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Mini-Review

Lesion mimic mutants

A classical, yet still fundamental approach to study programmed cell death

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Abbreviations: LMM, lesion mimic mutant; PCD, programmed cell death; HR, hypersensitive response; CNGC, cyclic nucleotide-gated ion channel; VPE, vacuolar processing enzyme; TMV, tobacco mosaic virus

Key words: lesion mimic mutants, PCD, cyclic nucleotide-gated ion channel, calcium, sphingosine, chloroplast

Over the last decade a substantial number of lesion mimic mutants (LMM) have been isolated and a growing number of the genes have been cloned. It is now becoming clear that these mutants are valuable tools to dissect various aspects of programmed cell death (PCD) and pathogen resistance pathways in plants. Together with other forward genetics approaches LMMs shed light on the PCD machinery in plant cells and revealed important roles for sphingolipids, Ca^{2+} and chloroplast-derived porphyrin-metabolites during cell death development.

In order to protect themselves against pathogen attack plants have evolved an elaborate defense system with a complex signaling network.[1] One of the most prominent responses is the hypersensitive response (HR), which is characterized by rapid death of cells at or surrounding the infection site. Over the last decade substantial effort has been made to unravel the signaling and to identify the genes involved.[2] One approach is the isolation of mutants that display a misregulation of cell death formation. These mutants are called lesion mimic mutants (LMMs) since they exhibit either constitutive or unregulated cell death formation which resembles the HR after pathogen infection. Many mutants have been identified mostly in Arabidopsis, rice, maize and barley.[3] Lorrain et al.,[3] defined two classes of LMMs: initiation mutants and propagation mutants. Initiation mutants show constitutive formation of lesions of variable sizes. On the other hand, the propagation mutants have defects that do not allow them to control cell death once it has started.

We know now that the HR is an active process and the dying cells undergo a type of programmed cell death (PCD).[4] Although it is possible that a mutation which does not directly regulate PCD induces cell death via non-specific cellular perturbation, still it is obvious that LMMs are valuable tools to identify genes that are involved in the regulation and execution of PCD in plants. The fact that we observe initiation and propagation mutants also suggests that there are separate pathways to initiate PCD and to control/suppress PCD once it has been started. Mutants such as *lsd1* (*lesions simulating disease resistance response 1*) display runaway cell death when inoculated with HR-inducing bacteria.[5] On the other hand mutants such as *acd5* (*accelerated cell death 5*) and *cpr22* (*constitutive expresser of PR 22*) show normal HR development.[6,7] The growing number of cloned LMM genes is revealing some of the important players in plant PCD: Ca^{2+} ion influx (*dnd1, dnd2/hlm1(defense no death 1,2; HR-like lesion mimic 1)*,[8-10] *cpr22*[7] *and cpn1/bon1* (*copine 1*, *bonzai 1*)[11,12]) ROS formation/sensing (*lsd1*),[5] sphingolipid metabolism (*acd5, acd11*)[6,13,14] and porphyrin/chlorophyll biosynthesis and catabolism (*acd1, acd2, lin2 (lesion initiation 2), lls1(lethal leaf spot 1), les22* and *flu1 (fluorescent 1*).[15-20] Additionally, at least some of the LMMs can be utilized as a tool to further dissect the affected pathways.

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Elucidating the Cell Death Machinery

Surprisingly none of the so-far-cloned LMMs codes for a protein that is clearly associated with the execution of PCD. However, some mutants such as *cpr22* (*AtCNGC11/12*) provide a useful tool to study PCD. Transient expression of *AtCNGC11/12* induces cell death in *Nicotiana benthamiana* leaves.[7] This cell death resembles the HR seen in an incompatible plant-pathogen interaction. A microscopic analysis of *AtCNGC11/12*-induced cell death revealed strong similarities to pathogen-induced as well as developmental PCD.[4,21-23] These include retraction of the plasma membrane from the cell wall, vesicle formation and degradation of the tonoplast.[23] Chromatin condensation suggested the occurrence of DNA degradation, which was confirmed by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. In animals DNA laddering, the cleavage of DNA into nucleosomal fragments of multiples of 180 base pairs, is a hallmark of PCD.[24] However in plants, laddering was only reported in some cases[25,26] but not in others.[27,28] These discrepancies might be due to the timing of sampling or there might be some differences between different types of PCD.

These observations suggest that the cell death observed in *cpr22* is not due to unspecific perturbation of cellular physiology but an activation of the PCD machinery. Further evidence came

from the demonstration that caspase-like activity was required for *AtCNGC11/12*-induced PCD. Caspases are cystein proteases that regulate PCD in animals.[29] There are no true orthologues of caspases in plants, but recently a related family of proteins, called meta-caspases was identified. However, so far the role of meta-caspases in plants is not well defined.[30] At present, the strongest candidates for caspase-like executioners of plant PCD are vacuolar processing enzymes (VPEs). Even though they display little sequence similarity, VPEs exhibit significant structural homology to animal caspases.[31] VPE possess similar substrate specificity as caspase-1.[32] Treatment with the caspase-1 specific synthetic inhibitors, Ac-YVAD-CHO or Ac-YVAD-CMK attenuated *AtCNGC11/12*-induced cell death in *N. benthamiana* significantly, whereas a non-specific protease inhibitor, PMSF, did not affect HR development. Furthermore, silencing of *VPE1a* and *VPE1b* by VIGS in *N. benthamiana* resulted in strong suppression of HR formation.[23]

These findings suggest that VPEs are functional homologs of animal caspases. Both, caspase inhibitors and silencing of VPE affected TMV-induced HR in tobacco in the same way,[33,34] indicating that AtCNGC11/12 can be used as a tool to elucidate pathogen-induced HR. Furthermore, transient expression of *AtCNGC11/12* can trigger cell death in a controlled and synchronized manner. This feature is a significant advantage to study the signal transduction to induce PCD, since the usage of LMM for PCD research has been hindered by the fact that cell death frequently develops in an unsynchronized way.

Containing Cell Death

The HR is an important feature for the plant to stop pathogen from spreading from the site of infection. However, the extent of the HR also has to be limited in order to keep the self-inflicted damage to a minimum. Therefore, plants also have sophisticated mechanisms to control the propagation of PCD. Failure to control PCD leads to uncontrolled damage to the plant. The lesions simulating disease resistance1 (*lsd1*) mutation causes a runaway cell death phenotype.[5] In this mutant, once cell death is induced it can not be controlled. *LSD1* codes for a zinc finger protein that may act as a negative regulator of a pro-death signal.[35] Recently, Kaminaka et al.,[36] suggested that LSD1 binds to the transcription factor AtbZIP10, thereby retaining it outside of the nucleus. Activation of AtbZIP10 or deactivation of LSD1 leads to dissociation from LSD1 allowing it to enter the nucleus where it induces the expression of HR-related genes. Epple et al.,[37] reported that LSD1 also interacts with LSD-One-Like 1 (LOL1). In the absence of LOL1 (in the *lol1* background) the *lsd1* LMM phenotype was abolished, indicating that the interaction with LOL1 is required for the onset of PCD.

One mechanism to control PCD is autophagy, where cytoplasmic components or damaged organelles are contained in vesicles called autophagosomes which are then sent to the vacuole or lysosomes for degradation.[38] Liu et al.,[39] demonstrated that the AuTophaGy (ATG) gene *AtATG6/Beclin 1* is required for the control of HR cell death. Silencing of *AtATG6/Beclin 1* leads to uncontrolled HR-PCD upon infection with TMV. PCD spread into uninfected tissue leading eventually to a collapse of the entire leaf. Autophagy might be induced during HR/PCD to eliminate "pro-death" signals in order to protect the uninfected leaf tissue. *AtATG6/Beclin 1* was identified in a screen for genes that affect initiation or execution of TMV-induced HR using virus induce gene silencing (VIGS).[39] In a similar screen aconitase was identified as a regulator of PCD.[40] Silencing of aconitase delayed the initiation of Pto-induced HR. Interestingly, once the HR started to develop, it could not be contained in aconitase-silenced plants leading to the collapse of the entire leaf. Thus, both LMMs and genetic screens start to shed light on the players that are required to control PCD.

A Role for Sphingolipids in PCD

Interestingly, at least two of the cloned LMM genes are involved in sphingolipid metabolism. *ACD5* encodes a ceramide kinase and *ACD11* codes for a sphingosine transfer protein.[13,14] *acd5* displays strongly reduced ceramide kinase activity resulting in an accumulation of precursor molecules, presumably ceramide or sphinganine.[13] *acd5* also displays enhanced cell death after infection with bacterial pathogens.[6] It seems that the balance between ceramides/sphingolipids and their phosphorylated derivatives is important for modulating cell death in plants. Phosphorylation of ceramides by ACD5 attenuates the proapoptotic effects of unphosphorylated ceramides. Similar observations have been reported in animals, where sphingosine-1-phosphate has been shown to suppress PCD in animals.[41] The sphinganine-analog mycotoxins fumonisin B_1 and AAL toxins are inhibitors of eukaryotic sphinganine *N*-acyltransferases. Treatment with AAL toxin or fumonisin B_1 led to the accumulation of free sphingoid bases such as 3-keto-dihydrosphinganine and dihydrosphinganine, which in turn lead to cell death.[42,43] Recently, Shi et al.,[44] reported that the Arabidopsis mutant *fbr 11-1* (*fumonisin B_1 resistant 11-1*), which is incapable of initiating PCD when the mutant is treated with fumonisin B_1, a specific inhibitor of ceramide synthase, encodes a long-chain base 1 (LCB1) subunit of serine palmitoyltransferase (SPT), which catalyzes the first step in the sphingolipid biosynthetic pathway. The authors also presented data that the free sphingoid bases dihydrosphingosine, phytosphingosine and sphingosine induce PCD whereas dihydrosphingosine-1-phosphate blocked sphinosine-induced PCD in a dose-dependent manner. C2-ceramides also have been shown to induce PCD in cell suspension cultures, where an increase in calcium influx was observed.[45,46] Inhibition of the calcium flux prevented cell death. These observations suggest that calcium signaling plays a role in ceramide-induced PCD.

The Ca^{2+} Connection

An influx of Ca^{2+} ions has been associated with HR/PCD for a long time.[21,47] Grant et al.,[48] showed an increase in cytosolic Ca^{2+} that was specific for HR-inducing bacteria. Interestingly so far five LMMs were identified as mutations in cyclic nucleotide-gated ion channel genes (CNGC)—*DND1* encodes *AtCNGC2*,[8] *DND2* and *HLM1* both were mapped to *AtCNGC4*.[9,10] The barley LMM *nec1* also has a mutation in the barley homolog of *DND2/HLM1*.[49] In these mutants the channel genes were knocked out making them loss of function mutants. On the other hand the aforementioned *cpr22* mutant gives rise to a novel chimeric protein derived from homologous recombination of two tandemly repeated CNGC genes. The N-terminal half of *AtCNGC11* and the C-terminal half of *AtCNGC12* fused to form a chimeric novel channel protein.[7] The chimeric *AtCNGC11/12* is in frame and expressed to normal levels. The phenotypes observed in *cpr22* are attributed to the expression

of this novel channel protein rather than the absence of *AtCNGC11* or *AtCNGC12*.[7] This differentiates this mutant from *dnd1, dnd2/hlm1*. CNGCs are a relatively uncharacterized family of ion channels comprising of 20 members.[50,51] *AtCNGC2* and *4* are the sole members of Group IVB, which is the most divergent group among the 20 member family of CNGCs. On the other hand, *AtCNGC11* and *12*, which are closest related to each other, belong to group I.[50] Several studies have shown that Arabidopsis CNGCs can function as non selective cation channels like their counterparts in animals. For examples, AtCNGC2 has been shown by patch clamp and yeast complementation analysis to be an inward rectifying Ca^{2+} and K^+ channel.[52,53] AtCNGC11 and 12 also conduct Ca^{2+} and K^+ as shown by yeast complementation assays.[7,23] AtCNGC4 is permeable for both Na^+ and K^+.[10] However, only Ca^{2+} channel inhibitors such as Gd^{3+} or La^{3+} significantly suppressed HR formation induced by *cpr22* (*AtCNGC11/12*), but not the K^+ channel blocker, tetraethylammonium chloride (TEA).[23] The same effects were reported by Ali et al.,[53] for AtCNGC2. This indicates that Ca^{2+} influx, at least in part mediated by CNGCs plays a crucial role in the induction of PCD.

Interestingly, two mutants *copine 1* and *bonzai 1* (*cpn1/bon1*) that are related to Ca^{2+} signaling were reported as LMMs, further supporting the connection between Ca^{2+} and PCD.[11,12] Copines are Ca^{2+}-dependent, phospholipid-binding proteins that repress cell death.[54] They are connected to membrane trafficking and Ca^{2+} signal transduction. They possess a von Willebrand A (VWA) domain for protein interaction and a C2 domain that mediates the membrane association.[55] Their function is still not cleat but they may play a role in defining the Ca^{2+} specificity required for proper signal transduction.

A Role for the Chloroplast

In both plants and animals there is growing evidence for a prominent role of the mitochondria in PCD.[4,56] However, several LMMs, e.g., *acd1* and *2,* also point to the chloroplasts as another player in PCD.[15,16] Light seems to be required for HR formation at least for some incompatible interactions.[57] Boccara et al.,[58] recently showed that after treatment with the elicitor harpin excess excitation energy (EEE) is being formed in chloroplasts leading to overexcited chlorophyll metabolites. They will react with $^3O_2$ and form singlet oxygen ($^1O_2$). $^1O_2$ will usually be quenched by carotenoids present in the chloroplasts. However, a release of activated chlorophyll catabolites from the photosynthetic reaction center can lead to oxidative damage. This may occur during the HR/PCD in plants. *ACD1* codes for pheophorbide a oxygenase, which oxidizes the toxic chlorophyll product pheophorbide; the accumulation of pheophorbide in the *acd1* mutant leads to the observed mutant phenotype.[15] The maize *LLS1* gene also codes for a pheophorbide oxigenase.[18] The product of *ACD1*/*LLS1*, red chlorophyll catabolite (TCC) is being removed by ACD2, a chlorophyll catabolite reductase.[16] The *acd2* mutant shows a similar phenotype to *acd1*. Further LMMs associated with porphyrin/chlorophyll biosynthesis and catabolism are *lin2* (coporphyrinogen III oxidase;[17] *les22* (accumulates uroporphyrinogen III;[19]) and *flu* (accumulates protochlorophyllide[20]). Interestingly, exogenous treatment with protoporphyrinogen IX also leads to PCD.[59] This suggests that porphyrin/chlorophyll catabolites may be one of the pro-death signals in plants.

Concluding Remarks

What are the signaling molecules that promote cell death during the HR and how can the HR be contained once it is started? Besides the above mentioned Ca^{2+} influx the most obvious candidates are reactive oxygen intermediates (ROS). The most likely source of ROS is a NADPH oxidase similar to the one found in mammals.[60] The gp91phox homologs in Arabidopsis, *AtrbohD* and *AtrbohF* have been implicated in HR formation.[61] However, ROS may also act as an anti-death signal in certain situations. The *rbohD lsd1* double mutant displayed reduced levels of ROS compared to *lsd1* but surprisingly showed increased cell death.[62]

Other players that are involved in HR induction are nitric oxide (NO) and salicylic acid (SA).[63,64] Van Camp et al.,[65] proposed a feed-forward cycle that included H_2O_2, SA and NO, which leads to the rapid development of PCD after pathogen infection. However, considering that both H_2O_2 and NO also have been shown to display anti-apoptotic features (reviewed in ref. 2), the stoichiometry of ROS, NO and SA may decide whether PCD is activated or suppressed.[63] One possible connection between ROS levels and SA could be the role of the redox status of the cell or certain compartments for the execution of PCD.[2,66]

Over the last decade, our knowledge about PCD has been significantly increased by numerous researchers' tireless work. However, only a few important players were identified as described here and a complete understanding of signal transduction pathway(s) for the induction of PCD and its control and propagation/suppression is still very far. Since the fist LMMs were described more than 30 years ago, the analysis of LMMs is already a classical, yet still important approach to identify essential players in PCD or as a tool to dissect the signaling involved in PCD and pathogen resistance in plants.

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