′’ | GGAACGAGAGAGACTCAAGATGTA |
| SlCysEP 5′’ | GGAACGAGAGAGACTCAAGATGTA |
| SlCysEP 3′’ | GGAACGAGAGAGACTCAAGATGTA |
| SlCysEP 5′  | GAACTGAAAAACTTGACTAAG |
| SlCysEP 3′  | GAACTGAAAAACTTGACTAAG |
| SlCysEP gDNA | TACCTCTAAAGAGATCGTGTGTAAC |
| SlCysEP gDNA | AACGTATCAAAACTTGAGCC |

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stages of PCD; only the mature proteinase is seen in later stages. Given that processing only occurs in acidic conditions in vitro, the in vivo observations support the premise of Gietl and Schmid (2001) that the collapse of the acidic central vacuole activates the enzyme, thus making it a late player involved in corpse processing (Jones, 2001).

According to the tomato flower development schedule used throughout this research (Polowick and Sawhney, 1993; Brukhin et al., 2003), tapetum degeneration begins at stage 13 and is complete by stage 18, which coincides very well with SlCysEP transcript and protein accumulation. As the tapetum is known to undergo PCD and ricinosomes and their marker proteinase predict PCD in some instances, we fully expected to find SlCysEP in this tissue. Surprisingly, the enzyme was not seen to accumulate in the tapetum but was found in the other sporophytic tissues, including those immediately adjacent to the tapetum. The specificity of localization in this case would have been lost had we relied solely on whole tissue extraction for analysis. We have not yet determined the substrate characteristics necessary for SlCysEP action, but castor CysEP is known to have a broad substrate specificity, as would be expected for a corpse-processing enzyme (Than et al., 2004). The tapetum, however, not only contributes to the nutrition of the developing pollen grains but also provides materials responsible for sculpting the surface and that can be important in pollen-recognition events (Vizcay-Barrena and Wilson, 2006). Having a broad-spectrum proteinase involved in the PCD of the tapetum would release the enzyme to the locular chamber, where it could potentially act in damaging or destroying these important pollen surface-located proteins (Wang et al., 2003). Thus, the gradual dismantling of the tapetum through PCD must employ mechanisms distinct from those used in the remaining sporophytic cell types.

While the data presented above clearly demonstrate that SlCysEP is present in ricinosomes within the stomium and the interlocular septum, they do not preclude the possibility that other enzymes may be present as well. Our data demonstrate that several other Cys proteinase genes are in fact expressed in anthers. These, however, are unlikely to be ricinosome-resident candidates. SICYSPro has a signal peptide suggesting that it targets through the endoplasmic reticulum, but it lacks an endoplasmic reticulum-retention signal, a feature that seems to be requisite for residence in ricinosomes. Furthermore, in western-blot analyses, antibodies directed against recombinant SlCysEP (rSlCysEP) do not detect SICYSPro, and those against rSlCYSPro do not detect SlCysEP; in total protein extracts of multiple tissues (Senatore, 2006), TDI-65 is reported to localize to the nucleus, chloroplasts, and cytosol (Tabaeizadeh et al., 1995), while both SGN-U321596 and SlMCA2 lack a signal peptide and therefore must localize outside of the endomembrane system. Since multiple Cys proteinases are expressed during instances of PCD, we fully expect that other enzymes are present and play a coordinated role in the PCD of sporophytic tissues in tomato (Trobacher et al., 2006).

Although not present in all incidents of PCD in plants, when they do occur, ricinosomes and the marker proteinase are only seen in cells that are going to die, and their occurrence predicts the cell’s death (Gietl and Schmid, 2001; Greenwood et al., 2005). The enzyme is involved in corpse processing, active only after the collapse of the central vacuole and cell death, and thus should only be expressed in cells that are fated to die. Ricinosomes accumulate within the staminal cells and those of the interlocular septum very early in the development of the anther. If the premise of Gietl and Schmid (2001) holds, then these cells have been fated to die at the same time. Both cell types lie immediately adjacent to each other, but the cells of the interlocular septum are among the first to undergo PCD, whereas those surrounding the stomium are among the last. This suggests that the initiation of PCD, as identified by the formation of ricinosomes, may be uncoupled from whatever may be acting as an effector to complete the process. In this case, one cell type may be competent and responding to some additional death signal that the other may be incapable of responding to until very late. One recurring issue that arises in studies of plant PCD is that we do not have any firm understanding of how the process is initiated and then regulated. Given that the expression of SlCysEP, and the concomitant formation of ricinosomes, can occur well prior to death and that these seem to invariably predict that the cells are going to die, examining the factors controlling the expression of SlCysEP may provide some insight into how PCD in plants is initiated.

MATERIALS AND METHODS

Plant Materials

Seeds of tomato (Solanum lycopersicum ‘Glamour’) were purchased from Stokes Seeds. Plants were grown in 3.1-plant cups in a growth chamber set to 14-h, 25°C days/10-h, 22°C nights (Brukhin et al., 2003). Lights were maintained at an intensity of 205 μmol m⁻² s⁻¹. Plants were watered three times per week and were supplemented once a month with 20:20:20 fertilizer (1 g L⁻¹, 500 mL per plant). The tomato flower development schedule used for this research was proposed by Brukhin et al. (2003) and is based on morphological landmarks in development. Entire flowers were directly ground in liquid nitrogen for developmental stage protein and RNA extraction; flowers were dissected into pedicel, sepal, petal, stamen, and carpel prior to grinding for floral tissue protein and RNA extraction.

Bright-Field Microscopy

Stamens were fixed and embedded in paraffin according to de Almeida Engler et al. (2001). Brieﬂy, androecia were removed from flowers at various stages of development and ﬁxed in ice-cold FAA solution (3.7% [v/v] formaldehyde, 5.0% [v/v] glacial acetic acid, and 63.7% [v/v] ethanol). Samples were dehydrated using a graded aqueous ethanol series of 50%, 70% (both at 4°C), 80%, 90%, 95%, and twice at 100% ethanol (at room temperature), then through 50:50 ethanol:SafeClear (Fisher Scientiﬁc Company) and 100% SafeClear, at room temperature. Paraffin was introduced as 50:50 SafeClear:Paraplast (Fisher Scientiﬁc) and then with three changes of 100% SafeClear, at room temperature. Paraffin was introduced as 50:50 SafeClear:Paraplast (Fisher Scientiﬁc) and then with three changes of

Sections (10 μm thick) were obtained using a rotary microtome (American Optical Company) and mounted on charged slides. Sections were deparaffinized in SafeClear and rehydrated through the reverse...
aqueous ethanol series. Some sections were stained with toluidine blue O (0.1% [w/v]) toluidine blue O, 7% [v/v] ethanol, and 0.9% NaCl, pH 2.3) for 5 min and then rinsed, and coverslips were mounted using immersion oil. Sections were observed using a Jena-Lumar Contrast (Zeiss-Jena) microscope under bright-field optics, and digital images were obtained.

Transmission Electron Microscopy

Androecia were harvested at different stages leading to dehiscence and prepared for transmission electron microscopy following the methods of Delonno and Greenwood (2000) with slight modifications. Briefly, androecia were immersed in cold primary fixative (2% [v/v] glutaraldehyde, 1% [v/v] acrolein, 10 mM MgCl$_2$, and 1% [w/v] caffeine in 0.025 M potassium phosphate buffer, pH 7.2) and subjected to vacuum infiltration three times for 2 min each. Samples were fixed for 2 h with gentle agitation at 4°C, washed in 0.025 M potassium phosphate buffer, pH 7.2, three times for 50 min each, and then postfixed for 2 h in 1% (w/v) OsO$_4$ in the same buffer. Samples were then rinsed overnight in double-deionized water, dehydrated through a graded aqeous ethanol series (50%, 70% [both at 4°C], 80%, 90%, 95%, and twice at 100% ethanol [at room temperature]), for 50 min at each step, then dehydrated further in 50:50 (v/v) ethanol-propylene oxide and again in 100% propylene oxide, each for 30 min. Samples were infiltrated overnight in 50:50 (v/v) propylene oxide:Spurr’s resin (Spurr, 1969; hard mixture; Canemco-Marivac) and then even through three changes in fresh Spurr’s resin overnight at 4°C, then cured at 65°C overnight. Sections of 80 to 90 nm thickness were cut using a diamond knife and collected on 200 mesh copper or nickel grids, depending on application. Those collected on copper grids were stained for 10 min with saturated aqueous uranyl acetate, rinsed in double-distilled water, and stained again for 3 min with Reynolds’ lead citrate (Reynolds, 1963). Sections were viewed with a Philips CM-10 transmission electron microscope at an accelerating voltage of 80 kV, and digital images were obtained. Sections collected on nickel grids were subjected to immunogold labeling (see below).

Cloning of SlCysEP cDNA and Genomic DNA

Total RNA was extracted from stage 13 flower buds with Tri Reagent (Sigma) according to the manufacturer. RT was carried out using primer SlCysEP R1 (Table I), complementary to the 5’ end of BT014302. Primers SlCysEP F1 (Table I) and SlCysEP R1 are complementary to the 5’ and 3’ extremities of the BT014302 mRNA sequence and were used to PCR amplify the SlCysEP cDNA from the RT sample. The cDNA was subsequently cloned into pGEM-T vector (Promega, Fisher Scientific) and sequenced. DNA was extracted according to Richards et al. (2001). Primers SlCysEP F1 and SlCysEP R1 (Table I) were used to PCR amplify the genomic DNA complementary to the BT014302 mRNA sequence. Sequences upstream and downstream of the BT014302 coding sequence were obtained using GenomeWalker (Clontech) according to the manufacturer’s instructions. Libraries were prepared using genomic DNA digested with EcoRV, PvuII, KspI, Stul, and DraI (blunt ending) and EcoRI, XbaI, BamHI, HindIII, and EcoRV, with any 5’ overhangs blunt ended prior to ligation with the GenomeWalker adaptor. Nested primers SlCysEP 5’ GW 1, SlCysEP 5’ GW 2, SlCysEP 5’ GW2-1, SlCysEP 5’ GW 2-1, and SlCysEP 5’ GW 2 (Table I) were used for PCR amplification of genomic DNA from cDNA-prepared libraries following thermal cycling parameters: 95°C for 2 min; 32 cycles of 94°C for 1 min, 58°C for 40 s, 72°C for 8 min, and 72°C for 10 min; and then 4°C hold. The entire genomic SlCysEP sequence was amplified with High-Fidelity PCR Enzyme Mix (Fermentas) using primers SlCysEP 5’ gDNA and SlCysEP 3’ gDNA (Table I) with the following thermal cycling parameters: 95°C for 2 min; 30 cycles of 94°C for 1 min, 54°C for 45 s, 72°C for 12 min, and 72°C for 10 min; and then 4°C hold. All PCR products were cloned into pGEM-T vectors (Promega) and sequenced. The assembled SlCysEP genomic sequence was subsequently deposited into GenBank under accession number EU122386.

In silico translation and molecular mass predictions were performed using the SDSC Biology Workbench. Multiple sequence alignments were performed using Tcofree (Poirot et al., 2003), and signal peptide prediction was performed using iPSORT (Bannai et al., 2002).

Southern-Blot and Northern-Blot Analyses

Southern-blot analysis (Southern, 1975) was performed according to Sambrook and Russell (2001). Genomic DNA was restriction digested with XbaI, HindIII, DraI, NcoI, and EcoRV (Fermentas), separated by electrophoresis through a 1% agarose gel, and transferred onto a positively charged nylon membrane (Roche) by capillary transfer. For northern-blot analysis, total RNA was isolated as described above and electrophoresed through 1% (w/v) formaldehyde-agarose gels (Sambrook and Russell, 2001). RNA was transferred onto a charged nylon membrane by capillary transfer. Hybridization was performed using a 479-bp digoxigenin-labeled probe, matching sequence within the first exon of SlCysEP, synthesized by PCR using primers SlCysEP F1 and SlCysEP R2 (Table I). Prehybridization, hybridization, washing, and detection were carried out as described by the manufacturer (Roche Diagnostics).

SICysEP Protein Expression and Antibody Production

Recombinant SlCysEP lacking 22 N-terminal amino acids (Vernet et al., 1989) but having a 6-His C-terminal tag was expressed in Escherichia coli BL21-CodonPlus(DE3)-RP (Stratagene), purified by nickel affinity chromatography, and refolded according to Siwali et al. (2001). Recombinant protein was sent to Cederlane Laboratories for production of polyclonal antibodies in rabbits. For affinity purification, recombinant SlCysEP was immobilized on a 900-cm² nitrocellulose membrane (Costar Scientific), which was then blocked over-night with TTBS (20 mM tris-hydroxymethyl aminomethane, 0.5 M NaCl, and 0.1% [v/v] Tween 20) containing 5% (w/v) skim milk powder, then cut into smaller squares. Three milliliters of anti-SICysEP antiserum was added to 7 ml of TTBS and 0.1 g of skim milk, and the antibody solution was incubated with 5 ml of 2× SICysEP squares at room temperature, washed twice with TTBS for 15 min, and then transferred to small trays. Purified antibodies were eluted using 3.0 ml of elution buffer (25 mM Gly, pH 2.3, 0.5 M NaCl, and 0.5% [v/v] Tween 20) repeatedly pipetted over each set of squares for 1 min. The antibody-containing supernatants were transferred to separate 15-ml centrifuge tubes each containing 3 ml of neutralization buffer (200 mM Tris, pH 7.8). Tubes were vortexed, sodium azide was added to 0.01% (w/v), and affinity-purified antibodies were stored at 4°C. Anti-SICysEP antibody cross-reactivity with other tomato anther proteinases was assessed by immunoprecipitation (see Supplemental Materials and Methods S1).

Recombinant SlCysEP Proteolytic Activity

To determine the pH optimum of rSlCysEP, citrate-phosphate buffers in a range from pH 3.0 to 8.0 were prepared by combining the appropriate amounts of 0.1 M citric acid, pH 2.0, and 0.2 M NaHPO$_4$, pH 8.4, in 1.5-Ml centrifuge tubes (Ruzin, 1999). To each tube, 600 µl of appropriate pH buffer was added, along with 90 µl of 5% (w/v) azocasein solution, 150 µl of double-distilled water, and 10.7 µg of recombinant protein (Charney and Tomarelli, 1947). The samples were incubated in the dark at 37°C overnight. The following day, 150 µl of double-distilled water and 150 µl of 50% (w/v) TCA were added. Tubes were vortexed and centrifuged at 13,000g for 10 min at 4°C to pellet undigested azocasein. Control samples were prepared similarly, with TCA being added prior to the addition of the enzyme. Aliquots (174 µl) from each sample were pipetted onto a microwell plate, and 26 µl of 10 M NaOH was added to each well. The plates were read at 490 nm using the THERMOMAX microplate reader (Molecular Devices).

Citrate-phosphate buffers were prepared (Ruzin, 1999) at pH 4.8 and 5.6 in order to accommodate the respective pH optima of SICysEP and human cathepsin B (Azarany et al., 1985). To 450 µl of the appropriate citrate-phosphate buffer was added 100 µl of 2.5% (w/v) azocasein, 0.136% (w/v) sodium azide, and 130 µl of 0.025 µg ml$^{-1}$ recombinant proteinase (or double-distilled water for control samples). Pairs of samples from each series were treated with 20 µl of 26.8 mM DTT, 20 µl of 10 mM E64 in dimethyl sulfoxide (Roche), or 20 µl of double-distilled water. Control reactions were treated with 100 µl of 50% (w/v) TCA immediately, and the volume of the experimental tubes was adjusted accordingly with 100 µl of double-distilled water. Reactions were carried out in the dark at 37°C overnight. Reactions were stopped in the experimental tubes with 100 µl of 50% (w/v) TCA, and volume was adjusted accordingly in the control tubes by the addition of 100 µl of double-distilled water. Tubes were vortexed and centrifuged at 13,000g for 15 min at 4°C, then triplicates of 174 µl were pipetted into wells on a microwell plate and 26 µl of 12% NaOH was added. Plates were read at 490 nm as indicated above.

SICysEP time-course self-hydrolysis experiments were conducted in citrate-phosphate buffer at pH 7.0 and 4.8. To 500 µl of the appropriate citrate-phosphate buffer, recombinant protein (11.65 µl) was added and the tubes were vortexed and left at room temperature for 3-min intervals. Hydrolysis
reactions were stopped by adding 128 μL of 5% protein sample buffer (0.31 mM Tris, pH 6.75, 10% [v/v] SDS, 50% [v/v] glycerol, 25% [v/v] β-mercaptoethanol, and 0.005% bromphenol blue) and immediately vortexing and heating to 90°C for 6 min.

**Western-Blot Analysis**

Proteins were extracted from liquid nitrogen-ground tissue using extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, and 1% [v/v] Nonidet P-40) supplemented with Complete, Mini, EDTA-free Protease Inhibitor Cocktail (Roche). SDS-PAGE was performed according to Laemmli (1970) using 12% acrylamide gels. Protein was transferred to a nitrocellulose membrane (Costar Scientific) as described by Towbin et al. (1979). Prior to immunoblottting, membranes were stained with Ponceau S (0.1% [w/v] Ponceau S and 5% [v/v] acetic acid) to ensure equal loading of protein. Anti-SiCysEP antibodies were used at approximately 14 μg mL⁻¹ for immunodetection (Burnette, 1981) followed by affinity-purified goat anti-rabbit IgG horseradish peroxidase-conjugated antibodies (Bio-Rad Laboratories) diluted to 1:10,000. ECL Plus western-blotting detection reagents (Amersham Biosciences) were used as substrate for the 2nd antibodies, following the manufacturer's instructions.

**Immunolocalization of SiCysEP**

De-paraffinized sections were submerged in TTBS containing 5% (w/v) skim milk powder for 1.5 h with gentle agitation, incubated in 1:200 (approximately 7 μg mL⁻¹) affinity-purified anti-SiCysEP antibodies in TTBS with 5% (w/v) skim milk for 1.5 h, and washed once with TTBS with 5% (w/v) skim milk powder for 15 min and twice more with TTBS for 15 min per wash. Slides were then incubated in 14,000 goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) in TTBS containing 5% (w/v) skim milk powder for 1 h with gentle agitation, then washed three times with TTBS for 15 min, and then incubated with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma) according to the manufacturer’s instructions. Control slides were treated identically using preimmune rabbit serum. Slides were observed and photographed using bright-field optics as described above.

Immunogold localization of SiCysEP was conducted on ultrathin sections of aldehyde-fixed, osmicated, Spurr’s resin-embedded samples mounted on nickel grids (see above) following the protocol of Craig and Goodchild (1984). Briefly, on drops arranged on Parafilm and at room temperature, sections were treated for 1 h with saturated aqueous sodium metaperiodate, washed through three changes of double-distilled water, treated for 5 min with 0.1 N HCl, and washed again. Blocking was performed on drops of 3X PBS-T-G-BSA (10 mM NaPO₄, pH 7.2, 0.45 mM NaCl, 0.02% NaN₃, supplemented with 0.05% Tween 20, 0.05% Glycerol, and 0.5% bovine serum albumin) for 10 min. Grids were then transferred to drops of affinity-purified rabbit-anti SiCysEP IgG, diluted 1:50 (approximately 28 μg mL⁻¹ in 3X PBS-T-G-BSA for 30 min, washed through six drops of 3X PBS-T-G-BSA for a total of 10 min, then transferred to 10-nm colloidal gold-conjugated goat-anti rabbit IgG (Sigma) diluted 1:50 in 3X PBS-T-G-BSA for 30 min. Grids were then washed through three changes of 3X PBS-T-G-BSA and three changes of 3X PBS, 3 min for each change, then incubated on 0.5% glutaraldehyde in 3X PBS for 3 min. Grids were then rinsed in double-distilled water, stained only with saturated aqueous uranyl acetate, and imaged on the CM-10 transmission electron microscope as above. Control grids were treated identically using either preimmune rabbit antisera or rabbit-anti SiCysEP IgG at approximately 28 μg mL⁻¹ preabsorbed with 200 μg mL⁻¹ rSiCysEP protein.

**RT-PCR**

Total RNA was extracted from dissected anthers using Tri Reagent (Sigma) according to the manufacturer. Each sample was treated with TURBO DNA-afro DNase (Ambion) according to the manufacturer and used immediately for cDNA synthesis. First-strand cDNA synthesis was performed using the lmProm-II Reverse Transcription System (Promega) with an oligo(dT) primer. RT-PCR was performed using actin as a control. Specific oligonucleotides were designed to amplify various candidate tomato Cys proteinase genes and/or unigenes that share some amino acid identity with SiCysEP as determined by a BLAST search of the SOL Genomics Network (Mueller et al., 2005). These as well as the annealing temperatures, number of cycles used, and product sizes are listed in Table II.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Immunoprecipitation of stage 18 to 20 anther proteins with anti-SiCysEP antibodies.

**Supplemental Materials and Methods S1.** Immunoprecipitation of anther proteins with anti-SiCysEP antibodies.

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Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EU122386 (SiCysEP gene, complete cds).
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