Chapter 4
Programmed Cell Death in Plant Immunity: Cellular Reorganization, Signaling, and Cell Cycle Dependence in Cultured Cells as a Model System

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4.1 Programmed Cell Death and Hypersensitive Responses in Plant Immunity Against Pathogen Attack

Programmed cell death (PCD) is a genetically regulated process of cellular suicide and is well known to play a fundamental role in a wide variety of developmental and physiological functions in multicellular organisms [1–3]. 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 [4]. 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,

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endosperm, anther tapetum, and abortive pollen in male sterility [4–7]. Evidence to date suggests plants lack homologs of most animal apoptosis-related genes and have evolved several specific mechanisms for PCD.

Plants also lack immune systems based on antibodies or phagocytosis. Instead, they have evolved multiple active defense responses including reorganization of the cell wall and production of pathogenesis-related (PR) proteins and antimicrobial secondary metabolites called phytoalexins. Initial cellular responses also include the production of reactive oxygen species (ROS) mediated by enzymes such as NADPH oxidases (Nox), as well as plasma membrane ion fluxes and increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$; [8]). This dynamic cellular reorganization 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 [9–11].

Notable differences of plant and animal cells are the presence of the cell wall, the plastids/chloroplasts, and the vacuole, which all play crucial roles in the regulation of plant immunity and PCD. Execution of PCD takes place with different morphological features from typical animal cell death programs such as apoptosis. Cellular morphological changes in animal cells undergoing apoptosis include cell shrinkage and nuclear fragmentation and 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 discussed extensively [12–14], the mechanisms for execution and regulation of plant PCD, including HR, still remain to be elucidated.

The execution of cell death in a regulated fashion accompanies dynamic reorganization of the cellular architecture. 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 had been the immunostaining of plant tissues infected with microbes [15–17]. However, it should be noted that the deformation of endomembrane systems, by chemical fixation, occurs with this technique. In vivo imaging based on various fluorescent probes such as GFP has allowed time-sequential observations of the endomembrane systems in plant-microbe interactions [18, 19]. Cellular morphological changes are often governed by cytoskeletons such as actin microfilaments (MFs) and microtubules (MTs) as well as the vacuole.

Reduction of growth accompanying cell cycle arrest is induced by various kinds of abiotic stresses such as oxidative damage mediated by menadione [20] or $KMnO_4$, hypoosmotic stress [21], flooding stress [22], and DNA damage [23, 24]. Such growth reduction is also seen during immune responses against pathogens. Treatment with pathogen/microbe/damage-associated molecular patterns (PAMP/MAMP/DAMPs) induces both defense responses and growth inhibition [25]. Pathogen-derived molecules called elicitors also induce downregulation of some cell cycle-related genes along with the induction of defense-related genes [26–28], suggesting that the downregulation of cell cycle-related genes may be involved in growth inhibition. No homologs of p53, involved in cell cycle regulation in animals, have been found in plants [29]. Although the molecular mechanisms for

immunity-related growth inhibition are largely understood, the molecular mechanisms of stress-induced cell cycle arrest are mostly unknown.

Plant cell cultures are useful simple model systems to monitor cellular events. Defense responses including intracellular reorganization and gene expression upon fungal infection are basically similar between cultured cells and *in planta* [30]. Treatment of cultured cells with purified signal molecules from pathogens (PAMP/MAMP/DAMPs) can mimic most defense responses, including the expression of defense-related genes, as effectively as microbial infections [31–33].

Here, we give an overview on the spatiotemporal dynamic changes of PCD triggered by signals from pathogens and compare the interrelationships among the reorganization of the cellular architecture, cell cycle, and signaling including ROS production, MAPK activation, and Ca^{2+} rise during innate immunity in cultured cells.

4.2 Effects of the Pathogenic Signal-Induced Reorganization of Cellular Architecture

Execution of cell death in a regulated fashion is accompanied by dynamic reorganization of cellular architecture. In plants, cellular morphological changes are governed by the cytoskeleton, including MFs and MTs [34] and the vacuole [35]. Rapid development of live cell imaging techniques has recently revealed novel aspects on the dynamics of these intracellular structures. Transgenic tobacco BY-2 cell lines expressing the GFP-markers for vacuolar membranes (VM; [36]) and cytoskeletons [37, 38] have been effective in monitoring dynamic changes in the cellular architecture during cryptogein-induced defense responses in vivo [39, 40].

4.2.1 Vacuole-Mediated Programmed Cell Death in Innate Immunity

The plant vacuole 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 [41]. In these processes, vacuoles show dynamic morphological changes including VM rearrangement [42].

Disintegration or collapse of the VM has been suggested to trigger the final step of PCD in several cell types [35, 43, 44]. 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 ([39]; Fig. 4.1). Tobacco mosaic virus-induced hypersensitive cell death in tobacco leaves involves vacuolar rupture, in which a vacuole-localized protease called vacuolar processing enzyme

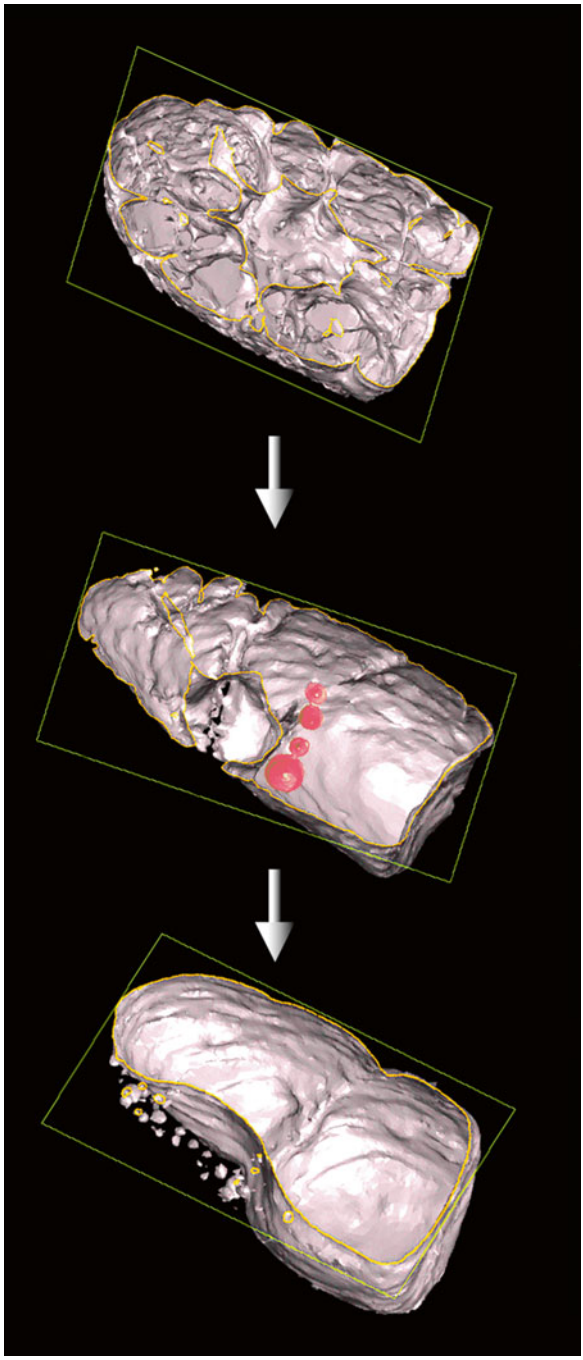

Fig. 4.1 Rearrangement of the vacuolar membrane structures during cryptogein-induced cell death in S-phase-synchronized tobacco BY-2 cells. Representative three-dimensional surface models reconstructed from serial sections of confocal microscopic images are shown. Experimental details are as described in Higaki et al. [39]. Many transvacuolar strands radially oriented from cell nuclei gradually decreased, and bulb-like structures (*pink*) appeared after the cryptogein treatment (from *top* to *middle*). Thereafter, the internal vacuolar membrane structures including bulb-like structures disappear before the disintegration of vacuolar membranes and execution of cell death (from *middle* to *bottom*)

(VPE) exhibiting caspase-1-like activity is involved [45, 46]. Disintegration of the VM and/or vacuolar collapse needs to be strictly regulated to accomplish PCD at 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 [47].

Cryptogein-induced PCD in BY-2 cells accompanies dynamic reorganization of the vacuole prior to the execution of cell death [40]. Cryptogein induces a decrease in the transvacuolar strands (TVS), tubular regions of the cytoplasm connecting the nucleus to the cell periphery, and formation of spherical intravacuolar structures called the "bulb" [39] that has been observed in a wide range of plant tissues [48–50]. 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 gets simpler. Simplification of vacuolar morphology is commonly observed in various PCD processes [51–55], suggesting a general role of VM rearrangement in vacuole-mediated PCD in plants.

The molecular mechanism of the vacuolar shape simplification is still ambiguous. A KEG (KEEP ON GOING) protein that contains functional RING-type E3 ligase domain [56] is involved in vacuolar expansion and cell growth [57]. The KEG proteins are localized at the trans-Golgi network and early endosomes, suggesting its critical roles in membrane trafficking to the vacuole [57, 58]. Interestingly, infection by a powdery mildew fungus *Golovinomyces cichoracearum* causes specific degradation of KEG proteins, suggesting KEG is targeted by fungus effectors to perturb the membrane traffic in the host cells [57]. This plausible story is supported by the putative roles of KEG in recruitment of EDR1 (ENHANCED DISEASE RESISTANCE1) kinase, which is involved in powdery mildew resistance [59], to the trans-Golgi network and early endosomes [58]. Future analysis on its target proteins would clarify the relationship between membrane trafficking and vacuolar morphological changes during defense responses.

4.2.2 Reorganization of Actin Microfilaments and Vacuole-Mediated PCD

MFs are involved in vacuolar morphogenesis including TVS formation in higher plants [60]. Live cell imaging has revealed that cryptogein-induced vacuolar reorganization accompanies MF reorganization in tobacco BY-2 cells [39]. In addition, a MF inhibitor promoted both the simplification of vacuolar structure and the induction of PCD [39]. Based on a series of these observations in the model system, we proposed a hypothetical model for a MF-regulated vacuole-mediated PCD. During the PCD process, MFs running through TVS disappear, but MF bundles appear on large vacuoles. MF rearrangement causes the conversion of intravacuolar

morphology from TVS to bulb-like structures. The MF bundles on the large vacuoles are necessary to maintain the bulb-like structures by covering the large vacuole surface. Disruption of the MF bundles triggers the simplification of the vacuoles, reduction of bulb-like structures, and formation of small spherical vacuoles derived from the bulb-like structures. The simple-shaped vacuoles are easy to rupture by water absorption and cause cell death defined by PM disintegration. In this model, the MF bundles that appear in the process negatively regulate vacuole-mediated PCD [35]. In other words, MF bundling may act as a safety lock against unexpected vacuolar rupture and cell death to keep the bulb-like structures intact.

Vacuolar rupture is observed in virus-induced hypersensitive cell death as well as other types of PCD in plants [46]. Possible involvement of actin MFs in the regulation and execution of the vacuole-mediated PCD in plants is an emerging important research topic. Fusion of the vacuolar and the PMs during bacterial infection-induced hypersensitive PCD has recently been reported by transmission electron microscopy and live imaging of GFP–PIP2a, a PM protein, and mRFP–AtVAM3, a VM protein in *Arabidopsis thaliana* [47]. This heterologous membrane fusion should accomplish quick discharge of vacuolar contents including antibacterial proteins into apoplastic space. A defect in proteasome subunit PBA1 abolishes the membrane fusion and the PCD. An attractive model has been proposed that proteasome-driven degradation of the unidentified heterologous membrane fusion inhibitor(s) triggers the bacterial infection-induced PCD [47, 61].

Functional modification and critical roles of the vacuole during the execution of hypersensitive cell death induced by viruses [45] and eukaryotic pathogens [39] seem to be different from those by bacterial pathogens. In cryptogein-treated tobacco BY-2 cells, the heterologous membrane fusion does not occur at least until VM disintegration because a vacuole-accumulated fluorescent probe leaks into the cytosol, and conversely free GFP-tubulin moves from the cytosol into the vacuolar region [39]. These observations are supported by recent findings on the modulation of a vacuolar protease by cytoplasmic proteins. Activity of a vacuolar papain-like cysteine protease RD21 (RESPONSIVE-TO-DESICCATION-21), which promotes cell death triggered by the necrotrophic fungus *Botrytis cinerea* or *Sclerotinia sclerotiorum*, is suppressed by overexpression of the gene for AtSerpin1, which is a cytoplasm-localized proteinaceous inhibitor of RD21 [62, 63].

In contrast to animals for which viruses and bacteria are major pathogens, the majority of plant pathogens are eukaryotes. These cell biological events in cryptogein-induced PCD in cultured cells may reflect common features of hypersensitive cell death induced by eukaryotic pathogens. Though dynamic functional modification of the VM seems to be a common critical step during execution of various PCD in plants, there are also a range of membrane dynamics and cell biological processes to execute cell death. In the future comparative cell and molecular biological studies of various pathways leading to the execution of the final steps of PCD in plants will be particularly important.

4.3 Early Signaling Events to Trigger Hypersensitive Cell Death

Upon recognition of signal molecules from pathogens, plant cells activate a widespread signal transduction network involving second messengers as early signaling events, which triggers inducible immune responses [64]. Characteristic early signaling events include influxes of Ca^{2+} and H^+; effluxes of K^+ and Cl^-; membrane potential changes, typically transient membrane depolarization [32, 65–68]; ROS production by enzymes such as NADPH oxidase [69, 70]; and activation of a MAPK cascade. These initial responses are followed by biosynthesis of phytoalexins, vacuolar collapse, and PCD.

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 [71, 72], followed by cell cycle arrest and PCD [73, 74]. 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 [71, 72].

NADPH oxidase-mediated ROS production has been suggested to play a crucial role in triggering and regulating PCD [75, 76]. Respiratory burst oxidase homolog (Rboh) proteins show ROS-producing activity synergistically activated by binding of Ca^{2+} to their EF-hand motifs and protein phosphorylation [77–79]. Potato StRbohB has been shown to be activated by phosphorylation by calcium-dependent protein kinases StCDPK4 and StCDPK5 [80]. *Arabidopsis* AtRbohF binds CIPK26, a protein kinase activated by binding of calcineurin B-like (CBL) Ca^{2+} sensor proteins, *in planta* [81], and is activated in the presence of Ca^{2+}, CBL1/CBL9, and CIPK26 [82].

Rise in cytosolic Ca^{2+} concentration $[Ca^{2+}]_{cyt}$ is one of the earliest common responses triggered by various pathogenic signals [64]. Correlation between the temporal pattern of $[Ca^{2+}]_{cyt}$ or Ca^{2+} signature and induction of downstream events including PCD has been discussed. For example, chitin fragments, a typical PAMP/MAMP, triggers a rapid/transient $[Ca^{2+}]_{cyt}$ rise without induction of PCD, while xylanase protein from a fungus *Trichoderma viride* (TvX) triggers a prolonged $[Ca^{2+}]_{cyt}$ rise followed by the induction of PCD in cultured rice cells [83].

Anion effluxes are often accompanied with the induction of immune responses and PCD [32, 84]. $[Ca^{2+}]_{cyt}$ rise is inhibited by several anion channel blockers, indicating the importance of the plasma membrane anion channel for the induction or amplification of $[Ca^{2+}]_{cyt}$ response [32, 84]. *Arabidopsis* SLAC1, an S-type anion channel, functions in cryptogein-induced early signaling events to trigger PCD in tobacco BY-2 cells. Functional characterization of *Arabidopsis SLAC1-overexpressing* lines suggests that SLAC1 mediates cryptogein-induced Cl^- efflux through the plasma membrane to positively modulate the elicitor-triggered activation of extracellular alkalinization, NADPH oxidase-mediated ROS production, and a wide range of defense responses including PCD [85].

4.4 Hypersensitive Cell Death and Cell Cycle

4.4.1 Pathogenic Signal-Induced Cell Cycle Arrest

The cell cycle is a tightly controlled process divided into four (S, M, G1, G2) distinct phases. During the S and M phases, the cell replicates its genome and separates the duplicated genome between the two daughter cells, respectively. Both phases are followed by a gap phase, designated G1 and G2 [86]. In animal cells, the crosstalk between cell cycle progression and apoptosis or immune responses has been well studied [87]. Similar crosstalk has also been suggested to exist in plant cells. Two major PAMPs, flg22 and elf18, induce immune responses including defense-related gene expression along with growth inhibition in *Arabidopsis* plants [88]. A variety of *Arabidopsis* and rice lesion mimic mutants expressing defense-related genes constitutively show dwarf phenotypes [89, 90], suggesting the presence of positive crosstalk between cell cycle progression and immune responses in plants as well as animals.

Synchronous culture of tobacco BY-2 cells using the aphidicolin or the aphidicolin/propyzamide synchronization method [91] has been developed to quantitatively evaluate the interrelationship between cryptogein-induced HR, including PCD, and the cell cycle [73]. The elicitation by cryptogein during S phase causes cell cycle arrest at G2 phase accompanying suppression of cell cycle-related genes *NtCycA1;1* and *NtCycB1;3* in BY-2 cells. In contrast, cells treated with cryptogein in late G2, M, or G1 phases progressed to M/G1 phase and arrested at G1 phase with suppression of *NtCycD3;1* and *PCNA* expression [71, 73]. Cyclins bind and activate cyclin-dependent kinases (CDKs), thus playing a central role in cell cycle regulation. A1- and B1-type cyclins are thought to be involved in the progression from G2 to M phase, and D-type cyclins and PCNA are crucial for the progression from G1 to S phase [92, 93]. Cryptogein suppresses the activity of CDKA and CDKB1 during G2 to M phase along with both suppression of expression of various cell cycle-related genes and degradation of CDKB1 and cyclin [74].

In animal and yeast cells, stress-induced cell cycle arrest is controlled by specific genes, and mutations in these genes often result in increased sensitivity to damaging reagents such as oxygen radicals. Moreover, these genes are commonly mutated in various kinds of cancers, highlighting their importance in maintenance of the cell cycle [94]. One of these genes encoding p53 protein harbors mutations in more than half of all human cancers [95]. p53 takes part in G1 arrest in response to DNA damage. DNA damage-induced cell cycle arrest in the G1 and S phases may partly involve inhibition of the activity of G1 CDKs by the specific CDK inhibitor p21 [96]. Furthermore, the mechanism underlying DNA damage-induced G2 arrest was shown to involve a specific inhibitory phosphorylation of the mitotic kinase CDK1 in human cells [97, 98]. However, no homologs of p53 involved in cell cycle regulation in animals have been found in plants [29]. Cryptogein induced a reduction in the level of NtWEE1, which is thought to be a negative cell cycle regulator [74], suggesting that cryptogein affects multiple targets to inactivate CDKA to induce G2

arrest by mechanisms distinct from known checkpoint regulation. Additional analyses of this system may provide further molecular links between signaling events and cell cycle regulation during stress responses in plants.

4.4.2 Cell Cycle Dependence of Immune Responses and Hypersensitive Cell Death

Plant innate immunity consists of two layers of responses. The first is governed by extracellular transmembrane receptors or pattern recognition receptors. By recognizing conserved PAMP/MAMP/DAMPs, pattern recognition receptors trigger a relatively weak immune response known as pattern-triggered immunity (PTI) that inhibits colonization by invading organisms [99]. The second layer of plant innate immunity is based on highly polymorphic resistance (R) proteins that are activated upon the recognition of highly variable pathogen molecules called avirulence effectors. This effector-triggered immunity (ETI) consists of a rapid and robust response, often associated with HR including PCD to control the spread of biotrophic pathogens [11, 100–103].

Cryptogein induces not only HR and PCD but also growth inhibition and cell cycle arrest in G1 or G2 phase [73]. The pattern of cryptogein-induced HR changes depending on the cell cycle stage. BY-2 cells in the S phase arrest the cell cycle at G2 phase and induce the expression of defense-related genes and PCD immediately [71]. In contrast, BY-2 cells in G2 or M phase arrest the cell cycle at G1 phase and induce these responses at the same time as cells treated with the elicitor in G1 phase, suggesting that HR and PCD, and cell cycle arrest, are induced only at specific phases of the cell cycle in BY-2 cells. In fact, a transient treatment with cryptogein during S or G1 phase induced cell death and growth inhibition in BY-2 cells. By contrast, similar transient elicitor treatment during G2 or M phase did not induce these responses (Fig. 4.2). The suppression of cryptogein-induced PCD during G2 or M phase suggests differences in some of the components involved in defense signaling in each phase of the cell cycle.

4.4.3 Cell Cycle Dependence of Signaling Events to Trigger PCD

4.4.3.1 ROS Production

ROS can function as signaling molecules during cell division, through complex signaling pathways [104], and ROS homeostasis has been suggested to be critical for plant cell division [105]. Oscillations in intracellular ROS level keep pace with respective antioxidant oscillations. Subsequently, ROS acting as signaling molecules contribute to the establishment of Ca^{2+} gradients and participate in the control

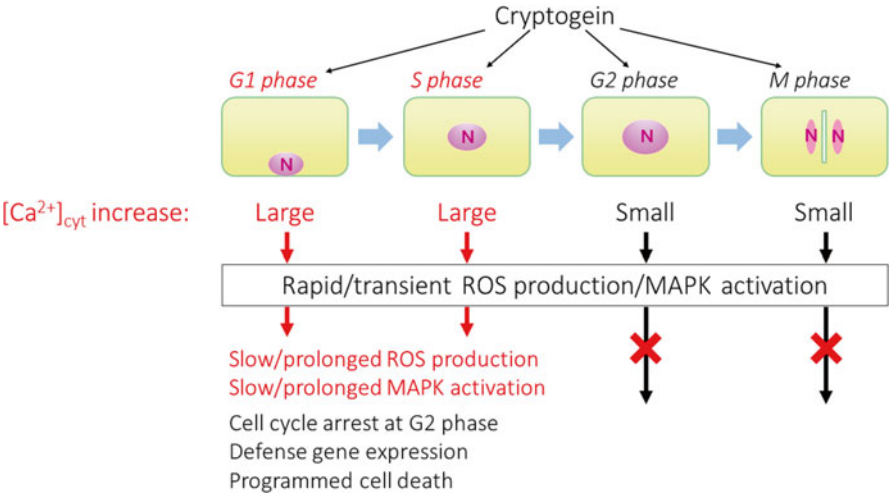

Fig. 4.2 Cell cycle-dependent regulation of cryptogein-induced defense signaling in tobacco BY-2 cells. The partial suppression of the cryptogein-induced $[Ca^{2+}]_{cyt}$ rise and the absence of sustained ROS production and MAP kinase activation at G2 and M phases are well correlated with the absence of induction of defense-related gene expression and PCD. *N* Nucleus. Treatment with cryptogein at G1 or S phase induces changes in $[Ca^{2+}]_{cyt}$, biphasic (rapid/transient and slow/prolonged) ROS production, and biphasic MAP kinase activation, followed by the cell cycle arrest at G1 or G2 phase and induction of defense gene expression and PCD. In contrast, treatment at G2 or M phase only induces smaller $[Ca^{2+}]_{cyt}$ changes, followed by only rapid/transient ROS production and MAP kinase activation (Kadota et al. [71]). The nucleus localizes at the cell periphery at G1 phase, but moves to the center at S/G2 phases (Higaki et al. [39])

of regulatory proteins such as CDKs and possibly aurora kinases. Rbohs have been identified as ROS-producing enzymes in plants, which have recently been shown to play key roles in numerous physiological processes such as tip growth of root hairs [78] and pollen tubes [106], hormonal responses, and abiotic and biotic responses [107]. A positive feedback mechanism involving Ca^{2+}-activated Rboh proteins and ROS-activated Ca^{2+}-permeable channels to regulate tip growth of root hairs has been proposed at the plasma membrane [78]. Rboh-mediated ROS production is implicated in the regulation of cell cycle progression, microtubule organization, nuclear envelope dynamics, and cell plate formation. In animal cells, ROS are involved in cell proliferation, regulating transition through specific cell cycle checkpoints [108].

Nox-/Rboh-mediated ROS production has been suggested to be involved in the regulation of PCD as a HR [109–111]. The rapid and transient phase (phase 1) of Rboh-mediated ROS production, triggered by cryptogein, occurred during any phase of the cell cycle, whereas the slow and prolonged phase (phase 2) was induced only by elicitation during S or G1 phase (Fig. 4.2). However, the relationship between the elicitor-induced oxidative burst and cell cycle regulation remain unclear, and these studies shed light on the novel aspect of the roles of Rboh-mediated ROS production and the immune signaling network in plants.

4.4.3.2 MAPK Activation

Cryptogein also induces biphasic activation of MAPK homologs, salicylic acid-induced protein kinase, and wounding-induced protein kinase [112]. The rapid and transient activation of both MAPKs occurs after elicitation during any phase of the cell cycle, whereas prolonged activation of MAPKs occurs only after elicitation during G1 or S phase [71]. The rapid/transient phase of cryptogein-induced ROS production and MAPK activation is induced at any phase of the cell cycle, suggesting that the elicitor is recognized throughout the cell cycle. In contrast, the slow/prolonged phase of ROS production (phase 2) and MAPK activation shows a strong correlation with the induction of immune responses including PCD (Fig. 4.2).

Suppression of HR during the G2 or M phase is correlated with the absence of prolonged production of ROS and prolonged activation of MAPKs. Other components participating in the induction of HR may be inactivated only during the G2 or M phase [71]. The elicitor-induced expression of *Rboh* genes is suggested to contribute to prolonged ROS production called the oxidative burst [71, 113]. The MEK^{DD} mutant, in which MAPKs are constitutively active, showed enhanced expression of *NbRbohB* and PCD in *Nicotiana benthamiana* [114], suggesting that the MAPK cascade positively regulates *Rboh* expression and ROS production.

Though PTI and ETI share downstream signaling machinery, activated immune responses in ETI are more prolonged and robust than those in PTI, and hypersensitive cell death are only induced by ETI signals. *Arabidopsis* MAP kinases 3 and 6, key regulators of immune responses, are activated rapidly and transiently during PTI, but activated for an extended period during ETI [100, 115]. Prolonged activation of oxidative burst and PCD are correlated with, and presumably require, prolonged activation of MAPKs [71, 116], suggesting that the cell cycle-dependent regulation of MAPK activation may be crucial for the induction of PCD. Suppression of the oxidative burst and PCD in cells treated with cryptogein during G2 or M phase may be attributed to the absence of prolonged activation of MAPKs.

4.4.3.3 Cytosolic Ca^{2+} Rise

Cryptogein induces a biphasic $[Ca^{2+}]_{cyt}$ rise in tobacco BY-2 cells [32]. It is induced at all phases of the cell cycle, but is significantly weaker at G2 and M phases than S and G1 phases in which hypersensitive cell death is induced (Fig. 4.2), suggesting that some signaling components upstream of $[Ca^{2+}]_{cyt}$ rise are regulated in a cell cycle-dependent manner [71]. Although the cryptogein receptor has not yet been identified, the expression of the receptor may be regulated differentially during the cell cycle phases and thus contribute to cell cycle-dependent regulation of immune responses. Transcriptomic analyses of a synchronized culture of *Arabidopsis* revealed that transcripts of two putative disease resistance proteins accumulate during G1 and S phases [117]. A cryptogein receptor may also be expressed more at G1 and S phases than at G2 or M phases. Alternatively, the receptor may be partially inactivated at G2 or M phases. Molecular mechanisms for the cell cycle-dependent

regulation of these signaling events are important issues to be elucidated in future research.

The partial suppression of the cryptogein-induced $[Ca^{2+}]_{cyt}$ rise at G2 and M phases is correlated with the absence of the oxidative burst and the prolonged activation of MAPKs at these phases (Fig. 4.2). Several studies have indicated that Ca^{2+} channel inhibitors and Ca^{2+} chelators inhibit pathogenic signal-induced ROS production and MAPK activation, suggesting that the Ca^{2+} influx is essential for the induction of these responses [32, 118]. The rapid/transient phase of ROS production and MAPK activation was induced even at G2 and M phases, in which $[Ca^{2+}]_{cyt}$ rise were partially suppressed. These results suggest that a relatively small increase in $[Ca^{2+}]_{cyt}$ at G2 and M phases is sufficient to induce the rapid/transient phase of ROS production and MAPK activation, which occurs independently of the slow/prolonged phase. The slow/prolonged phase of ROS production and MAPK activation is only induced at S and G1 phases, where the cryptogein-induced $[Ca^{2+}]_{cyt}$ rise is prominent (Fig. 4.2).

4.5 Conclusions and Future Perspectives

We have described the cell biological events, including intracellular reorganization and cell cycle regulation, during PCD as an immune response. Recent advances in GFP-based fluorescent molecular probes and microscopy have synergistically promoted our understanding on the structural changes in cytoskeletons and vacuoles during immune responses. The intracellular reorganization of organelles including the vacuole is suggested to be governed by cytoskeletons. Future molecular cell physiological studies should reveal the missing link between quantitatively detected early molecular events (e.g., ROS production, MAPK activation, Ca^{2+} influx) and intracellular structural changes (e.g., rearrangement of the cytoskeletons, vacuolar rupture). In this situation, quantitative evaluation of cell biological events must be of growing importance. Acquirement of enormous microscopic image data and its statistical analysis with numeric image features (e.g., skewness of fluorescent intensity distribution in GFP-labeled cytoskeletal images [119]) to evaluate cytoskeletal or vacuolar behaviors will become a growing area of research in the near future.

Besides apoptosis, autophagy is also involved in animal PCD [120, 121] and has also been suggested to play roles in several types of plant PCD including hypersensitive cell death against pathogen infection [122–125]. Autophagic cell death is characterized by the occurrence of double-membrane autophagosomes within the dying cells that remove the cell remnants [126]. In many eukaryotes, autophagy is required for normal development, for example, for dauer development in nematodes and preimplantation in mice [127–129]. Interestingly, autophagy-defective rice mutants show complete male sterility and limited anther dehiscence under normal growth conditions, suggesting that autophagy is crucial in reproductive development in rice [130]. A relatively simple method to quantitatively analyze autophagic fluxes has recently been developed in plants [131]. Such technical advances in combination with genetic analyses may reveal novel aspects of autophagy in PCD in plants.

The strict cell cycle dependence of pathogenic signal-induced immune responses and their suppression suggest that immune responses may be suppressed in dividing cells *in planta*, such as meristems, young leaves, and seedlings (Fig. 4.3). In fact, young leaves are less sensitive to pathogenic signals than mature leaves [132, 133]. In contrast, almost all mesophyll cells of mature leaves are at G0 or G1 phase [134] and induce strong immune responses including HR against pathogens [135], which is consistent with the finding using suspension-cultured cells showing that immune responses and hypersensitive cell death are strongly induced at G1 phase [73]. Molecular characterization of the relationship between the cell cycle and the immune responses in intact plants is a new frontier of research.

Some studies using synchronized suspension cells revealed that different cell cycle phases are associated with slightly different gene expression patterns [136] and several *R* genes exhibit peak expression at S or M phase. This differential expression pattern could have physiological consequences. Cells at different cell cycle phases exhibit different responses to elicitors, and defense gene induction by elicitors is cell cycle dependent [71]. Perturbation of cell cycle regulation triggers plant immune responses via the activation of disease resistance genes [137], suggesting that cell cycle regulation could have an impact on the expression of genes, including *R* genes, in plant immunity. Cell cycle progression is tied to the dynamics of not only DNA but also chromatin [138], which thus could have a profound effect on gene expression. Further investigation should reveal the regulatory mechanisms for gene expression during cell cycle phases and the increase our understanding of the interactions between plants and pathogens.

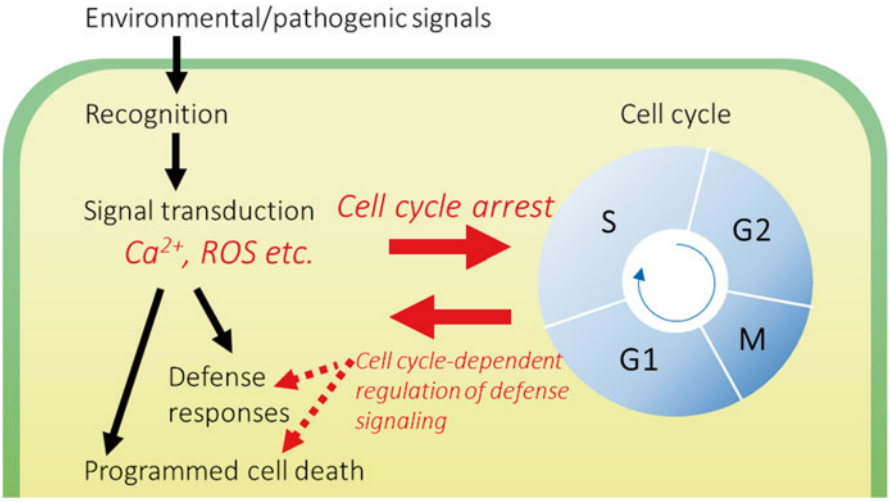

Fig. 4.3 The crosstalk between stress/defense signaling and cell cycle regulation. The strict cell cycle dependence of pathogenic signal-induced immune responses and their suppression in suspension-cultured cells indicate possible relationship between stress/defense signaling and cell cycle regulation in intact plants at various developmental stages

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