

# Chapter 36

## Regulation of Vacuole-Mediated Programmed Cell Death During Innate Immunity and Reproductive Development in Plants

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**Abstract** Programmed cell death (PCD), organized destruction of cells, is essential in development, maintenance of cellular homeostasis, and innate immunity in multicellular organisms. In most angiosperms, development of male and female organs involves spatial and temporal regulation of PCD. The tapetum, the innermost layer of the anther, provides both nutrient and lipid components to developing microspores, and has been proposed to be degraded by PCD during the later stages of pollen maturation. Plants lack homologues of most apoptosis-related genes in animals and have evolved specific mechanisms for PCD. PCD is also a crucial event in plant immune responses against microbial infection that prevents the spread of pathogens. Recent live cell imaging techniques have revealed the dynamic features and significant roles of the vacuole during defense responses and PCD. Disintegration or collapse of the vacuolar membrane has been suggested to trigger the final step of

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PCD in several cell types. We here overview spatiotemporal dynamic changes of the vacuole triggered by signals from pathogens and comparatively discuss PCD during innate immunity and reproductive development in plants.

**Keywords** Innate immunity • Programmed cell death • Reproductive development • Tapetum • Vacuole

## 36.1 Programmed Cell Death in Plants

Programmed cell death (PCD) is a genetically regulated process of cellular suicide that is well known to play a fundamental role in a wide variety of developmental and physiological functions in multicellular organisms (Bozhkov and Lam 2011; Fuchs and Steller 2011; Teng et al. 2011). In plants, PCD plays a critical role in the control of developmental processes such as xylogenesis, embryogenesis, pollen maturation, seed development, seed germination, and leaf senescence, as well as various stress responses including innate immunity against pathogen attack (Pennell and Lamb 1997). Reproductive development in angiosperms involves PCD in a variety of cells in reproductive organs, such as reproductive primordium abortion, style transmitting tissue, nonfunctional megaspores, synergids, antipodals, endosperm, anther tapetum, and abortive pollen in male sterility (Greenberg 1996; Pennell and Lamb 1997; Wei et al. 2002). Plants lack homologues of most apoptosis-related genes in animals and have evolved specific mechanisms for PCD. We here comparatively discuss mechanisms for PCD in innate immunity and reproductive development with special reference to the roles of the vacuole.

## 36.2 PCD in Plant Immunity Against Pathogen Attack

Plants lack immune systems based on antibodies or phagocytosis. Instead, they have evolved multiple layers of active defense responses including the deliberate production of reactive oxygen species (ROS), pathogenesis-related (PR) proteins, and antimicrobial secondary metabolites called phytoalexins. The dynamic reorganization of plant cells is triggered at the site of infection and often accompanies localized PCD, known as the hypersensitive response (HR), which is effective in preventing the spread of pathogens (Heath 2000; Mur et al. 2008).

The plant cells show different characteristics from animal cells, most notably the presence of the cell wall, the plastids/chloroplasts, and the vacuole, all of which play crucial roles in the regulation of plant immunity and PCD. This difference suggests that execution of PCD takes place with different morphological features from typical animal PCD such as apoptosis. Cellular morphological changes in animal cells undergoing apoptosis, including cell shrinkage and nuclear fragmentation, are followed by the fragmentation of cells and formation of apoptotic bodies, which are

then phagocytosed. Although the similarities and differences between PCD in plants and animals have been extensively discussed (Cacas 2010; Reape and McCabe 2010), the mechanisms for execution and regulation of plant PCD including HR still largely unclear.

Cell biological aspects of immune responses accompanying PCD have been studied in various experimental systems using a combination of plants and microbes. A major experimental approach is immunostaining of plant tissues infected with microbes (Kobayashi et al. 1994; Skalamera and Heath 1996; Kobayashi et al. 1997). However, the deformation of endomembrane systems by chemical fixation with this technique should be noted. Recently, green fluorescent protein (GFP)-based *in vivo* imaging has allowed time-sequential observations of the endomembrane systems in plant–microbe interactions (Takemoto et al. 2003; Koh et al. 2005).

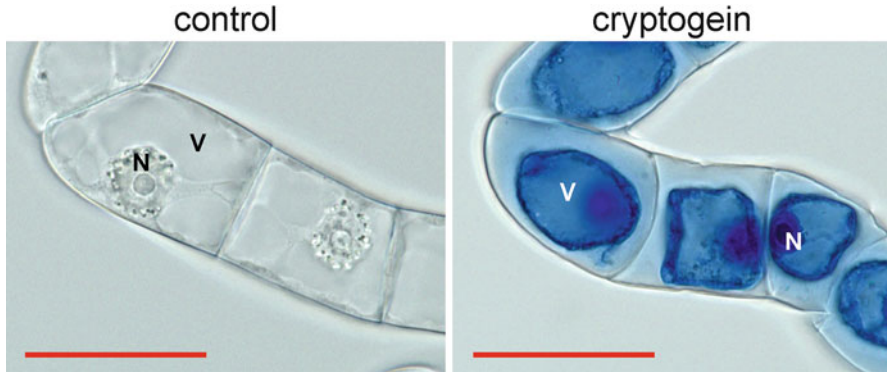
The execution of cell death in a regulated fashion should accompany dynamic reorganization of the cellular architecture. In plants, cellular morphological changes are often governed by cytoskeletons such as actin microfilaments and microtubules, as well as the vacuole, an organelle occupying most of the cell volume. In recent years, the rapid development of live cell imaging techniques has provided novel aspects on the dynamics of intracellular structures. We here focus on the dynamics of the vacuole and discuss its functional significance in innate immunity and PCD.

### ***36.2.1 Tobacco BY-2 Cells as an Excellent Model System to Study Immune Responses Accompanying Localized Plant PCD***

Plant cell cultures are useful simple model systems for the monitoring of cellular events. Defense responses including intracellular reorganization and gene expression upon fungal infection are basically similar between cultured cells and *in planta* (Gross et al. 1993). Treatment of cultured cells with purified signal molecules from pathogens can mimic most defense responses, including the expression of defense-related genes, as effectively as microbial infections (Lecourieux et al. 2002; Kadota et al. 2004a; Kadota and Kuchitsu 2006).

Treatment of tobacco (*Nicotiana tabacum*) BY-2 cells with cryptogein, a protein from the oomycete *Phytophthora cryptogea*, induces various immune responses such as membrane potential changes, ion fluxes, biphasic ROS production, and MAP kinase activation in a cell cycle-dependent manner (Kadota et al. 2005; Kadota et al. 2006), followed by cell-cycle arrest and PCD (Kadota et al. 2004b; Ohno et al. 2011). The slow prolonged phase, not the rapid transient phase, of ROS production shows strong correlation with downstream events including expression of defense-related genes and PCD (Kadota et al. 2005; Kadota et al. 2006).

NADPH oxidase-mediated deliberate ROS production has been suggested to play a crucial role in triggering and regulating PCD (Torres et al. 2005; Suzuki et al. 2011). Respiratory burst oxidase homologue (Rboh) proteins show ROS-producing activity synergistically activated by binding of Ca<sup>2+</sup> to their EF-hand motifs and



**Fig. 36.1** Cryptogein-induced programmed cell death in suspension-cultured tobacco BY-2 cells. Three-day-old cultured BY-2 cells were treated with 1  $\mu$ M cryptogein or distilled water (control) for 24 h. Dead cells were stained with Evans blue 24 h after cryptogein treatment (Higaki et al. 2007). V vacuole, N nucleus. Bar 50  $\mu$ m.

protein phosphorylation (Ogasawara et al. 2008; Takeda et al. 2008; Kimura et al. 2012). Potato StRBOHB has been shown to be activated by phosphorylation by calcium-dependent protein kinases StCDPK4 and StCDPK5 (Kobayashi et al. 2007). *Arabidopsis* AtRbohF has recently been shown to bind CIPK26, a protein kinase activated by binding of calcineurin B-like  $\text{Ca}^{2+}$  sensor proteins CBLs, *in planta* (Kimura et al. 2013), and be activated in the presence of CBL1/CBL9 and CIPK26 (Drerup et al. 2013).

Transgenic tobacco BY-2 cell lines expressing the GFP-markers for vacuolar membranes (VM; Kutsuna and Hasezawa 2002) have been effective in monitoring dynamic changes in vacuoles during cryptogein-induced defense responses *in vivo* (Higaki et al. 2007).

### 36.2.2 Regulation of Vacuole-Mediated PCD in Innate Immunity

The plant vacuole is an organelle that occupies most of the cell volume and contains many hydrolytic enzymes for digestive processes, similar to lysosomes in animal cells. The vacuole performs various functions essential for plant growth, development, and adaptation to both abiotic and biotic stresses (Marty 1999).

Disintegration or collapse of the VM has been suggested to trigger the final step of PCD in several cell types (Jones 2001; Hara-Nishimura and Hatsugai 2011; Higaki et al. 2011). At the final stage of cryptogein-induced PCD in tobacco BY-2 cells, disintegration of the VM is followed by the irreversible loss of plasma membrane integrity and cell shrinkage (Higaki et al. 2007; Fig. 36.1). Tobacco mosaic virus-induced hypersensitive cell death in tobacco leaves involves vacuolar rupture, in which a vacuolar-localized protease called vacuolar processing enzyme

(VPE) exhibiting caspase-1-like activity is involved (Hatsugai et al. 2004, 2006). Disintegration of the VM or vacuolar collapse needs to be strictly regulated to accomplish PCD with appropriate timing. Another mechanism involving fusion of the VM with the plasma membrane, resulting in the discharge of vacuolar antibacterial proteins to the outside of the cells where bacteria proliferate, has also been proposed for biotrophic bacteria-induced hypersensitive cell death (Hatsugai et al. 2009).

Cryptogein-induced PCD in BY-2 cells accompanies dynamic reorganization of the vacuole before the execution of cell death (Higaki et al. 2008). Cryptogein induces decrease in the transvacuolar strands (TVS), tubular regions of the cytoplasm connecting the nucleus to the cell periphery, and formation of a spherical intravacuolar structure called the 'bulb' (Higaki et al. 2007) that has been observed in a wide range of plant tissues (Saito et al. 2002). The bulb-like structure could be derived from the excess VM comprising the TVS. At the later stage of the PCD, the bulb-like structure disappears and the structure of the large central vacuole becomes simpler. Architecture of the vacuole is governed by actin filaments. Indeed, an actin polymerization inhibitor facilitates both the disappearance of the bulb-like VM structures and induction of PCD (Higaki et al. 2007). These findings suggest that the elicitor-induced reorganization of actin microfilaments followed by modification of the vacuolar structure to induce VM disintegration plays a key role in induction of the PCD.

### 36.3 Vacuole-Mediated PCD in Plant Vegetative Development

Vacuolar collapse plays a critical role at the final stage of execution of PCD during differentiation of the tracheary element in *Zinnia elegans* (Kuriyama 1999; Obara et al. 2001). PCD in the inner integument cell layers of developing *Arabidopsis* seeds also involves vacuolar rupture, in which a vacuolar-localized protease called VPE exhibiting caspase-1-like activity is involved (Nakaune et al. 2005).

The structural simplification of vacuoles has been commonly observed in various PCD processes including tracheary element differentiation (Obara et al. 2001), gibberellin-mediated PCD in central aleurone cells (Guo and Ho 2008), embryogenesis in a gymnosperm (Smertenko et al. 2003), and leaf formation in a lace plant (Gunawardena 2008). Decreases in the number of TVS and VM reorganization were confirmed in the process of developmental PCD in the lace plant *Aponogeton madagascariensis* (Wright et al. 2009), which suggests a general role of VM reorganization in vacuolar rupture-mediated PCD in plants.

## 36.4 PCD in Plant Reproductive Development

During plant reproduction, proper induction of PCD is prerequisite for reproductive success. Failure in PCD often results in sterility. In most angiosperms, development of male and female organs involves spatial and temporal regulation of PCD. For example, tapetal cells, the innermost layer of the anther, supply fundamental nutrients necessary for normal pollen development. Shortly after the microspore release from the tetrad and before mitosis, tapetal cells begin to degenerate by PCD. In female gamete development within the ovules, a typically diploid megaspore mother cell undergoes meiosis, giving four megaspores, and three of these megaspores undergo PCD.

Besides development of reproductive organs, PCD also has an important role in fertilization. For example, in self-incompatibility response in *Papaver*, an incompatible pollen tube is stopped by interactions with the pistil *S*-determinant by PCD (Bosch and Franklin-Tong 2008). In this way, PCD plays important roles in multiple steps of normal reproduction in plants from development of gametes to fertilization.

### 36.4.1 PCD as a Key System for Tapetum Degradation During Pollen Development

During pollen development, the tapetum is degraded to supply metabolites, nutrients, and sporopollenin precursors to developing microspores. Defects in the degradation of the tapetum cause the abnormal formation of the pollen coat and pollen grains and result in severe male sterility (Ku et al. 2003; Li et al. 2006; Zhang et al. 2008; Ariizumi and Toriyama 2011). The degradation of the tapetum is highly regulated and exhibits the hallmark features of PCD such as cell shrinkage, condensation of chromatin, swelling of the endoplasmic reticulum (ER), and persistence of the mitochondria (Rogers et al. 2005) as well as nuclear fragmentation (Wang et al. 1999; Vardar and Unal 2012).

The correct timing of tapetal PCD is important for normal pollen development. The initiation signal for tapetal PCD has been proposed to commence as early as the tetrad stage (Kawanabe et al. 2006). The *ms1* mutant showed delayed tapetal breakdown and a switch from PCD degradation to necrotic-based breakdown (Vizcay-Barrena and Wilson 2006). A rice mutant *tapetal degeneration retardation (Ostdr)* shows significantly delayed tapetal breakdown and PCD, resulting in the failure of pollen wall deposition and subsequent microspore degeneration (Li et al. 2006; Zhang et al. 2008). Degradation of the tapetum was also regulated by a plant hormone gibberellin (Cheng et al. 2004; Aya et al. 2009).

### 36.4.2 Regulation of Vacuole-Mediated PCD in Tapetum Degradation

Abnormal vacuolization in the tapetum during the tetrad stage causes abnormal tapetal PCD and results in male sterility (Wan et al. 2010). The vacuoles in the tapetum may supply tetrad wall-degrading enzymes before their secretion into the anther locules (Wu and Yang 2005). These results suggest important roles of tapetal vacuoles during anther development and PCD.

In the tapetum of *Lathyrus undulatus* L. at the vacuolated microspore stage, rupture of the VM and vacuolar collapse is induced, followed by possible release of hydrolytic enzymes and the degradation of cell components (Vardar and Unal 2012). Interestingly, the increment of VPE in the *Arabidopsis* anther has also been reported (Hatsugai et al. 2006), suggesting a possible role of VPE-mediated proteolysis during tapetal PCD. However, the molecular mechanisms for the dynamic reorganization of the vacuoles during tapetal PCD have not yet been elucidated.

## 36.5 Conclusions and Future Perspectives

Vacuolar collapse seems to be important in the triggering of plant PCD during innate immunity and development in both reproductive and vegetative tissues. Recent findings have shed light on the machinery for various plant PCD. Further live cell imaging may reveal novel dynamic aspects of the vacuole. In light of these circumstances, automated microscopy and image analysis techniques for the quantitative evaluation of cellular dynamics are of increasing importance.

Besides apoptosis, autophagy is also a participant in cell degeneration and PCD in animals (Shimizu et al. 2004; Tsujimoto and Shimizu 2005). Autophagic cell death is characterized by the occurrence of double-membrane autophagosomes within the dying cells that remove the cell remnants (Gump and Thorburn 2011). In *Drosophila melanogaster*, the destruction of the salivary glands and digestive tract was shown to be mediated by a marked upregulation of autophagy before and during cell death during metamorphosis (Melendez and Neufeld 2008).

Autophagy has recently been shown to be involved in various processes such as recycling of nutrients and senescence in plants (Yoshimoto 2012). Although autophagy-deficient mutants of *Arabidopsis* have been reported to show a normal life cycle, autophagy may also play roles in PCDs in development or stress responses in plants (van Doorn and Woltering 2010). A simple easy method to quantitatively analyze autophagic fluxes has recently been developed in plants (Hanamata et al. 2013). Such technical advances in combination with genetic analyses may reveal novel aspects of autophagy in PCD in plants.

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