Sphingolipid metabolism and programmed cell death in tomato

Spassieva, Stefanka Diankova

Summary

Programmed cell death is a process in multicellular organisms by which cells commit suicide. Programmed cell death is genetically determined. When the regulation of the process is disrupted it can have severe or lethal consequences for the organism. In mammals, cancer and neurodegenerative diseases are associated with abnormalities in programmed cell death. Development of an animal embryo is dependant on the correct execution of programmed cell death in certain cell types, which are no longer needed for the next developmental stage. In plants, programmed cell death occurs during development, as a response to pathogen attack (known as a hypersensitive response) or as a result of specific toxins released by a pathogen. The process of programmed cell death can be divided into three distinct phases, induction, effector and degradation. The focus of our research is the initial signalling events that take place in the induction phase of programmed cell death in tomato.

Tomato was chosen as a model organism because it is a genetically well characterised plant species and because of the well established *Asc*-AAL-toxin model system. *Asc* is a disease resistance locus in tomato that confers resistance to the disease *Alternaria* stem canker. The locus has two alleles, *Asc*, which confers resistance to the fungal pathogen *Alternaria alternata* f.sp. *lycopersici* and *asc*, which confers susceptibility. The fungus *Alternaria alternata* f.sp. *lycopersici* secretes a toxin, called AAL-toxin. The interaction between tomato plants and the fungal pathogen can be simplified to the interaction between the plant leaf and purified AAL-toxin. The *Asc* allele confers insensitivity to the toxin and conversely the *asc* allele confers sensitivity. Sensitive *asc/asc* tomato plants die, when challenged with AAL-toxin, by programmed cell death. AAL-toxin together with fumonisin, another toxin produced by a fungus (*Fusarium moniliforme*), are structural homologues of sphingoid long-chain bases and competitive inhibitors of (dihydro)ceramide synthase, a key enzyme of *de novo* sphingolipid biosynthesis. The *Asc-1* gene product is a member of a gene family of integral-membrane proteins that comprises homologues from all eukaryotic kingdoms. Compared to *Asc-1*, *asc-1* contains a few mutations, one of which, a 2bp deletion, results in a frame shift and most likely in absence of the protein. When the two yeast homologues of *Asc-1*, *LAG1* and *LAC1*, are deleted the result is a lethal or slow growth phenotype. The slow growth phenotype is associated with severe cell wall defects, delayed glycosylphosphatidylinositol- (GPI)-anchored protein transport and impaired sphingolipid biosynthesis. The two yeast homologues, *LAG1* and *LAC1*, have been shown to be necessary components of (dihydro)ceramide synthase. This suggests that sphingolipid signalling might be involved in AAL-toxin induced programmed cell death in *asc/asc* tomato plants.

Chapter 1 reviews the contemporary knowledge of plant sphingolipids. Sphingolipids are lipid molecules with a hydrophobic ceramide backbone and a hydrophilic head group. The ceramide backbone consists of a sphingoid long-chain base and a fatty acid linked via an amide bond. In plants, two groups of sphingolipids

have been described, glucosylceramides and inositolphosphorylceramides. The diversity of plant glucosylceramides is mainly due to the differences in the structure of the long-chain base and/or the fatty acid component of the ceramide backbone. Glucosylceramides are important structural components of tonoplast and plasma-membranes. Inositolphosphorylceramides are found as a part of the GPI anchor of plant proteins, the final destination of which is the outer leaflet of the plasma membrane, suggesting a role of the plant inositolphosphorylceramides in protein trafficking. In plants, a number of genes involved in the sphingolipid biosynthesis have already been cloned. The first report of a role of a plant sphingoid metabolite, sphingosine-1 phosphate, in signalling appeared recently.

The function of Asc-1p in sphingolipid metabolism in yeast is shown in Chapter 2. Expression of tomato *Asc-1* in the *lag1Δlac1Δ* yeast mutant partially complements the slow growth phenotype, restores the wild type morphology of the cell wall and importantly, restores sphingolipid biosynthesis. The *lag1Δlac1Δ* yeast mutant compared to wild type showed differences in the pattern of cell wall proteins. In yeast, a portion of the GPI-anchored proteins are detached from their anchor and attached to the sugar matrix of the cell wall. They, together with another group of proteins (proteins with internal repeats) form the outer protein layer of the yeast cell wall. When *Asc-1* was expressed in the *lag1Δlac1Δ* yeast mutant the wild type pattern of cell wall proteins was restored, most likely by negating the delay in GPI-anchored protein transport in the mutant.

In tomato, we have demonstrated that presence or absence of the *Asc-1* gene resulted in a different pattern of sphingolipid labelling when AAL-toxin was applied (Chapter 3). Firstly, the precursors of ceramide, 3-ketodihydrosphingosine and dihydrosphingosine, were elevated in the extracts from both genotypes but to a larger extent in the *asc* genotype. Secondly, AAL-toxin treatment affected sphingolipids. The sphingolipids were decreased in *asc* lipid extracts, while the *Asc* extracts showed wild type levels of sphingolipids and additional novel sphingolipid species. When tomato leaf discs were not treated with the AAL-toxin there was no difference between *Asc* and *asc* in their sphingolipids and sphingoid precursors as judged by thin-layer chromatography. In Chapter 4 labelling of the tomato leaf discs with *myo*-[^{3}H]inositol revealed that *de novo* synthesis of inositolphosphorylceramides is affected by AAL-toxin treatment. By using reverse phase liquid chromatography tandem mass spectrometry, we have identified 26 glucosylceramide species in tomato leaves. The relative abundance of the different glucosylceramides was not significantly affected by the genotype or the AAL-toxin treatment.

Long-chain bases, ceramide and their phosphorylated derivatives are known from yeast and mammalian systems as powerful signals involved in apoptosis, cellular proliferation and stress responses. Not only a decrease or increase of the individual sphingoid compound is important for cellular fate but also their relative proportion. In the case of tomato *asc*-AAL-toxin interaction, which results in programmed cell death, an increase in the level of the long-chain bases was observed

at the same time as a decrease in inositolphosphorylceramide synthesis. Myriocin, an inhibitor of the first step of *de novo* ceramide synthesis, substantially, though not completely, suppressed the cell death effect of the AAL-toxin on *asc* plants. This is additional evidence that the primary death signal derives from sphingolipid metabolites in AAL-toxin induced programmed cell death in tomato. It remains to be answered whether the signal is just the increased level of long-chain bases, or if there is a synergistic effect between the increased level of long-chain bases and the decreased level of inositolphosphorylceramides and possibly diacylglycerol.

Another form of programmed cell death in plants is the hypersensitive response. The hypersensitive response occurs as a result of recognition of a pathogen elicitor by a plant resistance gene. Subsequent signalling events lead to death of the tissue surrounding the pathogen. The pathogen remains contained and the plant resistant. The induction of programmed cell death in plants can be studied by analysing lesion mimic mutants. The lesion mimic mutants exhibit a hypersensitive response-like phenotype in the absence of a pathogen. A mutation in the maize *Lls1* gene results in such a lesion mimic phenotype. By cloning the tomato *Lls1* homologue and silencing it we were able to engineer a tomato lesion mimic mutant (Chapter 5). The analyses of the predicted Lls1 protein revealed that the protein is unique for photosynthesizing organisms. Light dependence of the *lls1* mutant phenotype and the homology of the Lls1 protein to proteins involved in photosynthesis suggest reactive oxygen species as possible death signals.

Programmed cell death is one of the basic processes in multicellular organisms important for the survival of the organism as a whole. Signals controlling this process and the genes involved in its regulation are conserved between species, as in the *Lls1* study. The conservation can even cross the border of kingdoms, as in the case of *Asc-1* and sphingolipid signalling. Asc-1p is part of a basic metabolic pathway, sphingolipid biosynthesis, disruption of which can have lethal consequences for the cell.