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Article Addendum

Scorched earth strategy

Grim Reaper saves the plant

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Programmed cell death is a common feature of developmental processes and responses to environmental cues in many multicellular organisms. Examples of programmed cell death in plants are leaf abscission in autumn and the hypersensitive response during pathogen attack. Reactive oxygen species (ROS) have been implicated in the regulation of various types of cell death.[1,2] However, the precise mechanics of the involvement of ROS in the processes leading to initiation of cell death and subsequent containment are currently unknown. We recently showed the involvement of an Arabidopsis protein GRIM REAPER in the regulation of ROS-induced cell death under stress conditions.[3] Our results indicated that the presence of a truncated protein primes plants for cell death in the presence of ROS leading to ozone sensitivity and increased resistance to hemibiotrophic pathogens.

Reactive oxygen species (ROS) have been implicated in the response to many biotic and abiotic stresses for example during pathogen attack, osmotic stress, excess light, wounding and ozone (O_3).[4] ROS are produced in different subcellular compartments and play far more complex roles than acting simply as cytotoxic compounds. For example, pathogens and ozone induce ROS production in the apoplast while high light or the herbicide methyl viologen induce ROS production in the chloroplast.[5] While it is clear that plants are able to distinguish different ROS with regard to type, timing and subcellular localization of ROS produced, the mechanisms of ROS perception are still largely unknown. Redox regulation is known for only few plant proteins, exemplified by heat-shock transcription factors (HSFs)[6] and NPR1, which confers redox regulation to the transcription factor TGA1 in response to salicylic acid (SA).[7]

ROS are critical to the regulation of programmed cell death. However, the precise role of ROS during these processes is not well understood.[4,5] The data available supports dual roles for ROS in lesion formation and spread as well as in the subsequent containment of cell death.[2,8] Detailed elucidation of how plants are able to use ROS for those opposing processes with such precision will answer many questions about signaling and signal integration during plant stress responses.

To identify novel components in ROS signaling in *Arabidopsis thaliana* we performed microarray gene expression profiling experiments to identify genes regulated by O_3. For genes with fast responses to O_3 (within 30 min to 2 hours) the corresponding knock-out mutants were exposed to O_3 and screened for sensitivity. One line with an O_3-sensitive phenotype was subsequently designated *grim reaper* (*gri*) and characterized in more detail.[3] The *GRI* gene encodes an Arabidopsis orthologue of the tobacco *Stig1* gene which encodes a small protein secreted in stigmatic lipid exudates.[9,10] The GRI protein has a signal peptide for secretion to the apoplast, and GRI-YFP localize partly to the apoplast. Since Arabidopsis flowers do not produce a stigmatic lipid exudate, comparisons between the functions in tobacco and Arabidopsis flowers are difficult. Flowers of the *gri* mutant have a normal appearence, however seed production was severely reduced in *gri* indicating a role for GRI in Arabidopsis fertility. LeSTIG1, the GRI orthologue in tomato, is able to promote pollen tube growth in vitro.[11] The Arabidopsis *gri* mutant does not differ from the wild type under normal growth conditions and no significant differences between *gri* and wildtype were found in the global gene expression profile under normal growth conditions (unpublished data).

Complementation of the O_3-sensitive phenotype of *gri* was only partially successful. Thus, *gri* is most likely not a simple recessive mutation and this prompted us to investigate alternative explanations for the O_3-sensitivity. The *gri* mutant has a transposon insertion at basepair 288 of the open reading frame in the intronless *GRI* gene. In *gri* a transcript is detectable with primers located upstream of the transposon insertion while primers located after the insertion did now show a transcript in the mutant.

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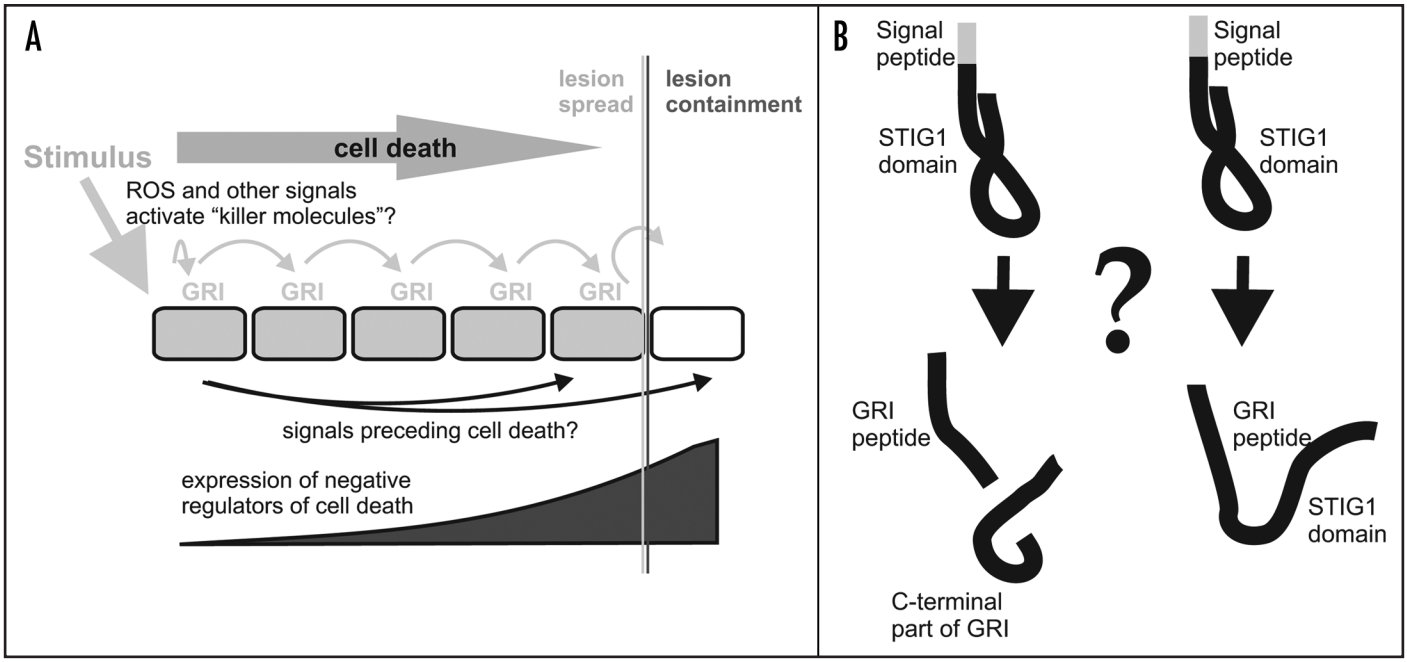

Figure 1. Hypothetical regulation mechanism of cell death and regulation of GRIM REAPER function. (A) after receiving a stimulus leading to the induction of cell death including hormone and ROS production, ROS function together with GRI to transmit the cell death signal to neighbouring cells. Simultaneously, cells start to transmit signals to neighbouring cells preceding the actual cell death front and express negative regulators of cell death. Ultimately, negative regulators of cell death reach sufficient levels for cell death containment. (B) GRI signal peptide is cleaved off and the protein is transported to the apoplast where it can receive signals. This could lead to proteolytic cleavage of GRI releasing active GRI peptide. Alternatively, apoplastic GRI protein refolds upon receiving a signal leading to exposure of the part of the protein corresponding to GRI peptide leading to GRI activation.

Consequently, a N-terminal fragment of the protein upstream the transposon insertion site could still be produced in *gri* and have functions related to the phenotypes observed. When GRI peptide, a truncated version of the GRI protein similar to the peptide likely to be present in *gri*, was infiltrated to leaves it induced cell death. The induction of cell death was dependent on the presence of superoxide; no cell death was apparent in co-infiltration of the GRI peptide with the $O_2^{\cdot-}$ scavenger superoxide dismutase or in infiltration into *AtrbohD* deficient in superoxide biosynthesis. This would make GRI peptide a candidate for ROS perception and subsequent induction of the processes leading to cell death in response to ROS. Figure 1A shows a hypothetical model for the regulation of cell death. ROS might activate cell death promoting molecules and thus propagate cell death from cell to cell. At the same time signals precede the death front and cause the transcriptional or post-transcriptional upregulation or activation of negative regulators leading to containment of cell death when a certain threshold is reached. Apoplastic ROS have previously been implicated in lesion spread but also in lesion containment.[2,8] This is consistent with the model presented in Figure 1A where ROS are participating in lesion spread and containment. Several other components involved in cell death regulation have been identified and include the plant hormones SA, jasmonic acid and ethylene.[4] The role of hormones in cell death can be studied using mutants deficient in their production or signaling pathways. Infiltration of GRI peptide into *sid2* plants deficient in SA biosynthesis showed a strict requirement for SA in activating cell death. Thus, SA or its derivatives are candidates for being cell death promoting molecules.

Overexpressing a C-myc/StrepII tagged version of GRI in planta suggested that GRI could be cleaved prior to activation. The presence of the truncated N-terminal fragment of GRI would prime cells for easier induction of cell death. However, it is still unclear if native GRI is cleaved. An alternative is that upon perception of a signal GRI refolds to expose the N-terminus in order to be able to activate cell death (Fig. 1B). It is yet unclear how GRI function is regulated but cell death induction of GRI peptide depends on the presence of superoxide in the apoplast. GRI could undergo direct oxidative modification and/or cleavage; or alternatively bind to an oxidized receptor. Whatever the mechanism of GRI activation, LeSTIG1—the tomato orthologue of GRI has been shown to bind to RLK receptors[11] in vitro, which suggests that activated GRI could bind to a plasma membrane based receptor to initiate further ROS signaling and cell death regulation. Further alternatives for the mechanism behind GRI peptide function are possible and will be a target of future investigation.

Taken together, the insertion in the *Gri* gene leads to faster induction of cell death resulting in ozone sensitivity. The tolerance to hemibiotrophic bacterial pathogens is another process that is heavily dependent on an accurate and well regulated cell death response. The fast induction of cell death in *gri* thus also led to an increased tolerance to a virulent strain of *Pseudomonas syringae pv.* tomato. The bacteria cannot grow in patches of dead cells, similar to the strategy of scorched earth in war. Understanding the mechanisms behind cell death induction by GRI peptide in concert with ROS will lead to novel insights into stress adaptation and pathogen defence, and also to understanding the links

and similarities between stress responses and development in the regulation of cell death.

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